DEVELOPMENT OF SINGLE NUCLEOTIDE POLYMORPHISM MARKERS FOR FINGERPRINT ANALYSIS OF TURKISH OLIVE (*Olea europaea* L.) CULTIVARS AND DETECTION OF ADULTERATION IN TURKISH OLIVE OIL

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ABSTRACT

DEVELOPMENT OF SINGLE NUCLEOTIDE POLYMORPHISM MARKERS FOR FINGERPRINT ANALYSIS OF TURKISH OLIVE (*Olea europaea* L.) CULTIVARS AND DETECTION OF ADULTERATION IN TURKISH OLIVE OIL

Olive (*Olea europaea* L.) tree and oil are signature figures of the Mediterranean culture. Because of its high economic value, olive oil is extremely vulnerable to fraud. The aim of this study was to develop molecular tests for authenticating cultivar and botanical origin in olive oils. In order to authenticate the botanical origin and detect adulteration, a plastid DNA region was utilized for standardizing a capillary-electrophoresis barcode assay. The performance of the assay was evaluated on series of olive oil : seed oil admixtures. The assay proved successful in identifying seed oils in olive oil down to a limit of 10%. The molecular assay described in this work enables adulteration detection regardless of compositional similarities between the adulterant and adulterated oil species, thus will complement the shortcomings of analytical chemistry approaches. In order to establish a DNA-based identification key to ascertain the cultivar origin of Turkish monovarietal olive oils, short fragments from five olive genes were sequenced for SNP (Single Nucleotide Polymorphism) identification. CAPS (Cleaved Amplified Polymorphic DNA) assays were designed for SNPs that alter restriction enzyme recognition motifs. When applied on the oils of 17 olive cultivars, a maximum of five CAPS assays were necessary to discriminate the varietal origin of the samples. Admixture detection threshold of the assays was identified as 20% when tested on olive oil admixtures. The SNP-based CAPS assays developed in this work can be used for testing and verification of the authenticity of Turkish monovarietal olive oils, for olive tree certification, and in germplasm characterization and preservation studies.

ÖZET

TÜRK ZEYTİN (*Olea europaea* L.) ÇEŞİTLERİNİN PARMAKİZİ ANALİZLERİ VE TÜRK ZEYTİNYAĞLARINDA TAĞŞİŞİN BELİRLENMESİ İÇİN TEK NUKLEOTİD POLİMORFİZM MARKÖRLERİNİN GELİŞTİRİLMESİ

Zeytin ağacı (*Olea europaea* L.) ve zeytinyağı, Akdeniz kültürüne özgü en önemli simgelerdendir. Yüksek ekonomik değeri nedeni ile, zeytinyağı tağşişe son derece açık bir gıda ürünüdür. Bu çalışma kapsamında zeytinyağlarında çeşit kimliği ve tağşiş belirlemede kullanılacak moleküler test yöntemlerinin geliştirilmesi hedeflenmiştir. Kloroplast genomunda bulunan bir DNA barkod bölgesi zeytinyağlarına tohum yağları ile yapılan tağşişi belirlemede kullanılmak üzere belirlenmiş ve bir kapiler elektroforez-barkod analiz yöntemi geliştirilip standardize edilmiştir. Analiz yönteminin performansı bir dizi zeytinyağı - tohum yağı karışımı üzerinde gösterilmiş, yöntemin zeytinyağlarında safsızlık belirleme limiti % 10 olarak belirlenmiştir. Bu çalışmada tanıtılan analiz yöntemi, farklı bitkisel yağlar arasındaki kimyasal kompozisyon benzerliklerinden bağımsız olarak tağşişin belirlenmesine olanak vermektedir ve dolayısı ile analitik kimya temelli yöntemlerin eksikliklerini tamamlamada faydalı olacaktır. Zeytin bitkisine özel gen bölgelerinin ekonomik öneme sahip Türk zeytin çeşitlerinde dizilenmesi ile tek nükleotid polimorfizmleri (SNP) tespit edilmiş ve bu polimorfizmler, tek-varyete Türk zeytinyağlarında çeşit kimliğinin doğrulanması amaçlı bir DNA-temelli tanımlama anahtarı oluşturmada kullanılmıştır. Restriksiyon enzimi tanıma bölgelerine denk düştüğü belirlenen SNP'leri genotiplemek üzere CAPS (kesilmiş çoğaltılmış polimorfik DNA) analizleri dizayn edilmiş ve 17 çeşide ait tek-varyete zeytinyağlarına uygulanmıştır. Farklı çeşit kimliğine sahip yağların SNP alellerine dayalı tanımlanmasında maksimum beş adet SNP yeterli olmuştur. CAPS analizlerinin farklı çeşit kökenine sahip zeytinyağı karışımlarını tespit etme etkinliği test edilmiş, karışım tespit limiti % 20 olarak belirlenmiştir. Bu çalışma kapsamında geliştirilmiş olan SNP-temelli CAPS analizleri, Türk tek-varyete zeytinyağlarının çeşit kimliğinin moleküler düzeyde belirlenmesi ve tesciline olanak sağlayacak, yanısıra fidan sertifikasyonu ve gen kaynaklarının karakterizasyonu ve korunması çalışmalarında kullanılabilecektir.

TABLE OF CONTENTS

LIST OF FIGURES viii

LIST OF TABLES x

CHAPTER 1. INTRODUCTION 1

1.1. Olive (*Olea europaea* L.) 1

1.2. Olive oil 2

1.2.1. Chemical composition, quality grades and health attributes 2

1.2.2. Authenticity and traceability issues of olive oil 4

1.3. Food genomics 6

1.3.1. Food genomics to establish the authenticity of olive oil 8

1.4. Aim of the study 10

CHAPTER 2. MATERIALS AND METHODS 11

2.1. Plant material and oil samples 11

2.2. DNA isolation 12

2.3. PCR-Capillary Electrophoresis (PCR-CE) barcode analysis 12

2.4. Gas chromatography analysis of olive oil : seed oil admixtures 12

2.5. Primer design, sequencing and SNP identification 13

2.6. Design and application of the CAPS assays for SNP genotyping 15

2.7. Statistical analysis 16

CHAPTER 3. RESULTS AND DISCUSSION 17

3.1. Authentication of botanical origin in olive oil by plastid non-coding DNA length polymorphisms 17

3.1.1. Determination of barcode DNA length polymorphisms among the ten plant species 17

3.1.2. Performance of the PCR-CE barcode assay to detect adulteration in olive oil 19

3.2. Cultivar Origin and Admixture Detection in Turkish Olive Oils by SNP-based CAPS Assays 29

3.2.1. SNP identification by sequencing ... 30
3.2.2. Design of CAPS assays for SNP genotyping ... 30
3.2.3. Fingerprinting monovarietal olive oils using SNP-based CAPS markers 39
3.2.4. Application of the SNP-based CAPS markers for detecting olive oil mixtures and to commercial oil samples ... 41

CHAPTER 4. CONCLUSION ... 44

REFERENCES ... 47

LIST OF FIGURES

Figure **Page**

Figure 3.1. Agarose gel electrophoresis image displaying the Barcode Pl-cd fragments amplified from ten plant species 18

Figure 3.2. Capillary electropherograms displaying the results of the PCR-CE barcode assays applied on a series of Olive oil : Sesame oil admixtures 20

Figure 3.3. Capillary electropherograms displaying the results of the PCR-CE barcode assays applied on a series of Olive oil : Soybean oil admixtures 21

Figure 3.4. Capillary electropherograms displaying the results of the PCR-CE barcode assays applied on a series of Olive oil : Sunflower oil admixtures 22

Figure 3.5. Capillary electropherograms displaying the results of the PCR-CE barcode assays applied on a series of Olive oil : Canola oil admixtures 22

Figure 3.6. Capillary electropherograms displaying the results of the PCR-CE barcode assays applied on a series of Olive oil : Corn oil admixtures 23

Figure 3.7. Capillary electropherograms displaying the results of the PCR-CE barcode assays applied on a series of Olive oil : Hazelnut oil admixtures 23

Figure 3.8. Capillary electropherograms displaying the results of the PCR-CE barcode assays applied on a series of Olive oil : Cottonseed oil admixtures 24

Figure 3.9. Capillary electropherograms displaying the results of the PCR-CE barcode assays applied on a series of Olive oil : Peanut oil admixtures 25

Figure 3.10. Capillary electropherograms displaying the results of the PCR-CE barcode assays applied on a series of Olive oil : Safflower oil admixtures 26

Figure 3.11. Capillary electropherograms displaying the results of the PCR-CE barcode assays applied on a series of Olive oil : Palm oil admixtures............ 26

Figure 3.12. Identification key displaying the discrimination of Turkish olive cultivars according to SNP-based CAPS assays 32

Figure 3.13. Electrophoretic patterns obtained by SNP-based CAPS assays for the monovarietal olive oils discriminated by two or three SNPs 35

Figure 3.14. Electrophoretic patterns obtained by SNP-based CAPS assays for the monovarietal olive oils discriminated by four SNPs 36

Figure 3.15. Electrophoretic patterns obtained by SNP-based CAPS assays for the monovarietal olive oils discriminated by five SNPs 37

Figure 3.16. Capillary electropherograms displaying the results of SNP3-HphI assay applied on olive oil admixtures 38
Figure 3.17. Capillary electropherograms displaying the results of the SNP1-HpyCH4III, SNP2-BsgI, SNP3-HphI, SNP4-Hpy166II, SNP5-HindIII and SNP6-EagI assays applied on commercial Sample No.1 42
Figure 3.18. Capillary electropherograms displaying the results of the SNP1-HpyCH4III, SNP2-BsgI, SNP3-HphI, SNP4-Hpy166II, SNP5-HindIII and SNP6-EagI assays applied on commercial Sample No.2 43

LIST OF TABLES

Table **Page**

Table 2.1. Olive varieties used in the study. 12

Table 2.2. List of primers used for SNP identification and sequencing. Unless indicated otherwise, primers were designed in this study 14

Table 3.1. Barcode Pl-cd amplicon sizes of plant species analyzed in this study 19

Table 3.2. Performance of the PCR-CE barcode assay and GC analysis to detect olive oil : seed oil admixtures 28

Table 3.3. Genotypes of the 17 olive varieties for the SNPs used for constructing the cultivar origin identification key 34

Table 3.4. Allele frequency, informativeness and reproducibility of the SNP markers, expressed as Probability of Identity and Allelic Error, respectively 34

CHAPTER 1

INTRODUCTION

1.1. Olive (*Olea europaea* L.)

Olive *(Olea europaea subsp. europaea* var. *europaea*) is an evergreen tree crop with a very long cultivation history that dates back to the third millenium BC (Zohary and Spiegel-Roy, 1975). *O. europaea* is the only cultivated member of the genus *Olea,* which comprises a total of 30 species (Bracci et al., 2011). The olive tree is mainly cultivated in the Mediterranean basin, with 98% of the cultivated olive trees present in Mediterranean countries (Gomes et al., 2012). Spain ranks first among the world's leading olive producers with 7,875,000 tonnes (t) of total production, followed by Spain (7,875,000 t), Italy (3,022,886 t), Greece (2,000,000 t) and Turkey (1,676,000 t) (FAOSTAT, Production statistics, 2013).

The cultivated (*O. europaea* subsp. *europaea* var. *europaea*) and wild (*O. europaea* subsp. *europaea* var. *sylvestris*) forms co-exist in the Mediterranean basin and the presence of oleaster (wild olives) populations is considered a characteristic of the Mediterranean floristic region. Cultivated olive and oleasters share the same chromosome number (2n = 46), they are interfertile and the two forms display good grafting compatibility. As a result, a continuous gene flow between the two populations has been reported, which results in phenotypic similarities that make it difficult to distinguish cultivated and wild olives. However, the wild germplasm still harbors a high abundance of rare alleles despite the gene flow, indicating the absence of genetic erosion in the wild germplasm (Belaj et al., 2007). Indeed, a high genetic diversity was also reported for both local cultivated germplasms and collections of cultivars representing different cultivation regions (Belaj et al., 2012). In this sense, the richness of olive genetic diversity represents a rare case among tree crops.

Olive tree is well-suited to the Mediterranean region with its tolerance to drought and salinity. Olive displays a higher drought and salinity tolerance compared to other woody crops grown in temperate climates (Cresti et al., 1994), and therefore, can be considered a model species to study tolerance against these two important abiotic

stress factors in tree crops (Gucci and Tattini, 1997). In parallel with increased recognition of its health benefits, olive oil consumption, as well as olive tree cultivation is expanding throughout the world (Perez-Jimenez et al., 2013), with the successful adaptation of the tree to regions such as California (USA), South America, Australia and South Africa, where drought and salinity are common problems in agricultural land (Gucci and Tattini, 1997).

1.2. Olive oil

Olive oil is among the most valuable agro-food products (Agrimonti et al., 2011) and has been a characteristic component of the Mediterranean diet and culture since ancient times with significant economic importance in the region (Consolandi et al., 2008; Pasqualone et al., 2012). Health claims associated with the Mediterranean diet are mainly attributed to the predominant consumption of olive oil in Mediterranean countries. According to the production statistics released by the International Olive Oil Council, the top olive oil producer countries are Spain, Italy and Greece, followed by Tunisia, Syria and Turkey. Mediterranean countries are not only the major olive oil producers, but also the major consumers; approximately 70% of the olive oil production is consumed in Mediterranean countries (Baldoni and Belaj, 2009).

1.2.1. Chemical composition, quality grades and health attributes

Triacylglycerols are the primary constituents in olive oil, representing almost 99% of the chemical composition. Secondary constituents are mono- and diacylglycerols, free fatty acids, lipid-soluble constituents including hydrocarbons, sterols, aliphatic alcohols, tocopherols and pigments, and a wide variety of phenolic and volatile compounds. Palmitic (C16:0), palmitoleic (C16:1), stearic (C18:0), oleic (C18:1), linoleic (C18:2), and linolenic (C18:3) acids are the fatty acids present in olive oil, with myristic (C14:0), heptadecanoic and eicosanoic acids represented only in negligible amounts. Olive oil fatty acid composition is highly affected by genotype, agricultural practices, climatic and soil conditions, and fruit maturity stage. As a result, there is wide variation in the relative abundances of the major fatty acids, palmitic (7.8 - 18.8%), oleic (58.5% - 83.2 %), linoleic (2.8% - 21.2%) and linolenic acid (0.42 –

1.91%). As a matter of fact, all physical and chemical indices are prone to environment and cultivar dependent variation and properties of an olive oil from the same production region may display variability among harvest years (Boskou et al., 2006).

Designated quality classes of olive oil defined by the International Olive Oil Council are: virgin olive oil (extra virgin, virgin, ordinary virgin, lampante), refined olive oil, and crude olive pomace oil. Virgin olive oils are extracted from olive fruits only by physical processes that include washing, decantation, centrifugation and filtration. Virgin olive oil grades suitable for consumption without a requirement for rafination are extra virgin and virgin, defined by a free acidity threshold of 0.8 g / 100 g (0.8%) and 2 g / 100 g (2%), respectively. Olive oils with a free fatty acid content between 2 - 3.3 g / 100 g (2 – 3.3%) are classified as ordinary virgin olive oil and legal permission to sell such oils without refining depends on the regulations in the country of retail sale. Lampante virgin olive oils have a free acidity that exceeds 3.3% and, unless refined, such oils are not suitable for consumption. Refined olive oils are obtained from virgin olive oils by rafination and are generally blended with virgin oils for marketing. Crude olive pomace oil is the oil recovered from olive pulp by solvent extraction. It is refined and blended with virgin olive oil to be suitable for consumption.

Protective effects against cancer and reduced risk of coronary heart disease are the most commonly pronounced health benefits of olive oil (Tuck and Hayball, 2002). These health promoting properties are attributed to a high oleic acid, carotenoid, α-tocopherol, squalene and, hydrophilic phenolic content that mainly includes three classes of potent antioxidants: secoiridoids, simple phenols and lignans (Owen et al., 2000; Servili et al., 2004; Bracci et al., 2011). While carotenoids and tocopherols are also found in seed oils, most of the antioxidants in the hydrophilic phenolic fraction are exclusive to olive oil, thereby, making olive oil a superior source of health beneficial phenolic antioxidant compounds (Servili et al., 2004; Bracci et al., 2011). However, it is important to note that since rafination processes significantly reduce the phenolic content, only unrefined, extra virgin olive oil represents a supply of health promoting phenolic compounds (Visioli et al., 2006).

1.2.2. Authenticity and traceability issues of olive oil

Olive oil accounts for only 2% of the world' vegetable oil consumption, whereas seed oils (palm, soybean, canola, sunflower, cottonseed and peanut) are produced and consumed in quantities that far exceed those of olive oil (Costa et al., 2012a). On the other hand, there is a constantly increasing demand for olive oil especially in developed countries, due to a growing interest in the consumption of natural, minimally processed food products rich in health beneficial phytochemicals (Ulberth and Buchgraber, 2000). Given its popularity as a healthy food and the significant price differential between cold-pressed extra virgin olive oils and refined seed oils, it is not surprising that olive oil is the most attractive target for adulteration among the edible plant oils (Costa et al., 2012a).

Olive oil is adulterated in two common ways: by mixing with seed oils of lower economic value or marketing under a false label of cultivar/geographical origin (Costa et al., 2012a). In addition to a violation of consumer rights, adulteration with seed oils is a potential health threat, since the adulterant species may be allergenic or in extreme cases, toxic. There is no better example than Spanish Toxic Oil Syndrome (TOS) to highlight the importance of establishing the traceability of edible oils. In 1981, rapeseed oil refined for industrial use was distributed in the local markets of Madrid as olive oil, leading to the outbreak of TOS. The multisystematic disease caused the deaths of over 350 people and resulted in permanent disorders in over 20 thousand affected individuals with more than 10% recorded as handicapped (WHO, 2004).

Because the quality characteristics of virgin olive oils such as color and taste are mainly defined by the olive cultivar from which the oil is extracted, there is consumer preference for oils of certain cultivars, and such products are sold at a price premium (Woolfe and Primrose, 2004). European Council Regulation (EC) No 510/2006 of 20 March 2006 on the protection of geographical indications and designations of origin for agricultural products and foodstuffs defines the labels PDO (Protected Designation of Origin) and PGI (Protected Geographical Indication). Olive oils awarded these labels command even higher market prices. Therefore, reliable methods to authenticate premium monovarietal olive oils are required to protect the consumer from fraud and prevent unfair competition in the olive oil market.

Analytical chemistry methods that analyze the fat phase constituents are conventionally used for investigating the authenticity of olive oils. Using mathematical models, the values measured in samples are compared to those of established standards to judge the oil's genuineness. The most commonly utilized analytical approach is analysis of the fatty acid composition by gas chromatography (GC). Analysis of the intact triacylglycerol composition by reversed-phase high-performance liquid chromatography (HPLC) is also proposed as a complementary approach to fatty acid profiling by GC. The analysis of minor constituents in the unsaponifiable fraction (e.g. tocopherols, sterols, hydrocarbons) by chromatographic methods is also used to complement fatty acid analysis (Ulberth and Buchgraber, 2000). Spectroscopic methods that require less complicated sample preparation steps compared to labor intensive chromatography analyses are also gaining popularity as analytical chemistry methods to determine edible oil authenticity (Costa et al., 2012a).

All of the above mentioned analytical approaches are either based on chromatographic or spectroscopic methods and rely on the assumption that the chemical composition of a blend will significantly deviate from that of olive oil due to the presence of a dramatic difference in the quantity of the analyte between olive oil and the adulterant oil species. However, this assumption does not hold especially in case of minor oil constituents (Ulberth and Buchgraber, 2000). In addition, the composition of the abundant oil constituents (fat fraction) may also be statistically indistinguishable, as it is the case for hazelnut oil and olive oil, which cannot be discriminated based on analytical chemistry approaches (Woolfe and Primrose, 2004). Moreover, the introduction of new oil seed varieties with desirable oil composition traits (e.g. high oleic acid content) through breeding or genetic modifications unquestionably stands as a challenge for the accurate assessment of the botanical origin of an oil sample. It is also important to note that fraudulent manufacturers are well aware of the chemical criteria used for authenticity assessment and take those into consideration for preparing blends that meet product specifications.

While the conventional authenticity assessment methods are relatively more efficient in identifying the botanical origin, they are not as successful in identifying the varietal composition of an olive oil sample as they are in identifying admixtures with seed oils (Costa et al., 2012a). This is mainly due to the fact that metabolite composition of a given variety's oil is highly affected by environmental conditions (Aguilera et al., 2005; Gomez-Rico et al., 2007; Erel et al., 2013; Dag et al., 2011), as well as

postharvest and postproduction treatments (Ben-Hassine et al., 2013). A promising approach for eliminating environmentally induced compositional variation is the recently emerging concept of food genomics (Agrimonti et al., 2011).

1.3. Food genomics

Food genomics relies on the assumption that remnant DNA in a food matrix can be recovered to serve as an analyte in food authenticity analyses. This assumption indeed proved accurate, as evidenced by several successful reports on the development of DNA-based food authenticity tests (Galimberti et al., 2013; Madesis et al., 2014). Because DNA is not subject to environmental influence, it directly reflects the species/varietal composition in a food product. By amplifying the residual DNA in a food matrix to useful quantities for downstream analyses, PCR-based techniques made an essential contribution to the newly emerging research field. All genetic analysis platforms can be utilized in food genomic analyses. Target DNA regions isolated from a food sample by PCR can be sequenced or analyzed by regular agarose gel electrophoresis or high-resolution capillary electrophoresis (CE) systems, depending on the research needs. Techniques that involve simultaneous PCR amplification and genotyping, such as quantitative real time-PCR or high resolution melting analysis (HRM) can be coupled to PCR amplifications.

DNA analysis made it feasible to detect trace amounts of allergen nuts in processed foods (Pafundo et al., 2009; Costa et al., 2012b; Madesis et al., 2013). Because nut species can trigger allergic reactions in susceptible individuals even when they are present in a food sample in trace amounts, sensitivity is a crucial factor in their detection. In this aspect, DNA analysis represents the most sensitive method in allergen detection, as the quantity of remnant DNA increases exponentially during PCR amplification, allowing the detection of minor constituents in a sample which are otherwise undetectable with analytical chemistry approaches. PCR-based methods were also adopted by food microbiologists, as they proved superior over culture-based methods in terms of sensitivity and specificity in detecting sub-dominant microbial populations in food samples (Postollec et al., 2011). Thus, food genomics offers the most powerful techniques to resolve food safety issues.

During the last fifteen years, significant progress was made in botanical origin detection in processed foods by DNA analyses. For example, substitution or admixing of high quality Arabica (*Coffea arabica*) coffee beans with the less economically valuable coffee species, *Coffea robusta*, could successfully be detected by a simple, SNP (Single Nucleotide Polymorphism) based restriction digestion assay (Spaniolas et al., 2006) that targets the mitochondrial genome. Detection of soft wheat (*Triticum aestivum* L.) adulteration in durum wheat (*Triticum turgidum* L.) semolina was achieved through a real time-PCR approach that detected the amplification of a soft wheat D-genome specific DNA region (Pasqualone et al., 2007a; Sonnante et al., 2009). Similarly, a soft wheat specific quantitative PCR approach and a SNP-based RFLP (Restriction Fragment Length Polymorphism) assay proved successful in identifying soft wheat (*Triticum aestivum* ssp. *vulgare*) adulteration in spelt (*Triticum aestivum* ssp. *spelta*) flour (Mayer et al., 2012). Species composition in fruit juices could successfully be identified by HRM analysis of the plastid *trn*L (UAA) intron barcode (Faria et al., 2013). Sequence Characterized Amplified Region (SCAR) markers that specifically amplify PCR products from the most commonly encountered adulterants in saffron (*Crocus sativus*) were introduced as a fast and cost-efficient alternative to analytical chemistry approaches for the detection of fraud in this high economic-value spice species (Marieschi et al., 2012). In other interesting work, the potential of a ribosomal DNA barcode sequencing approach to identify the floral composition in honey was demonstrated (Olivieri et al., 2012).

In contrast to botanical origin authentication approaches which commonly take advantage of interspecific polymorphisms in conserved DNA sequences, varietal discrimination requires intraspecific variability which is often absent in universal plant barcodes. The hypervariable nature of Simple Sequence Repeats (SSRs) and the high abundance of Single Nucleotide Polymorphisms (SNPs) in eukaryotic genomes make them the markers of choice to establish the provenance of agro-food products (Pasqualone et al., 2007b; Bazakos et al., 2012). Wine and olive oil are on top of the list of high economic value agro-food products, for which consumer perception favors products of certain cultivars. Therefore, quality grades and market prices are predominantly defined by the provenance of such products. SSR markers were proposed to provide the required discrimination efficiency for cultivar origin authentication in grape must by several authors (Siret et al., 2000, 2002; Baleiras-Couto and Eiras-Dias, 2006; Savazzini and Martinelli, 2006). In a more recent study, the performance of SSRs

and Inter Simple Sequence Repeats (ISSRs) was compared (Pereira et al., 2012). Results of the work confirmed the usefulness of SSRs in terms of cultivar discrimination efficiency and reproducibility and identified ISSRs as a marker system of poor reproducibility when applied on DNA isolated from grape must matrices.

Food genomic approaches used for adulteration detection and cultivar origin authentication in olive oil are discussed in the following section.

1.3.1. Food genomics to establish the authenticity of olive oil

The plastid *trnL* (UAA) intron represents a remarkably useful DNA barcode for species discrimination in agro-food products. Primers that amplify the universal barcode region are available since 1991 (Taberlet et al., 1991) and the universality of the *trnL* (UAA) intron was demonstrated on a diverse range of plant taxa (Taberlet et al., 1991, 2007; Gielly and Taberlet, 1994). Apart from the high copy number of the plastid genome that compensates for the low concentrations of intact DNA in processed food matrices, interspecific length polymorphisms make the *trnL* (UAA) intron an ideal barcode for identifying species composition in food samples based on amplified fragment length polymorphisms. The *trnL* (UAA) intron proved potentially useful for identifying the botanical origin in plant oils by simple and fast CE-based methodologies (Spaniolas et al., 2008, 2010). However, it remains to be determined whether *trnL* (UAA) intron length polymorphisms allow detection of the presence of minor quantities of seed oil in olive oil-seed oil admixtures. Another universal plastid barcode, the *rbcL* gene, was also proposed as an analyte DNA sequence to identify the presence of seed oils in olive oil. Zhang et al. (2012) used a CE approach to discriminate the botanical origin of plant oils using two fragments from the *rbcL* gene. Ganopoulos et al. (2013a) used HRM analysis as the genotyping strategy in order to demonstrate the potential of a *rbcL* fragment to identify canola (*Brassica napus*) oil adulteration in olive oil (Ganopoulos et al., 2013a).

During the past decade, several authors reported the use of DNA markers for the authentication of the varietal origin of olive oils. The most frequently used DNA markers in such work are SSRs (simple sequence repeats) (Breton et al., 2004; Testolin et al., 2005; Pasqualone et al., 2007b, 2012; Martins-Lopes et al., 2008; Perez-Jimenez et al., 2013; Raieta et al., 2014) because degraded DNA recovered from an oil sample

still allows the amplification of short PCR targets sufficient for SSR analysis (Bazakos et al., 2012). However, the most recent generation of DNA markers, SNPs, can be used for genotyping with even shorter DNA fragments than SSRs. In addition, the high frequency of SNPs in both coding and noncoding parts of the genome allows the differentiation of even closely related genotypes (Khletskina and Salina, 2006; Costa et al., 2012). For example, Ganopoulos et al. (2013b) were successful in discriminating 21 Greek sweet cherry cultivars by genotyping nine genic SNP loci. Moreover, a sufficient number of SNPs for varietal origin detection can be identified by analyzing relatively short DNA sequences. Reale et al. (2006) did the first work describing SNP marker development for olive cultivar identification and identified 22 SNPs with only 3440 bp (base pair) of olive genic sequence. Thus, they observed a high frequency of SNP occurrence (1 SNP per 156 bp). Using a total of 11 markers, 8 of which were SNP derived, Reale et al. (2006) discriminated 47 olive accessions. Following Reale et al. (2006), Consolandi et al. (2007) used 17 SNP loci to differentiate 49 olive varieties that are commonly cultivated in the Mediterranean region and developed a SNP-based ligation detection assay for varietal origin detection. The efficiency of the SNP-based ligation detection assay was displayed on eight monovarietal olive oils from Italy, Spain, France, and Portugal (Consolandi et al., 2008). Restriction profile-based genotyping, either referred to as CAPS or PCR-RFLP (PCR-restriction fragment length polymorphism), has been successfully used in a number of DNA-based food authentication studies. Dooley et al. (2005) were able to discriminate ten white fish species using three PCR-RFLP assays that target a 464 bp region of the *Cytochrome b* gene. In addition, the reproducibility of the assays was displayed by an interlaboratory study that involved five different food control laboratories in the United Kingdom. Spaniolas et al. (2006) described a single SNP-based PCR-RFLP assay that allows the discrimination of Arabica and Robusta coffee samples. Bazakos et al. (2012) were first to display the effectiveness of a PCR-RFLP (Restriction Fragment Length Polymorphism) approach to identify the varietal origin of olive oils based on their SNP alleles. The authors were able to discriminate eight olive varieties using five SNPs and showed the usefulness of this approach on five Greek monovarietal olive oils.

1.4. Aim of the study

Food genomic approaches are increasingly being used to resolve the traceability issues of agro-food products due to their improved reliability compared to conventional analytical chemistry analyses. Because olive oil is an attractive target for fraud, it is essential that robust DNA-based diagnostic tests are developed to ensure the authenticity and traceability of high economic value monovarietal olive oils. Such approaches have not yet been employed to authenticate premium Turkish olive oil products.

The aim of this work was to develop and standardize DNA-based tests for detection of adulteration in olive oil and establishing the traceability of cultivar origin in Turkish premium monovarietal olive oils. Utilization of a universal DNA barcode that allows polymorphism detection among a wide range of plant species would be an optimal choice for detection of adulteration with lower-economic-value plant oils in olive oil. In addition, the sequence of choice should preferentially be present in multiple copies in a plant cell, in order to enable robust amplifications from processed food products. The plastid genome meets these requirements, due to its high copy number in plant cells and the presence of highly conserved plastid DNA regions. Therefore, a universal non-coding plastid barcode sequence was utilized and evaluated in this work, for the development of a DNA-based test that detects adulteration in olive oil. Moreover, the performance of the DNA-based test was evaluated in comparison with that of the widely accepted standard analytical chemistry method.

Another objective of this study was to establish a DNA-based identification key that allows ascertaining the cultivar origin of Turkish monovarietal olive oils. In order to reach this objective, SNP loci polymorphic among the most commonly cultivated Turkish olive varieties were identified by sequencing fragments from olive genes. Genotyping assays that are applicable on DNA from processed foods were developed for SNP loci selected according to their discrimination efficiency. The efficiency of the SNP-based assays to identify cultivar origin was tested on Turkish monovarietal olive oils. In addition, the performance of the assays to detect olive oil admixtures was evaluated and applicability of the assays on commercial olive oils was demonstrated.

CHAPTER 2

MATERIALS AND METHODS

2.1. Plant material and oil samples

In order to develop a DNA-based test for the authentication of the botanical origin in olive oil, edible oil samples of 11 plant species were included in this work. Out of the 11 edible oil types, two were unrefined (olive and hazelnut oils) and the remaining nine were refined oils (soybean, sesame, sunflower, canola, corn, cotton seed, peanut, safflower and palm oils). The oil samples were purchased from retail stores. Reference olive and cotton leaf samples were obtained from Olive Research Institute (Izmir, Turkey) and Cotton Research Station (Aydin, Turkey), respectively. Reference tissue samples of the remaining nine plant species were obtained from a private seed company (Agromar Seed, Bursa, Turkey).

Leaf and oil samples of 17 commonly cultivated Turkish olive varieties were used in this study in order to develop SNP-based assays for cultivar origin identification in Turkish monovarietal olive oils. Leaf samples of each olive variety were collected from three individual plants. Cultivar names and primary usage information are provided in Table 2.1. Leaf samples and filtered monovarietal oils produced by a laboratory-scale mill were obtained from the Olive Research Institute (Izmir, Turkey). Commercial olive oil samples were obtained from a retail market.

Table 2.1. Olive varieties used in the study.

Olive Variety	Common Usage[1]	Region of Cultivation
Memecik	Oil	Southern Aegean
Gemlik (Trilye)	Table/Oil	Marmara
Ayvalik	Oil	Northern Aegean
Uslu	Oil/Table	Southern Aegean
Erdek Yaglik	Table/Oil	Marmara
Kilis Yaglik	Oil	Southeast Anatolia
Nizip Yaglik	Oil	Southeast Anatolia
Tesbih Celebi	Oil	Southeast Anatolia
Tekirdag Siyah Salamura	Table/Oil	Marmara
Domat	Table/Oil	Aegean
Yun Celebi	Oil/Table	Southeast Anatolia
Cekiste	Table/Oil	Aegean
Mavi	Oil	Southeast Anatolia
Sinop No:5	Oil	Black Sea
Patos	Oil/Table	Black Sea
Maraş No:7	Oil	Southeast Anatolia
Saurani	Oil	Mediterranean

[1]Primary usage is indicated first for the varieties that have both uses.

2.2. DNA isolation

DNA from the leaf tissue of three replicate plants was isolated from 200 mg of liquid nitrogen-frozen ground leaf tissue using a CTAB method (Doyle and Doyle, 1990). For isolating DNA from monovarietal olive oil samples and seed oils, Wizard® Magnetic DNA Purification System for Food (Promega Corp., Madison,WI, USA) was used according to the manufacturer's instructions. The concentration of DNA from leaf and oil samples was measured using a Qubit® 2.0 Fluorometer (Life Technologies, Thermo Fisher Scientific Inc., Waltham, MA, USA) with dsDNA BR Assay Kit (Life Technologies). DNA integrity was checked on a 1.5% agarose gel.

2.3. PCR-Capillary Electrophoresis (PCR-CE) barcode analysis

Primers that amplify the plastid *trnL* (UAA) intron (Primers c & d), were introduced by Taberlet et al. (1991). For simplicity, the plastid barcode is named after

the amplification primers as Plastid-cd (Pl-cd). Barcode region was amplified in 20 µL reaction mixtures containing 1X PCR buffer, 1.5 mM $MgCl_2$, 0.25 mM of each deoxyribonucleotide triphosphate (dNTP) (Promega Corp., Madison, WI), 1U Taq polymerase, 0.25 µM of each primer, and 5 ng template DNA. Thermal cycling conditions consisted of one cycle of initial denaturation for 10 min at 94 °C, followed by 35 cycles (40 cycles for oil DNA) of 94 °C for 30 s, 62 °C for 30 s, 72 °C for 60 s, with a final extension step of 10 min at 72 °C. Barcode amplicons were separated on a Qiaxcel Advanced capillary electrophoresis system (Qiagen, Valencia, CA) using a Qiaxcel DNA High Resolution Kit (Qiagen). QX DNA Size Marker 50−800bp, v2.0 (Qiagen) was used as the size standard, and QX Alignment Marker 15 bp/1000 bp (Qiagen) was used for aligning the size standard fragments. The high resolution run method OM800 was applied with a sample injection time of 10 s. QIAxcel ScreenGel Software (Qiagen) was used for visualization of the barcode profiles. PCR-CE barcode assays were performed in three replicates for each DNA sample.

2.4. Gas chromatography analysis of olive oil : seed oil admixtures

Fatty acid compositions of olive oil : seed oil admixtures were analyzed by gas chromatography (GC) analysis using a GC (Agilent 6890, Agilent Technologies, Santa Clara, USA) equipped with an auto-sampler (Agilent 7863 & FID). Column specifications were as follows: 100 m*0.25 mm ID, 0.2 µm HP-88 (J&W 112-88A7). Analytical conditions applied were: inlet temperature, 250 °C; injection volume, 1 µL; split ratio, 1/50; carrier gas, helium; head pressure, 2 ml / min constant flow; oven temperature, 175 °C, 10 min, 3 °C / min, 220 °C, 5 min; detector temperature, 280 °C; detector gas, hydrogen: 40 ml / min, air: 450 ml / min, helium make-up gas: 30 ml/min.

2.5. Primer design, sequencing and SNP identification

Primers that amplify targets from the *Cycloarthenol synthase* and *Lupeol synthase* genes were designed based on the partial coding sequences Genbank Acc. No. AY847065 and AY847066, respectively (http://www.ncbi.nlm.nih.gov/genbank) (Table 2.2). The primer design process was carried out using Primer3 software (Rozen and Skaletsky, 2000). The primers Cyc6-F and Lup1-F&R were previously reported by

Bazakos et al. (2012) (Table 2.2). Primers that target the *Anthocyanidin synthase* (Ant-F&R), *Calcium binding protein* (Cbp-F&R) and *Chalcone synthase* (Chs-F&R) genes were reported by Consolandi et al. (2008) (Table 2.2).

Table 2.2. List of primers used for SNP identification and sequencing. Unless otherwise indicated, primers were designed in this study.

Primer[1] Name	Sequence (5' to 3')	Product Size	Assay Name	SNP Alleles	Motif[2] (5' to 3')
*Ant-F**	GCCCAGCAACAAGTGAGTATG	274 bp	SNP3-HphI	C/T	GG**T**GA
*Ant-R**	GACCCATTTTCCCTCACAGA				
*Cbp-F**	CAGGAATTCCAAGCCTTCAG-	328 bp	SNP6-EagI	C/G	CGG**C**CG
*Cbp-R**	GCTCGACGGATGAGATCAAT				
*Chs-F**	TCGGAGATTACTGCGGTTACTT	297 bp	SNP2-BsgI	C/T	C**T**GCAC
*Chs-R**	CTAGAGGCTGGAATGCTTCA				
Cyc1-F	TTTGCTCCCAGGTCATTA	165 bp			
Cyc1-R	GGATGCTGTAGCATGACA				
Cyc2-F	TGGTTTGCCTTTTCATTT	168 bp	SNP4-Hpy166II	C/T	GTNNA**C**
Cyc2-R	ACCAAATTCTTCCCCAAG				
Cyc3-F	AGCTTGGGGAAGAATTTGGTC	198 bp			
Cyc3-R	ATATCGCCTGGGCAATCCTC				
Cyc4-F	TCTTTTCTTTAGGCGGTTC	173 bp			
Cyc4-R	AGGTGGAAACATCACAATG				
Cyc5-F	AACAGCAATTGTTGGTGAA	163 bp	SNP5-HindIII	C/T	AAG**C**TT
Cyc5-R	ATCCCTTTCCGAAAACAA				
Cyc6-F**	TTCTGTTGTTTTCGGAAAGG	220 bp			
Cyc6-R	TGGAGCCATCTTTTTCCAAT				
Lup1-F**	GCAACTCAAATGAATGAATC	125 bp			
Lup1-R**	TCTAAGAAACGGACTTGCTAT				
Lup2-F	AGTAGCATACCCTGTGTTTC	204 bp			
Lup2-R	TTGCTTGTTGGGTAGAAGAT				
Lup3-F	ACCCTGTGTTTCGATTTGCT	107 bp	SNP1-HpyCH4III	A/T	ACNG**T**
Lup3-R	ATGCAGGTTATTTCTTTCATCA				

[1] *Primers reported by Consolandi *et al*. (2008); **Primers reported by Bazakos *et al*. (2012). Primers used in CAPS assays are given in italics.
[2] Nucleotides in bold indicate SNP loci in motifs.

Leaf DNA from the 17 olive varieties was used for dye-terminator sequencing reactions. Sequencing targets were amplified in 20 µL reaction mixtures containing 1X PCR buffer, 1.5 mM $MgCl_2$, 0.25 mM of each deoxyribonucleotide triphosphate (dNTP) (Promega Corp.), 1 U Taq polymerase, 0.25 µM of each primer and 5 ng

template DNA. Thermal cycling conditions consisted of one cycle of initial denaturation for 10 min at 94 °C, followed by 35 cycles of 94 °C for 30 sec, 57 °C for 30 sec, 72 °C for 45 sec, with a final extension step of 10 min at 72 °C. For each olive variety, PCR reactions were performed in five replicates and combined during the purification step with the DNA Clean & Concentrator™-5 Kit (Zymo Research, Irvine, CA, USA). Purified PCR products were used as template in dye-terminator sequencing reactions, prepared using GenomeLab DTCS Quick Start Kit (Beckman Coulter, Brea, CA, USA) according to the manufacturer's instructions. Sequencing reaction thermal cycling conditions were 30 cycles of 96 °C 20 sec, 50 °C 20 sec, 60 °C 4 min. The reaction mixture for each amplicon was then purified using ZR DNA Sequencing Clean-up Kit™ (Zymo Research), resuspended in 30 μL of sample loading solution (Beckman Coulter) and run on a Beckman CEQ8800 capillary electrophoresis device using the LFR-c method (injection voltage 2.0 kV for 10-15 sec, separation temperature 60 °C, separation voltage 7.4 kV, separation time 45 min). Sequence peaks were visualized by Beckman CEQ8800 software version 8.0 (Beckman Coulter) and SNP detection was done by visual inspection of sequence peaks from different varieties.

2.6. Design and application of the CAPS assays for SNP genotyping

Sequences obtained by the dye-terminator method were analyzed with the NEBcutter Version 2.0 software (http://tools.neb.com/NEBcutter2/) (New England Biolabs Inc., Ipswich, MA, USA) for the allelic variants of each SNP identified. SNPs with alleles that alter recognition motifs of restriction enzymes were selected for genotyping by a restriction digestion-based analysis.

Fragments that harbor SNP loci were amplified in 20 μL reaction mixtures containing 1X PCR buffer, 1.5 mM $MgCl_2$, 0.25 mM of each deoxyribonucleotide triphosphate (dNTP) (Promega Corp.), 1 U Taq polymerase, 0.25 μM of each primer and 5 ng of olive leaf DNA or 5 ng of oil DNA stock solution as template. Thermal cycling conditions consisted of one cycle of initial denaturation for 10 min at 94 °C, followed by 40 cycles (35 cycles for leaf DNA) of 94 °C for 30 sec, 57 °C for 30 sec, 72 °C for 45 sec, with a final extension step of 10 min at 72 °C. PCR products were directly subjected to restriction digestion without any purification step. Digestion reaction mixtures of the CAPS assays (Table 2.2) contained 20 μL of PCR product, 1

μL of restriction enzyme (New England Biolabs Inc.) and 2.5 μL of CutSmart™ reaction buffer (New England Biolabs Inc.) in a total volume of 25 μL. Reaction mixtures of the enzyme EagI used NEBuffer 3.1 instead of the CutSmart™ buffer and 0.25 μL of S-adenosylmethione was added to the BsgI reaction mixtures. Incubation temperature was constant (37 °C) for all enzymes and incubation time was standardized as six hours. Following the incubation step, digestion fragments were run on a Qiaxcel Advanced capillary electrophoresis system (Qiagen) using a Qiaxcel DNA High Resolution Kit (Qiagen). QX DNA Size Marker 25–500bp, v2.0 (Qiagen) was used as the size standard and QX Alignment Marker 15 bp/600 bp (Qiagen) was used for aligning the size standard fragments. The high resolution run method OM800 was applied with a sample injection time of 20 sec. QIAxcel ScreenGel Software (Qiagen) was used for the visualization of the digestion profiles. The six CAPS assays developed in this work were applied on the oil DNA of each olive variety in three replicates.

2.7. Statistical analysis

The frequency of each SNP allele was determined by calculating the proportion of the occurrence of the allele to the total number of alleles at a given SNP locus. Probability of identity for individual SNP loci and the combined, multilocus probability of identity were calculated according to Paetkau et al. (1995). Allelic error was calculated according to Pasqualone et al. (2013).

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Authentication of botanical origin in olive oil by plastid non-coding DNA length polymorphisms

Because the production quantities of certain seed oils, such as palm, soybean, canola, sunflower, cottonseed and peanut oils, far exceed that of olive oil, they constitute the most easily accessible substitutes to olive oil. Indeed, all types of seed oils are reported as adulterants in olive oil (Agrimonti et al., 2011). In this work, we tested the performance of plastid non-coding DNA length polymorphisms to authenticate the botanical origin of olive oil. The plastid *trnL* (UAA) intron used in this work (Barcode Pl-cd) was reported as a region of significant size variation, when sequences flanked by primers c and d (Taberlet et al., 1991) were compared for 706 plant species corresponding to 366 genera and 116 families (Taberlet et al., 2007). Therefore, the plastid intron represents an optimal analyte DNA region that will enable resolving species composition in a given sample of plant origin.

3.1.1. Determination of barcode DNA length polymorphisms among the eleven plant species

Barcode Pl-cd was amplified using DNA from reference tissue samples as template. Barcode amplicons were initially run on a 2% agarose gel in order to assess the potential of the agarose gel to resolve barcode fragments from different plant species (Figure 3.1). PCR amplicons from all species displayed different mobilities and were all distinguishable on the agarose gel. However, because the main objective of this work was to evaluate the performance of Barcode Pl-cd length polymorphisms to identify adulteration in olive oil, we assessed if the barcode fragment amplified from olive could readily be distinguished from those of the remaining plant species. Problems in resolving *trnL* (UAA) intron barcode amplicons from olive and sesame were reported

previously by Spaniolas et al. (2008). In agreement with Spaniolas et al. (2008), we observed that barcode Pl-cd fragments from olive and sesame were similar in size (Figure 3.1, Lanes 1 & 2), highlighting the requirement for a high-resolution separation system for distinguishing barcode amplicons from the two species in case they co-exist in admixtures.

Figure 3.1. Agarose gel electrophoresis image displaying the Barcode Pl-cd fragments amplified from 11 plant species. L: 100bp ladder, 1: Olive, 2: Sesame, 3: Soybean, 4: Sunflower, 5: Canola, 6: Corn, 7: Hazelnut, 8: Cotton, 9: Peanut, 10: Safflower, 11: Oil Palm.

Therefore, we employed a high-resolution capillary electrophoresis (CE) system to determine barcode amplicon sizes of the reference tissue samples and to test the performance of Barcode Pl-cd length polymorphisms to identify edible oil admixtures. Barcode amplicon sizes of the 11 plant species are provided in Table 3.1. A single fragment amplification pattern was observed for all of the tested species, except for cotton which yielded double bands of 649 and 723 bp. All 11 species displayed distinctive barcode fragments with no two species yielding fragments of identical size. The Barcode Pl-cd amplicon size ranged between 388 (canola) and 723 bp (cotton) among the 11 species. Olive barcode fragment size was 551 bp and the species with the closest barcode amplicon size to olive was sesame, which produced a barcode Pl-cd fragment of 560 bp. Nevertheless, a size difference of 9 bp was far above the minimum

fragment size resolution (2-3 bp) supplied by the CE system and therefore, was reproducibly detected in all replicate CE runs of the reference barcode amplicons.

Table 3.1. Barcode Pl-cd amplicon sizes of plant species analyzed in this study.

Plant species (common name)	Barcode Pl-cd
Olea europaea (olive)	551 bp
Glycine max (soybean)	585 bp
Sesamum indicum (sesame)	560 bp
Helianthus annuus (sunflower)	507 bp
Brassica napus (canola)	388 bp
Zea mays (corn)	527 bp
Gossypium hirsutum (cotton)	649 bp & 723 bp
Corylus avellana (hazelnut)	608 bp
Arachis hypogaea (peanut)	641 bp
Carthamus tinctorius (safflower)	499 bp
Elaeis guineensis (oil palm)	578 bp

3.1.2. Performance of the PCR-CE barcode assay to detect adulteration in olive oil

We tested the performance of the Barcode Pl-cd analysis on a series of olive oil admixtures with ten seed oil species. In order to set a limit of detection for the PCR-CE barcode analysis, we applied the assay on replicate DNA samples from oil admixtures prepared in four different ratios. Olive oil : seed oil admixing ratios were 60 : 40%, 75 : 25%, 90 : 10% and 95 : 5%. DNA was extracted from the admixtures for PCR-CE barcode analysis in three replicates. A DNA concentration of approximately 2 ng/µL was measured by fluorometer for the oil admixtures, which constituted a sufficient quantity of template DNA for further PCR amplifications. Barcode Pl-cd was amplified using DNA from the admixtures as template and barcode fragments were run on the CE system simultaneously with the barcode amplicons from the relevant reference tissue samples.

Olive and sesame reference samples yielded barcode amplicons of 551 bp and 560 bp, respectively (Table 3.1). When olive oil : sesame oil admixtures were subjected

to the PCR-CE barcode analysis, the sesame-specific barcode Pl-cd fragment was reproducibly detected down to a limit of 10% in all three replicate experiments (Figure 3.2). However, the 95 : 5% admixture displayed only the barcode fragment from the major ingredient in the blend (olive oil) and the sesame specific 560 bp Barcode Pl-cd fragment could not be detected in any of the replicate experiments (Figure 3.2).

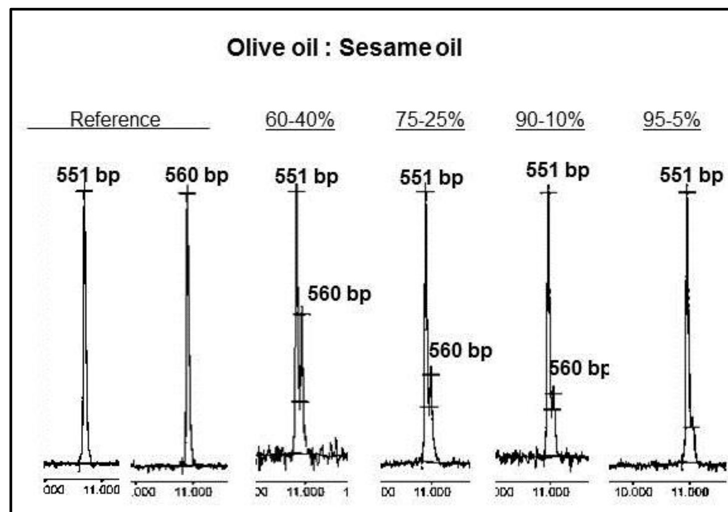

Figure 3.2. Capillary electropherograms displaying the results of the PCR-CE barcode assays applied on a series of Olive oil : Sesame oil admixtures.

The size of the Barcode Pl-cd fragment amplified from reference soybean tissue was 585 bp (Table 3.1). When the PCR-CE barcode assay was tested on olive oil : soybean oil admixtures, both olive and soybean specific barcode fragments were identified in case of all four admixing ratios, resulting in the successful detection of soybean oil in olive oil in all of the tested admixtures (Figure 3.3). The minimum soybean oil admixture ratio of 5% was detectable in all three replicate experiments.

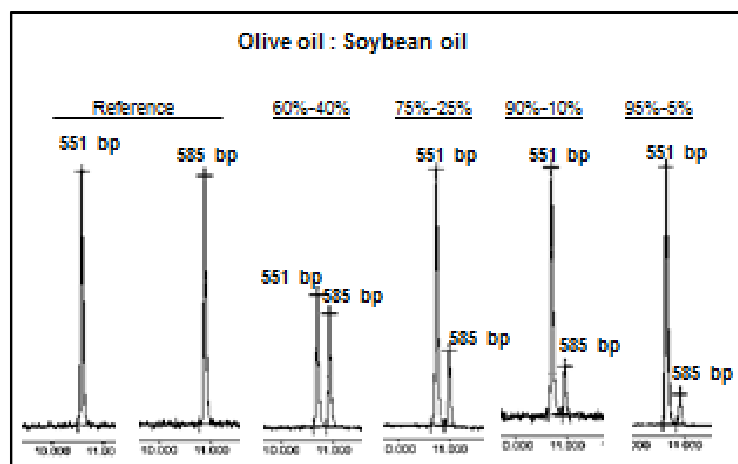

Figure 3.3. Capillary electropherograms displaying the results of the PCR-CE barcode assays applied on a series of Olive oil : Soybean oil admixtures.

Barcode Pl-cd amplicon size of sunflower was detected as 507 bp (Table 3.1). PCR-CE barcode analysis of olive oil : sunflower oil admixtures resulted in the successful identification of both olive and sunflower-specific barcode fragments for the 60 : 40%, 75 : 25% and 90 : 10% admixtures (Figure 3.4). Similar results were obtained in all three replicate experiments, except for the 95 : 5% admixture for which the sunflower-specific barcode amplicon was detectable only once out of the three replicate analyses. Otherwise, as seen in Figure 3.4, the 95 : 5% admixture displayed only the olive-specific 551 bp barcode fragment, thus, a 5% sunflower oil contribution in the admixture could not be detected.

The canola reference sample produced a Barcode Pl-cd amplicon of 388bp (Table 3.1). Both olive and canola-specific barcode fragments were readily visible in the barcode electropherograms of their oils' admixtures (Figure 3.5). As a result, the series of olive oil : canola oil blends were all correctly identified as the admixture of the oils of the two species by the PCR-CE barcode analysis.

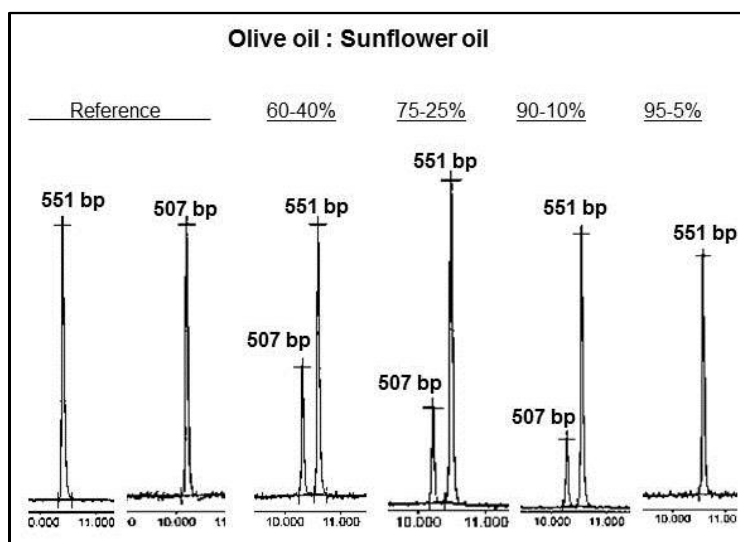

Figure 3.4. Capillary electropherograms displaying the results of the PCR-CE barcode assays applied on a series of Olive oil : Sunflower oil admixtures.

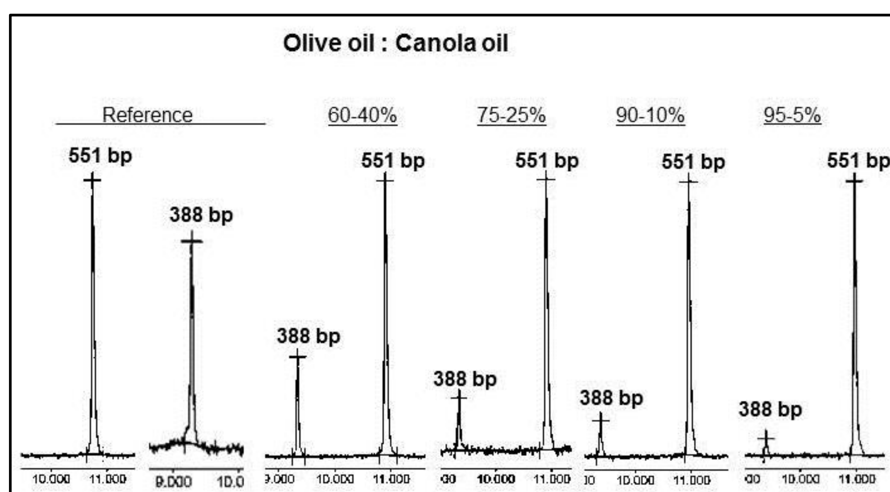

Figure 3.5. Capillary electropherograms displaying the results of the PCR-CE barcode assays applied on a series of Olive oil : Canola oil admixtures.

The Barcode Pl-cd fragment size of the reference corn tissue sample was 527 bp (Table 3.1). The corn-specific fragment was reproducibly detected alongside the olive-specific 551 bp amplicon in the electropherograms of all four olive oil : corn oil blends (Figure 3.6), enabling the identification of admixing with corn oil down to a limit of 5%.

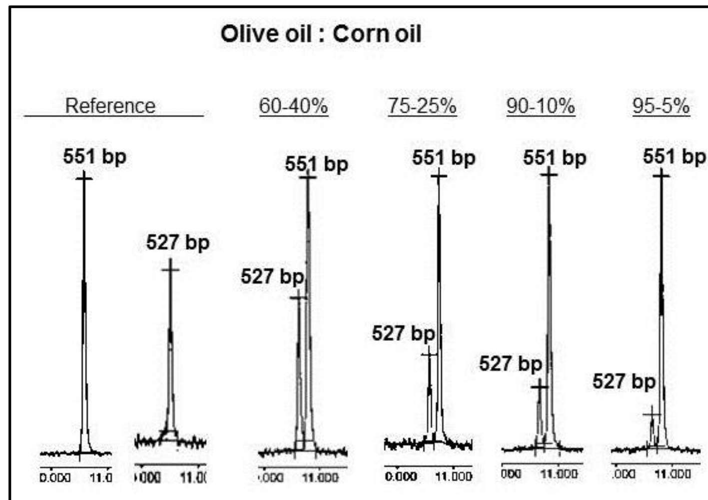

Figure 3.6. Capillary electropherograms displaying the results of the PCR-CE barcode assays applied on a series of Olive oil : Corn oil admixtures.

Hazelnut reference tissue displayed a Barcode Pl-cd size of 608 bp (Table 3.1). PCR-CE barcode assay reproducibly detected both olive and hazelnut specific barcode fragments in all four admixtures (Figure 3.7), thereby correctly identifying olive oil : hazelnut oil admixing down to a limit of 5%.

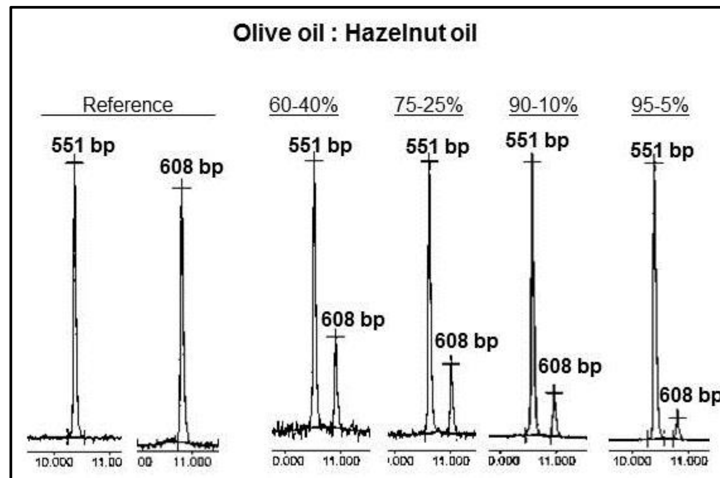

Figure 3.7. Capillary electropherograms displaying the results of the PCR-CE barcode assays applied on a series of Olive oil : Hazelnut oil admixtures.

Cotton was distinguished from the rest of the plant species included in this study with a two-fragment Barcode Pl-cd profile (Table 3.1). The two cotton-specific amplicons of 649 and 723 bp were successfully detected in olive oil : cottonseed oil admixtures down to a limit of 10%. We observed only the olive-specific 551 bp amplicon for the 95 : 5% admixing ratio for two out of the three replicate experiments (Figure 3.8), therefore a 5% cottonseed oil contribution could be detected only once in three parallel experiments.

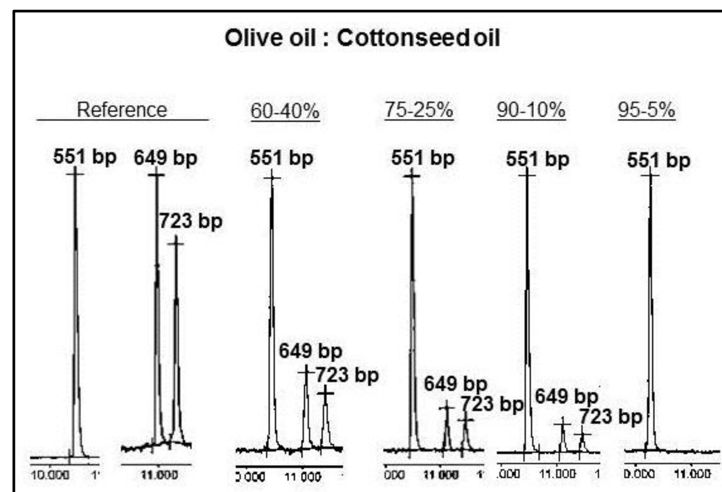

Figure 3.8. Capillary electropherograms displaying the results of the PCR-CE barcode assays applied on a series of Olive oil : Cottonseed oil admixtures.

Peanut reference tissue produced a Barcode Pl-cd amplicon of 641 bp (Table 3.1). When the PCR-CE assay was tested on a series of olive oil : peanut oil admixtures, both species-specific fragments were detectable for all four admixing ratios (Figure 3.9). As a result, the PCR-CE barcode assay was efficient in identifying peanut oil in olive oil down to a limit of 5%.

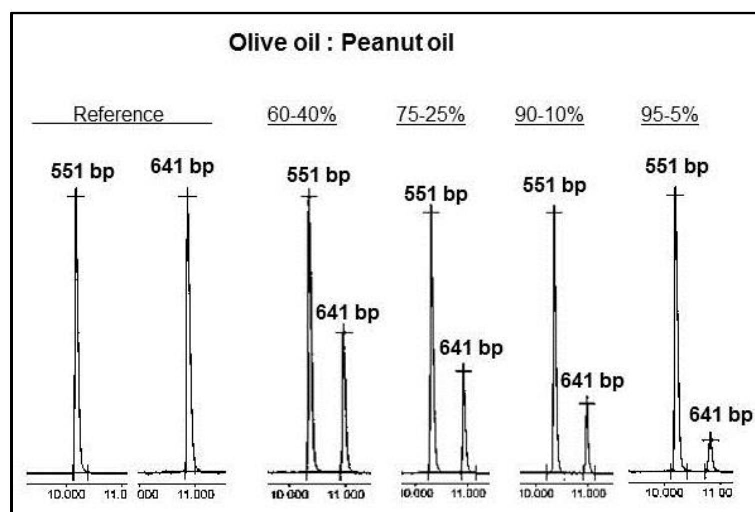

Figure 3.9. Capillary electropherograms displaying the results of the PCR-CE barcode assays applied on a series of Olive oil : Peanut oil admixtures.

Barcode Pl-cd fragment size of the safflower reference sample was 499 bp (Table 3.1). Testing the PCR-CE assay on olive oil : safflower oil admixtures proved successful in detecting both olive and safflower-specific barcode amplicons in the admixtures down to a limit of 10% (Figure 3.10). Because the 95 : 5% admixture could be correctly identified only once out of the three replicate experiments, we consider a 5% safflower oil contribution in an olive oil sample as below the limit of detection of the PCR-CE barcode analysis.

Oil palm reference tissue displayed a Barcode Pl-cd amplicon size of 578 bp (Table 3.1). When the series of olive oil : palm oil admixtures were analyzed with the Barcode Pl-cd assay, all four samples were correctly identified as admixtures of olive and palm oil (Figure 3.11). In addition, it was feasible to consistently detect the 95 : 5% admixture as a blend. Thus, the Barcode Pl-cd was efficient in detecting palm oil in olive oil down to a limit of 5%.

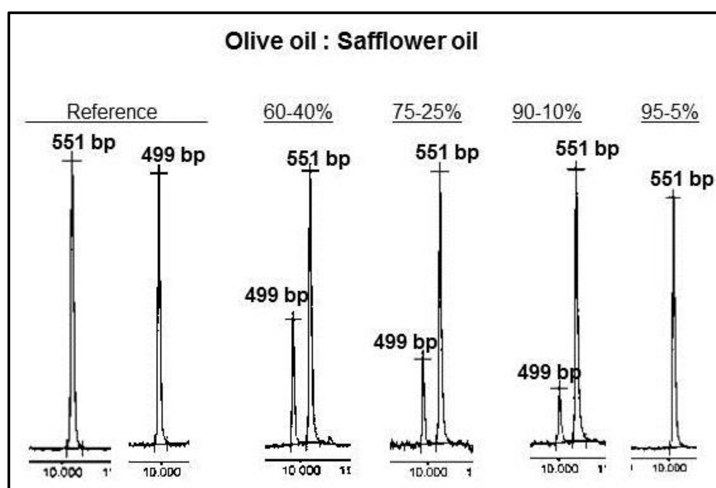

Figure 3.10. Capillary electropherograms displaying the results of the PCR-CE barcode assays applied on a series of Olive oil : Safflower oil admixtures.

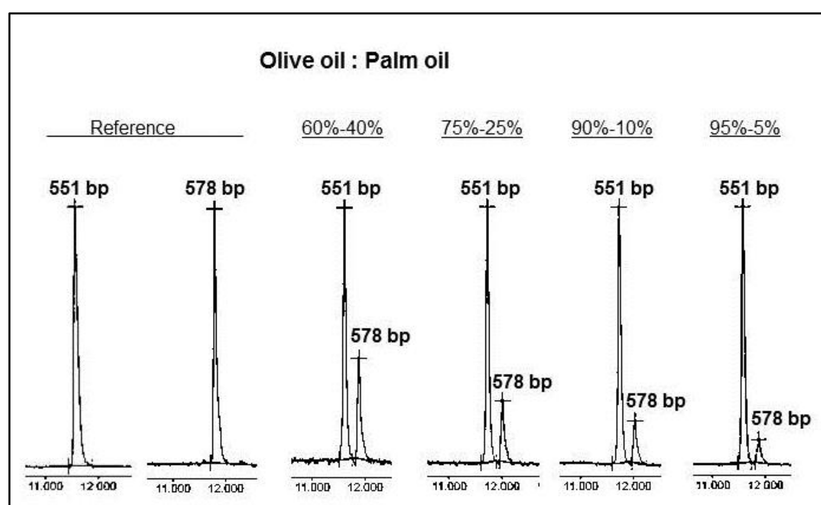

Figure 3.11. Capillary electropherograms displaying the results of the PCR-CE barcode assays applied on a series of Olive oil : Palm oil admixtures.

To summarize, the PCR-CE barcode assay proved successful in reproducibly detecting a 5% contribution of soybean, canola, corn, hazelnut, peanut and palm oils in olive oil (Figures 3.3, 3.5-3.7, 3.9 and 3.11). However, for admixtures with sesame, sunflower, cotton and safflower oils, the minimum ratio of admixing that could be consistently detected was 10% (Figures 3.2, 3.4, 3.8 and 3.10). Because we set reproducibility as the criterion to determine the sensitivity of the PCR-CE barcode assay, the limit of the assay to confidently detect adulteration in olive oil was set as

10%. Zhang et al. (2012) also reported a sensitivity threshold of 10% for their CE-SSCP (Capillary Electrophoresis Single Strand Conformation Polymorphism) assay combination to detect admixing in plant oils. However, the authors did not consider reproducibility across replicate experiments and different types of oil admixtures while setting a limit of admixture detection for the assays they proposed. For all ten series of olive oil : plant oil admixtures, the sizes of the Barcode Pl-cd amplicon peaks from the minor constituents gradually declined in parallel with the decrease in the ratio of the relevant species' oil in the admixtures (Figures 3.1-3.10), as expected. The rafination process is generally considered to significantly reduce the quantity of intact DNA in the end product (Costa et al., 2012). However, similar fluorometric DNA concentration measurements were obtained for both unrefined and refined oil samples. In addition, we did not observe a correlation between barcode amplification efficiency and processing state of the oils under study (Figure 3.1). Moreover, relative barcode amplicon quantities from the five refined oils (soybean, canola, corn, peanut and palm) were sufficient to reproducibly detect the presence of the relevant species even in the 95 : 5% (olive oil : seed oil) admixtures. This result can be attributed to the high copy number of the plastid genome in plant cells, which makes it an ideal target for use in food genomic analyses.

Gas chromatography analysis of fatty acid composition is a widely accepted standard assay to determine the authenticity of plant oils. Therefore, the ten series of olive oil : seed oil admixtures were subjected to GC analysis, in order to evaluate the performance of the PCR-CE barcode assay comparative to the standard method. The performance of both methodologies to detect plant oil admixtures is summarized in Table 3.2.

As shown in Table 3.2, the PCR-CE barcode assay and GC analysis of the fatty acid profile were equally efficient in identifying soybean, canola and palm oils in olive oil down to a limit of 5% (Table 3.2). However, while our DNA-based assay failed to reproducibly identify a 5% contribution of sesame, cottonseed and sunflower oils in olive oil, GC analysis correctly detected admixing in these samples (Table 3.2). In the case of olive oil : safflower oil admixtures, GC analysis failed to identify admixing when the ratio of safflower oil in the admixture was as low as 10 and 5%. Whereas, the PCR-CE assay enabled the correct identification of the sample with 10% of safflower oil contribution as an admixture (Table 3.2).

Table 3.2. Performance of the PCR-CE barcode assay and GC analysis to detect olive oil : seed oil admixtures.

Type of admixture	Analysis method	(60 : 40%)	(75 : 25%)	(90 : 10%)	(95 : 5%)
Olive oil : sesame oil	GC	D	D	D	D
	PCR-CE	D	D	D	ND
Olive oil : Hazelnut oil	GC	ND	ND	ND	ND
	PCR-CE	D	D	D	D
Olive oil : soybean oil	GC	D	D	D	D
	PCR-CE	D	D	D	D
Olive oil : cottonseed oil	GC	D	D	D	D
	PCR-CE	D	D	D	ND
Olive oil : Safflower oil	GC	D	D	ND	ND
	PCR-CE	D	D	D	ND
Olive oil : Peanut oil	GC	D	D	ND	ND
	PCR-CE	D	D	D	D
Olive oil : Canola oil	GC	D	D	D	D
	PCR-CE	D	D	D	D
Olive oil : Sunflower oil	GC	D	D	D	D
	PCR-CE	D	D	D	ND
Olive oil : corn oil	GC	D	D	D	ND
	PCR-CE	D	D	D	D
Olive oil : palm oil	GC	D	D	D	D
	PCR-CE	D	D	D	D

D: Admixture was detected; ND: Admixture was identified as pure olive oil.

Similarly, 90 : 10% and 95 : 5% olive oil : peanut oil admixtures were identified as pure olive oil according to their fatty acid profiles obtained by GC analysis. However, PCR-CE barcode assay proved successful in identifying all four samples as olive oil : peanut oil admixtures (Table 3.2). The DNA-based assay also proved superior over GC analysis in identifying a 5% contribution of corn oil in olive oil (Table 3.2). Most striking results were obtained from the analysis of olive oil : hazelnut oil admixtures. GC analysis failed to identify adulteration for all of the four admixing ratios. This was indeed an expected result, as hazelnut oil and olive oil are reported to display indistinguishable fatty acid compositions (Woolfe and Primrose, 2004). In contrast to GC analysis, PCR-CE barcode assay was successful in detecting hazelnut oil contribution in olive oil for the entire series of olive oil : hazelnut oil admixtures. Thus, the PCR-CE barcode analysis proved superior over the widely accepted analytical approach in identifying an otherwise undetectable plant oil species in olive oil. Indeed,

the ability to identify trace amounts of a nut oil is extremely important in edible oil authenticity analyses because ingestion of nut derived products can trigger serious allergic reactions in susceptible individuals. Therefore, the DNA-based assay proposed in this work complements an important shortcoming of analytical chemistry approaches. Overall, the PCR-CE barcode assay correctly identified 90% (36 out of 40 samples) of the samples as olive oil : seed oil admixtures, whereas the success rate of the GC analysis to detect botanical origin was 77.5% (31 out of 40 samples). Thus, comparative evaluation of GC and DNA barcode analyses showed that DNA-based tests are more efficient than the conventional analytical chemistry approaches in authenticating the botanical origin of edible oils. Moreover, a food genomic assay can detect the presence of an adulterant with a highly similar chemical composition with the adulterated oil species. Thus, complementing the widely used analytical methods with DNA-based tests will unquestionably improve the precision and reliability of edible oil authenticity analysis protocols.

While utilization of the barcode assay was demonstrated on the basis of detecting adulteration in olive oil, the plastid barcode can be employed to investigate the authenticity of all types of plant oils. By maintaining the DNA samples of common oil crops as standard references, plant oil samples of unknown identity can be analyzed in comparison with reference plastid barcode profiles, and can be attributed to their correct botanical origins.

3.2. Cultivar Origin and Admixture Detection in Turkish Olive Oils by SNP-based CAPS Assays

As an agro-food product with great economic value, virgin olive oil is an attractive target for adulteration and mislabeling. While olive oils with mislabeled cultivar origin do not constitute a health threat, traceability protocols to verify the authenticity of genuine products should be established to protect basic consumer rights. Several researchers reported that the chemical composition of oil extracted from the same olive variety is highly affected by climatic conditions (Aguilera et al., 2005), agricultural practices (Gomez-Rico et al., 2007; Erel et al., 2013), time of harvest (Dag et al., 2011), extraction procedure and packaging and storage conditions (Ben-Hassine et al., 2013). In contrast, DNA-based assays are not influenced by compositional variations

due to external effects. Therefore, either alone (Bazakos et al., 2012) or in combination with analytical chemistry methods (Pasqualone et al., 2012), DNA-based assays are increasingly used to authenticate monovarietal olive oils. We used SNP markers in our study as their abundance in the genome makes them ideal for identifying differences between closely related genotypes (Costa et al., 2012).

3.2.1. SNP identification by sequencing

Five olive genes, *Anthocyanidin synthase* (*Ant*), *Calcium binding protein* (*Cbp*), *Chalcone synthase* (*Chs*), *Cycloarthenol synthase* (*Cyc*) and *Lupeol synthase* (*Lup*) were targeted for SNP identification by sequencing. Leaf genomic DNA from the 17 olive varieties (Table 2.1) was used as template for the dye-terminator sequencing reactions. Total lengths of the sequencing targets from *Cyc, Lup*, *Cbp, Chs* and *Ant* genes were 1087 (six amplicons), 436 (three amplicons), 328 (one amplicon), 297 (one amplicon) and 274 bp (one amplicon), respectively (Table 2.2). Sequencing of these 12 PCR amplicons that spanned 2422 bp of coding sequence resulted in the identification of 17 SNPs, corresponding to a frequency of 1 SNP per 142.5 bp of coding sequence. Among these SNPs, five, one, two, six and three SNPs were identified in the *Ant*, *Cbp*, *Chs*, *Cyc synthase* and *Lup* sequences, respectively. The SNP frequency we identified in olive coding sequences (1 SNP per 142.5 bp) was similar to the results of Reale et al. (2006) who reported a frequency of 1 SNP per 156 bp in the *Cycloarthenol synthase*, *Lupeol synthase* and *Cu-Zn-superoxide dismutase* genic sequences. The high frequency of single nucleotide polymorphisms identified in olive coding sequences suggests that olive germplasm harbors a high level of genotypic diversity. Indeed, Belaj et al. (2012) reported high genetic diversity in both local olive germplasm and collections of cultivars representing different cultivation regions. Despite clonal propagation, a high genetic variability is not unexpected in olive because *Olea europaea* L. is an outcrossing species and interfertile with oleasters (Belaj et al., 2007; 2012).

3.2.2. Design of CAPS assays for SNP genotyping

Restriction digestion-based SNP genotyping is a highly reproducible strategy, producing results that are not prone to variable interpretation in different hands. Very

high reproducibility is due to the fact that genotyping is based on observing digestion patterns and does not involve any fluorescence signal intensity measurements or fragment size comparisons. In addition, there is no requirement for SNP-specific probe design or sophisticated equipment (Spaniolas et al., 2014). Regular PCR primers are sufficient for the application of SNP-based CAPS assays. Restriction enzymes recognizing a wide variety of sequence motifs are available, making restriction digestion-based SNP genotyping a highly feasible strategy.

In order to develop a restriction digestion-based SNP genotyping strategy, sequences obtained from the five olive genes by dye-terminator method were analyzed to identify SNPs that reside in restriction enzyme recognition motifs. In the analysis, allelic variants were taken into account for each SNP, so that restriction enzymes with allele-dependent digestion patterns could be identified. As a result, allelic variants of 12 of the 17 SNP loci (71%) were found to alter restriction enzyme recognition motifs. CAPS assays were designed for each SNP and applied on leaf DNA from three replicate plants for SNP verification. Out of the 12 CAPS assays, a subset of six was sufficient to provide the discrimination power to distinguish 17 olive varieties. As a result, those six assays (Table 2.2) were used to establish an identification key for the monovarietal olive oils (Figure 3.12). Among the six SNPs used in the identification key, three were reported in previous work. SNP2, identified in the *Chalcone synthase* gene was reported by Consolandi et al (2007). SNP5, identified in the *Cycloarthenol synthase* gene was also reported by Consolandi et al. (2007) and Bazakos et al. (2012). In addition, we found that the SNP in the *Calcium binding protein* gene first described by Reale et al. (2006), was also polymorphic in Turkish germplasm and is referred to as SNP6 in this study. These results demonstrate that SNPs in olive coding sequences are conserved across the geography of olive distribution. Therefore, the SNPs identified in this work can be utilized for estimating ancestral alleles via phylogenetic analysis.

Figure 3.12. Identification key displaying the discrimination of Turkish olive cultivars according to SNP-based CAPS assays.

According to long-term observation of agro-morphological traits, two groups of varieties included in this work were suggested as synonyms by the experts of the Olive Research Institute (Izmir, Turkey). The first group of putative synonyms consisted of Gemlik, Erdek Yaglik and Tekirdag Siyah Salamura, while the second group included Kilis Yaglik and Tesbih Celebi (personal communication). These varieties were included in our analysis in order to investigate if the DNA-based assay produced false positive results by identifying clones as distinct varieties. As a result, we found that the putative synonyms carried identical alleles for the 17 SNP loci identified in five genes and they were grouped as indistinguishable varieties in the identification key (Figure 3.12). This result can be attributed to the fact that the SNP loci identified in this work reside in coding sequences. However, performing a detailed molecular genetic characterization involving markers from both coding and non-coding regions may result in distinguishing the putatively synonymous varieties.

SNP1, identified in the *Lup* gene, had A and T variants. Based on SNP1 alleles, the olive varieties were separated into three groups (Table 3.3). A total of five varieties were homozygous for the A allele, ten varieties were heterozygous carrying both A and T alleles, and two varieties were homozygous for the T allele. The T allele of SNP1 resulted in an intact HpyCH4III recognition motif, while the A allele disrupted the motif. As a result, genotypes homozygous for the T allele were distinguished by a homozygous digestion pattern with two restriction fragments, whereas genotypes homozygous for the A allele produced a single undigested fragment. Heterozygous genotypes yielded a three fragment digestion pattern with two digestion fragments and the undigested amplicon (Figures 3.13-3.15). SNP2, identified in the *Chs* sequence separated the olive varieties into two groups. While 13 homozygous genotypes carried only the C allele, the remaining four genotypes were heterozygous and carried C and T alleles (Table 3.3). The C allele disrupted the recognition site for the restriction enzyme BsgI, leading to a single undigested fragment for homozygous genotypes. In contrast, heterozygous genotypes carried one copy of the intact BsgI restriction site and were distinguished by a three fragment digestion pattern in the capillary electropherograms (Figures 3.13-3.15).

Table 3.3. Genotypes of the 17 olive varieties for the SNPs used for constructing the cultivar origin identification key.

Olive Variety[1]	**SNP1-HpyCH4III**	**SNP2-BsgI**	**SNP3-HphI**	**SNP4-Hpy166II**	**SNP5-HindIII**	**SNP6-EagI**
Memecik	TA	CC	CC	TT	CC	GG
Gemlik/Trilye	TA	CC	CT	TC	CC	GG
Ayvalik	AA	CT	CT	TC	CC	GG
Uslu	TT	CC	CT	TT	CT	GG
Erdek Yaglik	TA	CC	CT	TC	CC	GG
Kilis Yaglik	TA	CC	CC	TT	CT	GC
Nizip Yaglik	AA	CT	CC	TT	CC	GG
Tesbih Celebi	TA	CC	CC	TT	CT	GC
Tekirdag SS*	TA	CC	CT	TC	CC	GG
Domat	TA	CC	CT	TT	CT	GG
Yun Celebi	AA	CC	CT	TT	CC	GC
Cekiste	AA	CC	CC	TT	CC	GG
Mavi	TA	CT	CT	TT	CT	GC
Sinop No:5	TT	CC	CC	TT	CT	GC
Patos	TA	CC	CT	TC	CT	GG
Maraş No:7	TA	CC	CC	TT	CT	GG
Saurani	AA	CT	CT	TT	CC	GG

[1] *Variety name Tekirdag Siyah Salamura is abbreviated as Tekirdag SS.

Table 3.4. Allele frequency, informativeness and reproducibility of the SNP markers, expressed as Probability of Identity and Allelic Error, respectively.

SNP Locus	**AF**[1]		**PI**[2]	**AE**[3]
	T	**A**		
SNP1	0.41	0.59	0.383	0.000
	C	**T**		
SNP2	0.88	0.12	0.649	0.000
	C	**T**		
SNP3	0.71	0.29	0.428	0.000
	C	**T**		
SNP4	0.15	0.85	0.593	0.000
	C	**T**		
SNP5	0.76	0.24	0.475	0.000
	G	**C**		
SNP6	0.85	0.15	0.593	0.000
Multilocus PI			0.018	

[1]AF, Allele Frequency; [2]PI, Probability of Identity; [3]AE, Allelic Error

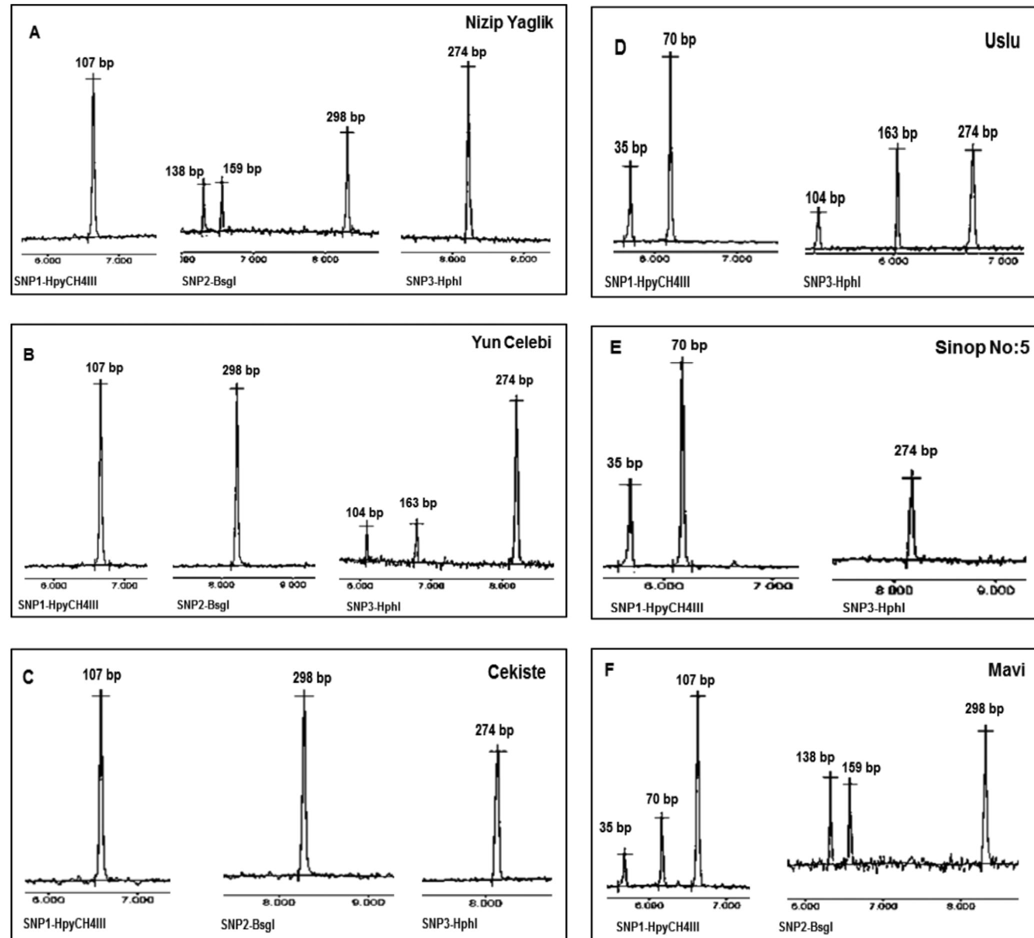

Figure 3.13. Electrophoretic patterns obtained by SNP-based CAPS assays for the monovarietal olive oils discriminated by two or three SNPs.

SNP3 was identified in the *Ant* sequence and allowed separation of olive varieties into two groups. Genotypes homozygous for SNP3 (seven varieties) carried only the C allele (Table 3.3) and were distinguished by a single undigested fragment. Whereas, due to the presence of the T allele, heterozygous genotypes (ten varieties) (Table 3.3) carried one copy of an intact HphI recognition motif and produced a three fragment HphI digestion pattern (Figures 3.13-3.16). SNP4 and SNP5 were both identified in sequences from the *Cyc* gene. Olive genotypes were either homozygous (TT) (12 varieties) or heterozygous (TC) (five varieties) for the SNP4 locus (Table 3.3).

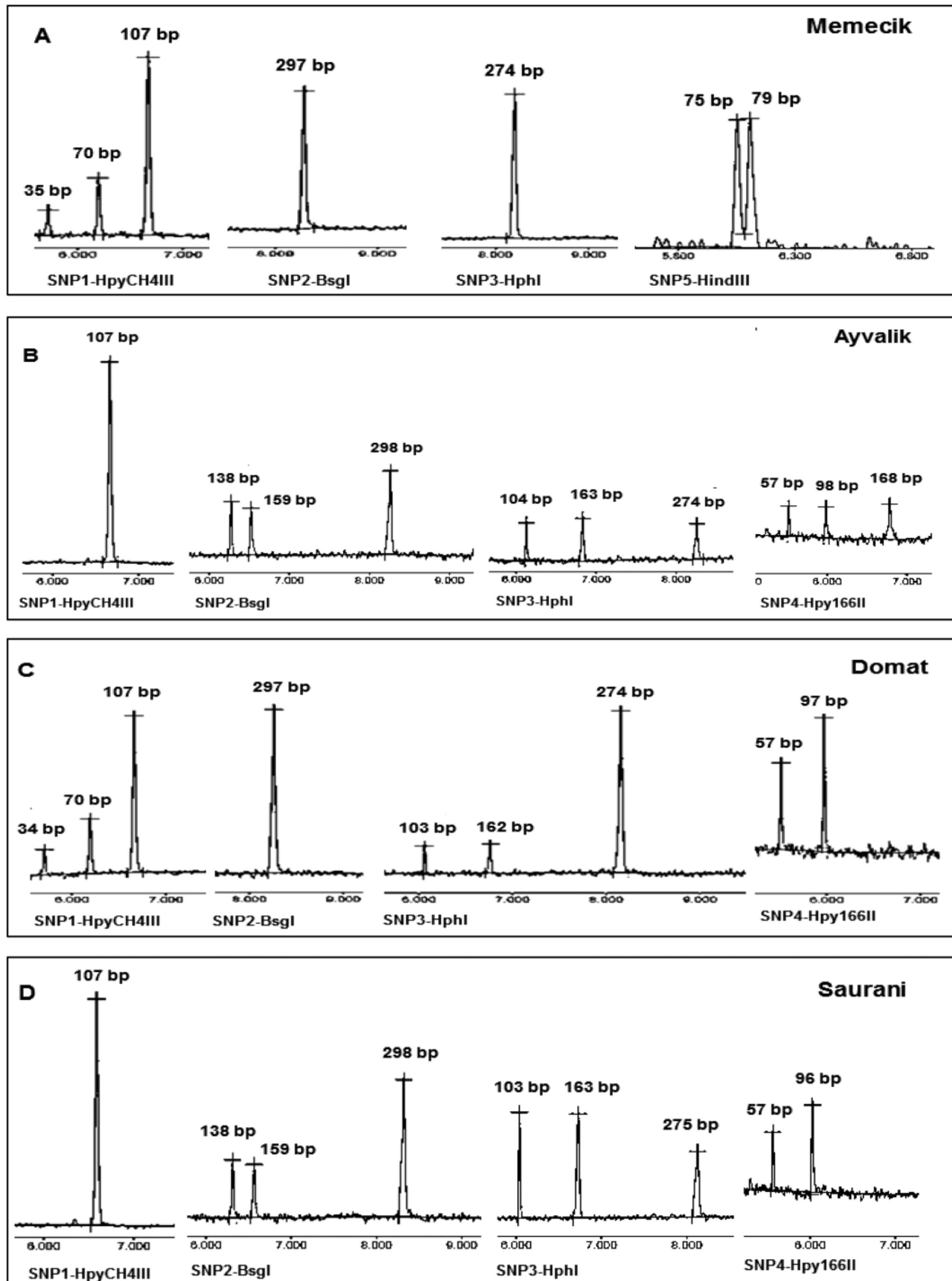

Figure 3.14. Electrophoretic patterns obtained by SNP-based CAPS assays for the monovarietal olive oils discriminated by four SNPs.

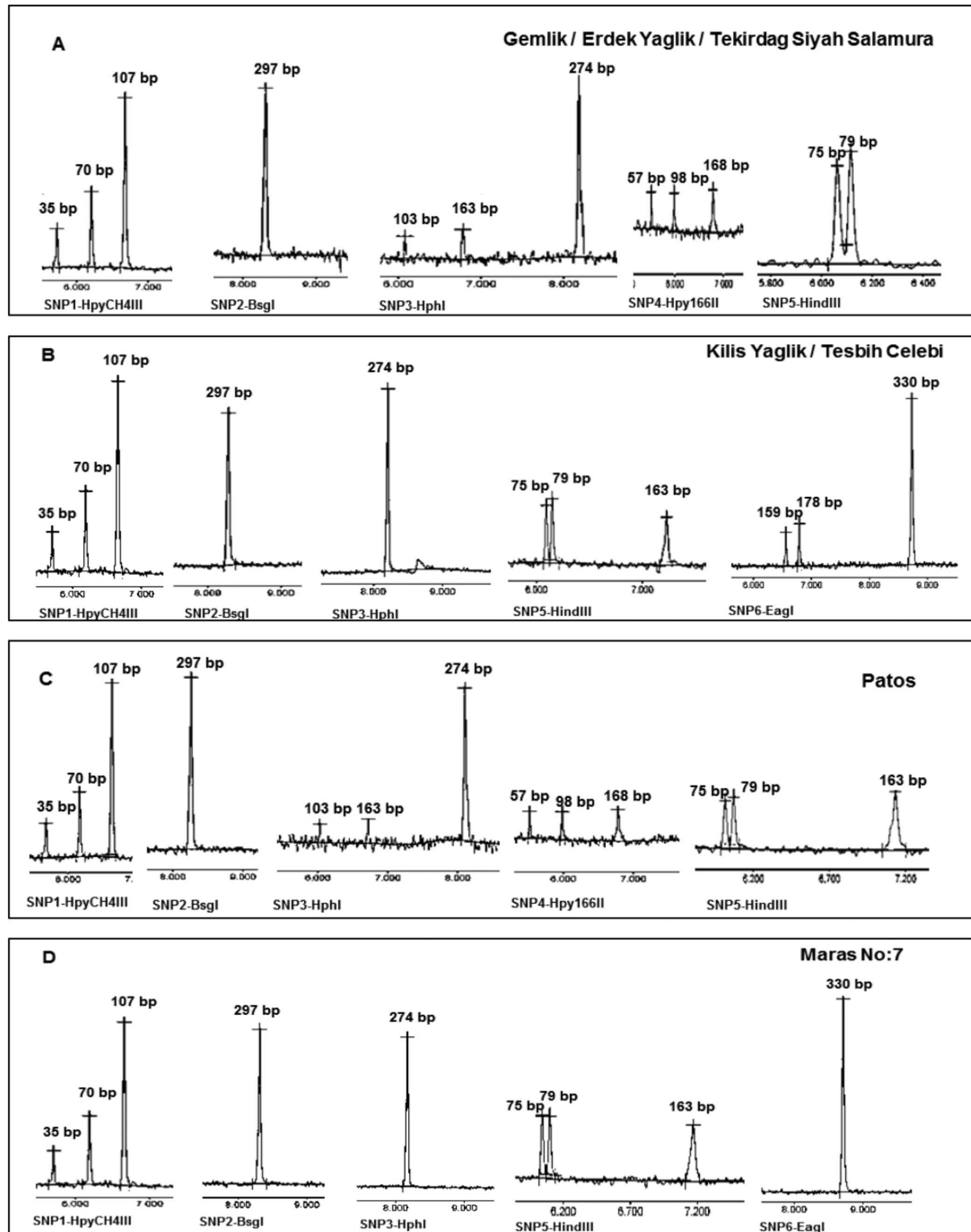

Figure 3.15. Electrophoretic patterns obtained by SNP-based CAPS assays for the monovarietal olive oils discriminated by five SNPs.

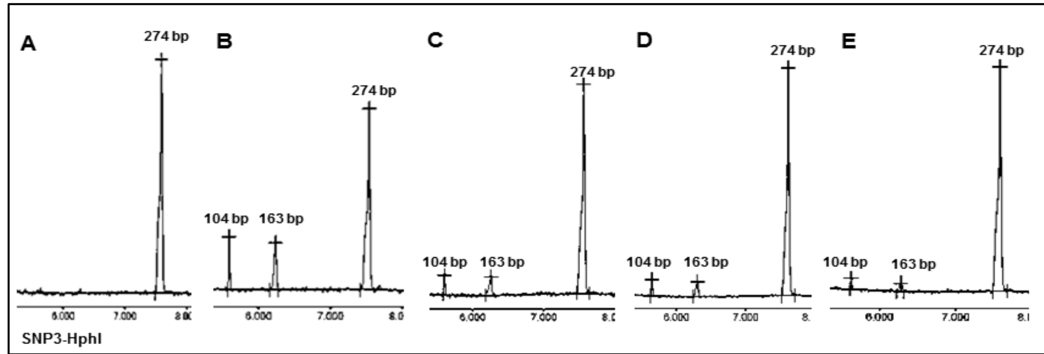

Figure 3.16. Capillary electropherograms displaying the results of SNP3-HphI assay applied on olive oil admixtures. A; 100% Memecik oil, B: 50% Memecik oil : 50% Uslu oil, C; 60% Memecik oil : 40% Uslu oil, D; 70% Memecik oil : 30%, E; 80% Memecik oil : 40% Uslu oil.

Homozygous genotypes were distinguished by a homozygous Hpy166II digestion pattern with two restriction fragments. Because the C allele disrupted the recognition motif for the enzyme, heterozygous genotypes were identified by three fragments in their capillary electropherograms (Figures 3.14, 3.16).

SNP5 separated olive varieties into two groups of nine homozygous (CC) and eight heterozygous (TC) genotypes (Table 3.3). Homozygous genotypes (CC) displayed a homozygous digestion pattern with two HindIII digestion fragments in their capillary electropherograms. Heterozygous genotypes (TC) were distinguished by a three fragment digestion pattern including the two digestion fragments and the undigested amplicon (Figures 3.14, 3.15). Olive varieties were either homozygous (GG) (12 varieties) or heterozygous (CG) (five varieties) for SNP6, identified in the *Cbp* gene (Table 3.3). Because the G allele disrupted the EagI recognition motif, homozygous genotypes displayed a single undigested fragment in their capillary electropherograms. Heterozygous genotypes carrying one copy of an intact EagI recognition site were characterized by a three fragment restriction pattern (Figure 3.15). Allelic frequencies of the six SNP loci used for the discrimination of the olive varieties are given in Table 3.4.

3.2.3. Fingerprinting monovarietal olive oils using SNP-based CAPS markers

The recovery of a sufficient quantity and quality of DNA is a key step in the success in DNA-based olive oil authentication. Different authors recommend various commercial kits or in-house protocols (Busconi et al., 2003; Breton et al., 2004; Testolin et al., 2005; Consolandi et al., 2008).When we compared different commercial kits and an in-house CTAB based protocol, we found that commercial kits are faster and more consistent in yielding amplifiable DNA from olive oil samples. While a DNA concentration that ranges between 100 – 200 ng/µL was obtained from olive leaf samples with the CTAB (Doyle and Doyle, 1990) method, the DNA extracted using the same method from oil samples was often below the limit of detection of the fluorometer. In contrast, commercial kits were consistent in yielding a DNA concentration of 2- 4 ng/µL from the oil samples, with no visible sign of degradation on the agarose gel. Therefore, our experience suggests using food-specific commercial kits to ensure successful downstream applications.

Among the six SNPs used to design CAPS assays, combinations that involved two, three, four or five SNPs were sufficient to discriminate the olive varieties (Figure 3.12). Therefore, variable sets of CAPS assays were applied to the oil DNA samples of the 17 varieties. SNP1-HpyCH4III assay was common in the discriminatory assay combinations for all varieties. However, different combinations of the remaining five assays (SNP2-BsgI, SNP3-HphI, SNP4-Hpy166II, SNP5-HindIII and SNP6-EagI) were used for the identification of the olive varieties.

Only two SNPs were sufficient to discriminate Uslu, Mavi and Sinop No:5 varieties. Discrimination of both Uslu and Sinop No:5 oils involved using the SNP1-HpyCH4III and SNP3-HphI assay combination (Figure 3.13). The SNP2-BsgI assay was used instead of SNP3-HphI for the discrimination of the Mavi variety (Figure 3.13). Identification of the varieties, Nizip Yaglik, Yun Celebi and Cekiste involved using three SNPs. A common set of CAPS assays (SNP1-HpyCH4III, SNP2-BsgI and SNP3-HphI) was used to discriminate the monovarietal oils of these varieties (Figure 3.13). The varieties Memecik, Ayvalik, Domat and Saurani could be discriminated from the rest using four SNPs. Ayvalik, Domat and Saurani oils were discriminated using an identical set of assays that included SNP1-HpyCH4III, SNP2-BsgI, SNP3-HphI and

SNP4-Hpy166II (Figure 3.14). Whereas, the SNP5-HindIII assay was required instead of SNP4-Hpy166II for the discrimination of Memecik oil (Figure 3.14). Among the 17 commonly cultivated Turkish olive varieties included in this work, two groups of cultivars were suggested as synonyms by the experts of the Olive Research Institute (Izmir, Turkey) based on long-term agro-morphological observations. The first group of varieties that are suggested as synonyms was Gemlik, Erdek and Tekirdag Siyah Salamura. A combination of five CAPS assays (SNP1-HpyCH4III, SNP2-BsgI, SNP3-HphI, SNP4-Hpy166II and SNP5-HindIII) allowed the discrimination of these three monovarietal oils from the rest of the collection (Figure 3.15). The second group of varieties suggested as synonyms was Kilis Yaglik and Tesbih Celebi. Using a set of five CAPS assays (SNP1-HpyCH4III, SNP2-BsgI, SNP3-HphI, SNP5-HindIII and SNP6-EagI), the oils of these two varieties were discriminated from the rest of the monovarietal olive oils (Figure 3.15). Olive varieties Patos and Maras No:7 were also distinguished from the rest using five SNPs. Thus, a set of five assays (SNP1-HpyCH4III, SNP2-BsgI, SNP3-HphI, SNP4-Hpy166II and SNP5-HindIII) was used for the discrimination of Patos oil (Figure 3.15), while the SNP6-EagI assay was interchanged with the SNP4-Hpy166II assay for the discrimination of Maras No:7 oil (Figure 3.15).

When we applied SNP-based CAPS assays on monovarietal olive oils, we were able to obtain the expected digestion profiles. Indeed, SNP-based CAPS analysis proved highly reproducible, and produced identical banding patterns in all three replicate experiments. As a result, the allelic error was found to be 0.000 for the six SNP-based CAPS markers (Table 3.4). As expected for SNP markers due to their biallelic nature (Ganopoulos et al., 2013b), the multilocus probability of identity value of the SNP loci was moderately low (0.018). By following the identification key (Figure 3.12) introduced in this work, the cultivar origins of oils from the 17 Turkish olive varieties can be identified and authenticated. Given that the genotypes included in this work represent the most commonly cultivated olive varieties in Turkey, we expect that the variety-specific assay combinations would largely meet the expectations of the Turkish olive oil industry for authenticating high-economic value, premium monovarietal olive oils.

3.2.4. Application of the SNP-based CAPS markers for detecting olive oil mixtures and to commercial oil samples

The ultimate goal of cultivar origin detection analyses is to determine if the origin of an oil sample is true to its labelling. Therefore, such an assay should enable the detection of undeclared impurities in a sample. Due to the fact that both the adulterant and the adulterated food commodity are extremely similar materials, the detection of mislabeled cultivar origin is a challenging task, making it attractive for unscrupulous manufacturers or vendors to gain profit from such fraudulent practice (Ulberth and Buchgraber, 2000; Costa et al., 2012). In addition, it is reasonable to expect that fruits of varieties cultivated in the same region are commonly processed together for oil extraction, leading to unintended mislabeling of cultivar origin. Thus, in addition to establishing a cultivar origin identification key, it was important for us to test the ability of our SNP-based CAPS assays to detect olive oil admixtures. The Memecik and Uslu varieties were specifically chosen for this kind of analysis, as the oil of the Memecik variety is well-known for its sensory qualities and its area of cultivation (South Aegean region of Turkey) overlaps with that of Uslu. Therefore, we can expect that fruits of these two varieties are commonly used together for oil production.

We prepared admixtures of Memecik and Uslu oils at different ratios, extracted DNA from those admixtures and applied the SNP3-HphI assay to determine if we could detect the contribution of DNA from the Uslu oil in the admixtures. When applied on the Memecik variety, the SNP3-HphI assay yielded a single, undigested fragment (Figure 3.14). Because Uslu was heterozygous for SNP3, application of the same assay yielded a three-fragment digestion pattern when applied on the oil of this variety (Figure 3.13). Therefore, any digestion fragment alongside the undigested band would be proof of an impurity in a Memecik oil sample. The olive oil samples prepared for the admixture detection assay contained 50%, 40%, 30%, 20% and 10% of Uslu oil added to Memecik oil. In order to assess the limit of detecting olive oil admixtures confidently, SNP3-HphI assay was carried out in three replicates on each DNA sample. As a result of the analysis, we successfully detected Uslu oil contribution down to a limit of 20% (Figure 3.16). Because the admixture with 10% Uslu oil was detected only once out of the three replicate analyses, a 10% contribution by an undeclared variety was considered to be below the limit of detection of the CAPS analysis (data not

shown). Thus, we set the limit of admixture detection as 20%. We consider this to be a good threshold of detection because, due to economic concerns, fraud generally involves much higher proportions of adulterants.

We further tested the applicability of the SNP-based CAPS markers on two commercial olive oil samples. While Sample No.1 had its cultivar origin declared on the label as “Memecik”, Sample No.2 was not labelled as a monovarietal product. A DNA concentration of approximately 2 ng/μL was measured by fluorometer for both of the commercial samples, which constituted a sufficient quantity of template DNA for further PCR amplifications. The entire set of six CAPS assays (Table 2.2) was applied on DNA from both samples in three replicates. For Sample No.1, the digestion profiles resulting from the six assays (Figure 3.17) were in agreement with the genotype of the Memecik variety (Table 3.3), and did not provide any evidence to suspect the declared varietal origin of the product.

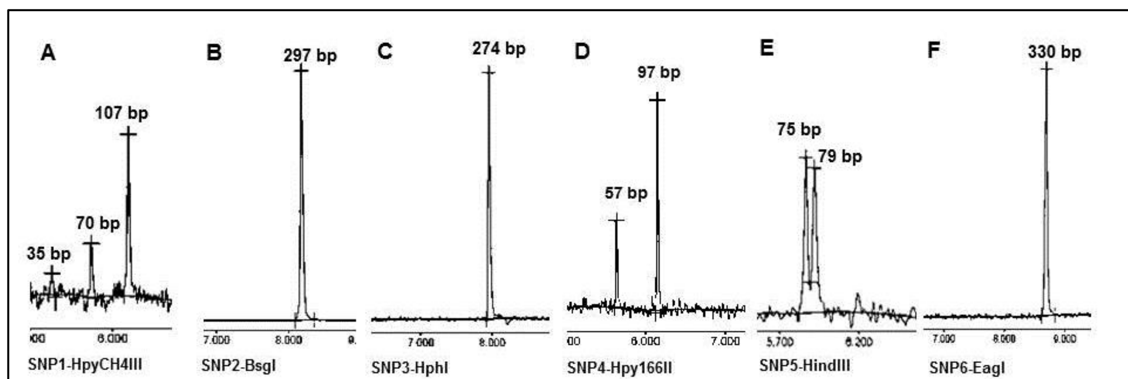

Figure 3.17. Capillary electropherograms displaying the results of the SNP1-HpyCH4III SNP2-BsgI, SNP3-HphI, SNP4-Hpy166II, SNP5-HindIII and SNP6-EagI assays applied on commercial Sample No. 1.

In case of Sample No.2, the digestion profiles obtained from the six assays (Figure 3.18) matched the combined SNP genotypes of none of the 17 varieties (Table 3.3). In addition, the result of the SNP2-BsgI assay (Figure 3.18) excluded the four varieties, Ayvalik, Nizip Yaglik, Mavi and Saurani, as these varieties were heterozygous for this locus.

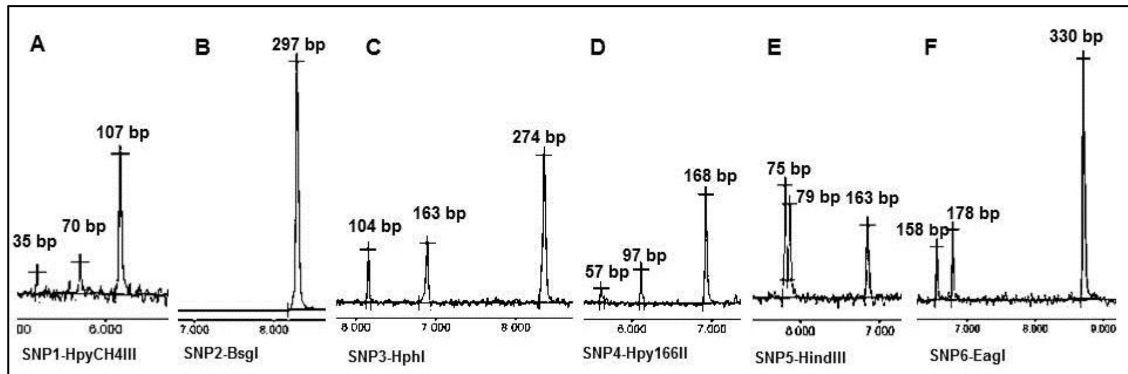

Figure 3.18. Capillary electropherograms displaying the results of the SNP1-HpyCH4III SNP2-BsgI, SNP3-HphI, SNP4-Hpy166II, SNP5-HindIII and SNP6-EagI assays applied on commercial Sample No. 2.

As a result, it was concluded that either the cultivar origin of Sample No.2 was not represented in the set of 17 varieties or the product was a mixture of the oils from different varieties. The latter seemed to be a more viable explanation, because Sample No.2 was not declared as a monovarietal product on the label and the genotypes included in this work represent the most commonly cultivated Turkish olive varieties.

CHAPTER 4

CONCLUSION

Olive oil is one of the most valuable products of the agro-food industry, thus, one of the most attractive targets for adulteration. Fraud is generally committed either by mixing olive oil with lower economic value seed oils or by marketing lower grade olive oils under false labels that claim a well-reputed cultivar origin.

Edible oil authenticity is not only an issue of food quality but also an issue of food safety, since seed oils used as adulterants can trigger allergic reactions in susceptible individuals. Moreover, consumption of fraudulent oils can even lead to permanent health problems if the adulterant seed oil is refined for industrial use and not intended for human consumption. While Turkey is among the world's leading olive and olive oil producing countries, there has been no systematic study to establish traceability procedures in order to protect the authenticity of premium Turkish monovarietal olive oils.

Food genomics is a recently emerging discipline that has found a wide range of applications in food authenticity analyses. In contrast to conventional food authenticity tests which rely on the analysis of chemical constituents, food genomic approaches analyze the remnant DNA in a food sample and produce results that are independent from environmentally induced compositional variations. In this work, food genomic approaches were used for developing DNA-based tests for botanical origin authentication in olive oil and to establish the traceability of Turkish monovarietal olive oils. Because the rafination process is considered to reduce the DNA yield significantly, it was important for us to utilize a target DNA region that enables robust PCR amplifications from seed oil samples. Therefore, the plastid genome was selected as the analyte target, due to its high copy number in plant cells. Using an optimal barcode region (plastid *trnL* (UAA) intron) that displays length polymorphisms among botanical species, it was feasible to standardize a PCR-CE barcode assay that enabled the reproducible detection of seed oil presence in olive oil down to a limit of 10 %. Given that fraud is committed in order to make economic profit, it is reasonable to expect much higher ratios of adulterants in fraudulent olive oil products. Thus, we consider 10

% a good limit for detecting seed oils in olive oil. On the other hand, it is important to note that the DNA-based assay was successful in identifying admixtures that contained the adulterant seed oil at a ratio of 5% for more than half of the seed oil species. In addition, comparative evaluation of the results obtained via the DNA-based assay and gas chromatography analysis showed that the PCR-CE assay was superior over the widely accepted standard method in identifying adulteration in olive oil. Moreover, admixtures that were identified as pure olive oil by GC analysis were correctly identified as blends of olive oil and hazelnut oil by the PCR-CE barcode assay, displaying the usefulness of complementing analytical chemistry methods with genomic approaches in order to improve the precision and sensitivity of the existing edible oil authentication protocols. The PCR-CE barcode analysis described in this work is a highly reliable, fast and cost-efficient method for the authentication of the botanical origin in olive oil and is expected to complement the shortcomings of conventional olive oil authenticity tests, such as the inability to discriminate plant oils with similar fatty acid compositions.

In order to develop DNA-based assays for cultivar origin authentication in Turkish monovarietal olive oils, fragments from five olive genes were sequenced to identify single nucleotide polymorphisms. In this work, SNPs were the markers of choice, as they are the most frequent type of polymorphisms in the genome and enable the discrimination of closely related genotypes. The SNP survey resulted in the identification of 17 SNP loci polymorphic among 17 commonly cultivated Turkish olive varieties. Out of the 12 SNP loci amenable to genotyping by CAPS strategy, six proved sufficient to establish an identification key for the monovarietal olive oils. In addition, not all six assays were required for the discrimination of the olive varieties but combinations of two, three, four or five assays were sufficient to ascertain the cultivar origin of the monovarietal olive oils. When applied on the monovarietal oils of the 17 Turkish olive cultivars, the SNP-based CAPS assays proved successful in correctly identifying the cultivar origin. Moreover, it was feasible to display the applicability of the SNP-based assays on olive oil admixtures and commercial olive oil samples.

To our knowledge, this is the first report that describes the utilization of SNP-based CAPS assays for the detection of admixtures of oils from different olive varieties. Moreover this is the first time that SNP markers were applied on Turkish olive oils for establishing a varietal identification key and detecting olive oil admixtures. The applicability of the CAPS assays on commercial olive oil samples was also

demonstrated. In addition to their use for addressing the authenticity issues of monovarietal olive oils, the CAPS assays introduced in this work will find several uses including olive tree certification, germplasm characterization and preservation studies.

It is expected that current food authentication protocols will be extended with genomic assays in the near future. Such assays will improve the confidence in verifying the authenticity and detecting adulteration in food products, thus ensuring food quality and safety. Therefore, the botanical origin authentication and cultivar origin detection methods introduced in this work are expected to be adopted by the food industry and public institutions in the near future.

While it is important that traceability protocols are established to verify the cultivar origin of monovarietal olive products in order to protect consumer rights and elevate the economic value of Turkish olive oils in the international market, it is also crucial that olive genetic diversity is protected via systematic germplasm characterization and preservation efforts. Therefore, future work will involve characterization of Turkish olive germplasm using the SNP-based CAPS assays developed in this study. Identified and registered wild olive resources will be included in such work, with an aim to contribute to efforts to prevent genetic erosion in this ancient tree crop species. The results of this study demonstrated that SNPs in olive coding sequences are conserved across the geography of olive distribution. Thus, SNP-based CAPS assays will be used in future work toward characterization of other olive germplasm preserved in the Mediterranean basin.

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