UNIVERSITÀ DEGLI STUDI DELLA TUSCIA DI VITERBO

DIPARTIMENTO DI SCIENZE DELL'AMBIENTE FORESTALE E DELLE SUE RISORSE (DIBAF)

CORSO DI DOTTORATO DI RICERCA IN

BIOTECNOLOGIA DEGLI ALIMENTI - XXIII CICLO

Identification of molecular markers for the characterization of hazelnut oil

BIO/10

Coordinatore: Prof. Marco Esti

Firma ……..

Tutor: Prof. Nicolò Merendino

Firma……

Dottorando: Sabrina Lucchetti

Firma

……..

CONTENTS

SUMMARY

In this project it has been set up a simple method for molecular traceability of hazelnut oil through microsatellite markers.

With the aim to achieve the purpose, first of all an amplification/ extraction methodology has been set up on 2 pools of Tonda Gentile Romana hazelnut (TGR). The best technique it is subsequently used to analyze other four hazelnut varieties.

Amplification tests performed on DNAs, extracted through the different methodologies, permitted us to evaluate samples quality and to identify the best PCR conditions for microsatellites analysis.

Study of 9 microsatellites, performed on the best DNAs, has permitted us to define a fingerprint for each variety.

Data obtained from hazelnut varieties compared with a data bank have shown that varieties surely correspond to those declared, except for 2 pools of TGR. The study of two pools with CAC B028 microsatellite has shown that four alleles, instead two, are present in each sample. This result could indicate a non homogeneity of the two matrices.

Molecular approach, set up on hazelnut matrix, has shown to be effective for the study of hazelnut oil matrix too, prepared with the two hazelnut pools of TGR. Molecular analysis of oil matrix, in fact, confirm data obtained from hazelnut matrix, include the anomalous result about CAC B028 marker.

To evaluate the homogeneity of one of the TGR pool, analysis on individual hazelnut have been performed, instead on pool, as before. The results show that, for each marker, the global allele number identified in all samples is always more than two expected.

This suggests that the matrix isn’t homogeneous and that any individual nut analyzed belongs to the TGR cultivar.

RIASSUNTO

In questo progetto è stato messo a punto un metodo per la tracciabilità molecolare dell'olio di nocciola attraverso l'uso dei microsatelliti.

Per tale scopo innanzitutto sono state messe a punto le tecniche di estrazione/amplificazione su 2 pool di nocciole di Tonda Gentile Romana (TGR). La migliore tecnica è stata poi utilizzata per l'analisi di altre quattro varietà di nocciole.

I test di amplificazione effettuati sui DNA, estratti attraverso le diverse procedure, hanno permesso di valutare la qualità dei campioni e di individuare le miglior condizioni di PCR per l'analisi dei microsatelliti.

Lo studio dei dei 9 microsatelliti effettuato sui migliori DNA ha permesso di definire un fingerprint per ciascuna varietà.

Il confronto dei fingerprint con una banca dati suggerisce che le varietà possano corrispondere a quelle dichiarate, ad eccezione dei 2 pool di TGR. Nei pool di TGR infatti, Lo studio dei due pool con il marker CAC B028, ha messo in evidenza quattro forme alleliche, anziché due, in ogni campione facendo presupporre una disomogeneità di ciascuna delle due matrici.

L'approccio molecolare messo a punto sulla matrice nocciola ha mostrato di essere efficace anche nello studio della matrice olio di nocciola, preparato a partire dai due pool di nocciole TGR, tanto da confermare i dati ottenuti da nocciola compresi quelli relativi al marker CAC B028.

Per valutare l'omogeneità di del pool TGR1, sono state eseguite delle analisi su singole nocciole, anziché su pool, come fatto precedentemente. I risultati hanno evidenziato che, per ciascun marker, i campioni nel loro insieme mostrano più dei due alleli attesi.

I risultati suggeriscono che la matrice TGR1 non è omogenea e nessuna delle singole nocciole analizzate appartiene alla cultivar TGR.

INTRODUCTION

1. HAZELNUT

The hazelnut crop in Italy is a growing sector of great economic importance and employment. Italy, with a production of about 100,000 tons per year (14% of world production), is, in fact, the second largest producer after Turkey which has always been the undisputed leader (Dossier/Il Nocciolo, a cura di CRPV, Cesena, 2008).

Among the different species of the genus Corylus, the European hazelnut (*Corylus avellana*) is the most important species commercially, and is, therefore, undoubtedly the most widespread species in Europe and Asia Minor.

The long selection process which occurred both naturally and by man over the centuries has created numerous cultivars. These have developed and adapted to the climactic characteristics of the various distribution areas, entering the local agricultural traditions having new technical and commercial properties (productivity, vigor, and better quality of fruits).

This adaptation phenomenon is evident worldwide, and can be observed even within homogeneous regions. Moreover, it is also visible even in Italy, where the cultivation of different varieties has been developed according to various environmental and agronomic characteristics. Regional examples are the Tonda Gentile Romana and Nocchione in Lazio (Rome and Viterbo area), Tonda Gentile delle Lange in Piedmont (Lange area), and Mortarella and Tonda di Giffoni in Campania (Caserta, Naples, Salerno and Avellino areas).

As for Italian production, it has been reported that some varieties of Italian hazelnuts also enjoy great acclaim as well as qualifying for IGP certification. For example the Tonda Gentile delle Lange, Tonda di Giffoni, or DOP (recently acquired by the Tonda Gentile Roman).

2. HAZELNUT AND ITS CHEMICAL CHARACTERISTIC

Hazelnuts are considered an important source of nutrition and health. They are mainly composed of lipid (60%), with 14% of proteins and 6% of carbohydrates. They are also an important source of many bioactive constituents and functional nutrients (Maguire *et al*., 2004).

From a nutritional standpoint, the fat composition of hazelnuts is favourable, with high monounsaturated fatty acid (MUFA) and low saturated fatty acid (SFA) percentages.

Many studies demonstrate in fact that MUFA determines the reduction of LDL (low density lipoprotein), a contemporary induction of HDL (high density lipoprotein) and a consequent reduction of atherosclerosis risk and cardiovascular diseases (Elvevoll et al., 1990, Rajaram, et al. 2001, Mercaligil, S. M. et al. 2007).

CHEMICAL COMPOSITION OF HAZELNUTS	
TOTAL FATS	60 %
SFA	4,16 %
MUFA	38,62 %
PUFA	5,20 %
PROTEINS	14 %
CARBOHYDRATES	6 %
Insoluble sugar	4 %
Vit A	30 μg ret. eq./100g of edible part
Vit E	15 mg/100g of edible part
Vit C	4 mg
FIBERS	8,1 g
POTASSIUM	466 mg
IRON	3,3 mg
CALCIUM	150 mg
PHOSPHORUS	322 mg
TIAMINA	0,51 mg
RIBOFLAVINA	0,10 mg
NIACINA	2,80 mg

Tab. 1- Chemical hazelnut composition (Tabelle di Composizione degli Alimenti, INRAN, 2000).

Moreover, hazelnuts also contain many antioxidant and nutraceutical molecules, such as polyphenos, vitamins E, A and C, phytosterols and squalene, besides minerals and fibers (Tab. 1) (Tabelle di Composizione degli Alimenti, INRAN, 2000). Several studies have demonstrated that antioxidant and nutraceutical molecules and a particular lipids composition, such as that identified in hazelnuts, are beneficial for the prevention of some cardiovascular diseases , some forms of cancer, aging and the regulation of immunitary system (Alphan *et al*., 1997; Durak *et al*., 1999; Awad *et al.,* 2001, Bouic *et al,*. 2001, Plat *et al,*. 2001)**.**

The awareness that hazelnuts, as well as all nuts, contain several bioactive/healthy principles (Contini *et al*. 2010) has compelled some countries (like the United States, Spain, Canada) to include them in their dietary guidelines as being useful for the prevention of the above-mentioned diseases (Dreher *et al*. 1996, Kris-Etherton *et al*. 1999, Maguire *et al*. 2004, Chen *et al*. 2008; Bullò *et al*, 2011). In particular, in the United States, the Food and Drug Administration authorizes companies to write the following health claim on a product's label with the aim of reducing heart desease: "*Scientific evidence suggests but does not prove that eating 1.5 ounces per day of most nuts as part of a diet low in saturated fat and cholesterol may reduce the risk of heart disease"*. (Alphan *et al*., 1997).

3. HAZELNUT OIL

In the past, hazelnuts were mainly consumed as transformed byproduct confectionery, but awareness that the hazelnut is a good health source has grown, coupled with an increase in interest on the market. In fact, marketing strategies are encouraging more consumption of healthy products with respect to the past, so that hazelnuts are starting to be consumed also as fruit or as its main byproduct: oil.

In regard to the latter, hazelnut oil is an extremely interesting product, able to replace, within a proper and balanced diet, other fat dressing more traditionally used. In addition, the launch of the hazelnut oil market would open new prospects economically as it could be used in a convenient form, for example in the production of snacks and sweets in general.

At present, hazelnut oil is largely used in the Black Sea region, particularly Turkey, the largest producer of hazelnuts in the world. In Turkey, hazelnut oil is largely consumed for cooking, deep frying and salad dressing (Alasalvar *et al*., 2003). On the contrary, in Italy, hazelnut oil

consumption is growing very slowly and with many difficulties, because of lack of publicity and the high price of the product. For these reasons, hazelnut oil is being used only by a very refined and health conscious consumer: who looks upon it as a gourmet item.

4. HAZELNUT OIL, ITS QUALITY AND THE SOPHISTICATIONS

Hazelnut oil, when it is produced by mechanical cold methods and when good raw materials are used, is a precious product, as it maintains the beneficial properties of the original fruit, with the additional advantage that it can easily be employed in the daily diet (Alasalvar *et al*. 2006).

However, as for the production of oil extracted from olives, only a correct application of extraction technologies can ensure that the molecules of nutritional interest (antioxidants) contained in the hazelnuts are transferred to the oil, turning it into a fatty seasoning of great nutritional interest. (Crews *et al*., 2005;Alasalvar et al., 2003; Alasalvar *et al.,* 2006).

In fact, it is absolutely true that the oil produced according to correct principles can really be a useful tool as part of a healthy balanced diet, similar to extra virgin olive oil, which is modeled almost faithfully to the composition. It is equally true that it loses its beneficial feature when it is produced through incorrect technological processes from raw materials rather than quality processes.

Regarding the extraction processes, note that the extraction with Hexane is not always able to transfer to the final product all the bioactive molecules useful to make a product with all the nutritional and health properties previously reported. Also, more importantly, remember that nuts not in good condition, or stored improperly, can easily promote the development of high doses of Mycotoxin,. (Shephard, 2008; Ibanez *et al*., 2011), meaning that it has lost many of its beneficial properties, and is even potentially dangerous to the health of the consumer.

In addition bad oil can be employed for another nefarious purpose too, namely fraud. In fact hazelnut oil is often prepared with waste raw material and then added to olive oil fraudulently, and finally sold as 100% olive oil. As hazelnut oil added to olive oil can exceed 10% of the total amount, it is possible to imagine the dimension of this type of business (Baeten, *et al.,* 2005). This fraud causes an image problem, and economic damage to the hazelnut and olive oil market. This not only contravenes the law, but leads to a poorer nutritional option, as low-quality hazelnuts and low quality olives are usually used in these cheats.

The adulteration of extra virgin olive oil by the addition of hazelnut oil is still a very difficult fraud to identify due to the almost complete overlap of the constituents, normally used for recognition, in the two types of matrices. (Agiomyrgianaki *et al*., 2010, Chen *et al*., 2011).

Despite the considerable efforts made to date, the appropriate cost-effective methods to solve this thorny problem still have not been found. (Garcia-Gonzalez *et al,*. 2007, Aparicio *et al*., 2007, Sayago *et al.*, 2007, Vlahov *et al*., 2006).

This scenario makes it so essential to develop new methods of tracking that can clearly characterize the raw material from which oil originates. Such will help to protect the quality of the emerging market of hazelnut as well as the already developed but still mild extra virgin olive oil market.

5. TRACEABILITY

5.1 Introduction to the traceability

The traceability of food, in the classical sense, makes use of techniques well-known and widely used, and which are, however, only recognized in the specification of certified products by the law. The guarantee of a product is often only associated with a form of tracking and tracing paper, with the resulting uncertainty for this type of traceability.

From the point of view of control, until a few years ago, foodstuffs were studied on a physical-chemical plane, while approaches were analyzed through phenotypic markers (seed color, leaf shape, flower color, etc..). These were insufficient approaches because the parameters used were not peculiar to each non-processed or processed food. They were sensitive, moreover, to environmental variations, agronomic techniques, and, most importantly, applied to the processes of transformation.

With the chemical-physical or phenotypic approach, which uses classical traceability, one can definitely make a first quality screening. Analysis of these types are essential for safety verification, as well as the organoleptic constitution step, essential for traceability. The drawback of these procedures is not being able to identify with certainty the origin of the raw materials used, nor carrying out studies characterization. Basically, the major limitation of these techniques is that the parameters measured are strongly influenced by environmental factors, in addition to having a low discriminating power in the characterization of species or varieties, rendering them ineffective as a result of old and new generational issues.

It is clear that the new requirements now seeking other safety standards cannot be reached from the traditional approach. For these reasons, it is therefore necessary to develop new methods, procedures and protocols to track food, protect its quality, and give an adequate response to other parallel aspects of great interest. These “parallel aspects” include the growing interest in biodiversity, the identification of the description of new species, the recognition of synonyms in

the current nomenclature, the characterization and tracking of complex food matrices, the entry into the market of many products whose origin and quality are hardly detectable, and the need to protect local products from unfair competition or fraud by the development of strong competition on typicality and territoriality that bind products to a territory.

5.2 Molecular traceability

Today, molecular traceability, in light of the innovative techniques used, and the problems successfully addressed, has a versatile use, lending itself to such fields as the legal, medical and genetic.

The new frontiers opened by molecular traceability are making great strides, especially because they offer more reliable and reproducible results in the characterization of species (animals and plants), and food matrices, especially where classical traceability has many uncertainties. (Peres *et al*., 2007). Today molecular methodology helps us to identify the origin of raw materials used to produce whatever foodstuffs. It is also a valid tool to guarantee quality and safety of foodstuffs from frauds to contrast the insalubrious potentialities of foodstuffs when they are related to their possible dubious manufacture.

Through this tool, it is possible to check food with the aim of assuring free food choices, avoid frauds/falsification and alleviate allergy from people.

Over the last few years, the appearance of the molecular approach has allowed us to analyze any type of complex matrix, avoiding all the limits existing in this field with the traditional approach. The focal point of molecular traceability is based on the study of DNA. DNA is, in fact, the best parameter to be studied for identifying foodstuffs, as its characteristic is to be totally independent from any type of environmental influence or technological intervention, it is stable in time, and it needs a very few amount of samples to be analyzed. The advent of rapid DNA sequencing has brought the discovery of DNA-based markers (molecular markers) which are used as markers of choice for the characterization of a genome. Molecular markers are based on DNA polymorphism as a result of mutation, and they are characterized by high heritability. Their main advantage is that they are much more numerous and polymorphic than morphological and biochemical markers.

Thus, molecular analysis is becoming a very important tool to identify the real identity of a simple or complex matrix without doubts or perplexity. The result of a matrix's molecular characterization through molecular markers is its "fingerprint", a sort of a identity card, unique and unequivocal invariably along its life and different from the other matrices.

It is certain that despite the new propulsion generated by the molecular systems to the most demanding challenges, it is necessary that the old and new methods of traceability work in concert via the integration of the systems. This is my vision in order to insure the quality and safety of the product.

In some cases, the synergism which is obtained by using the innovative approach with the traditional one has become almost indispensable. For example, the molecular approach, although not legally required, is often useful in the analysis of products of high quality and strong connections to the origins and traditions of the territory where it is applied. In the case of PDO (Protected Designation of Origin), DOC and/or DOCG (Controlled and Guaranteed Denomination of Origin), the use of molecular methods is valuable for guaranteeing the origin and healthiness of the product, as well as figuring out suspicious cases of adulteration (Cappellehi *et al*., 2007; Martins-Lopes *et al*., 2008; Pasqualone *et al*., 2007).

In the food industry, molecular techniques made their entrance a few years ago, but have already gained the trust of experts, having proven to be extremely useful to the sector as it is versatile, reliable and safe, especially where the chemical and physical analyses are lacking or insufficient.

In some cases, the use of the molecular approach has become quite indispensable. This is the case for extremely complex matrices in which the technological treatments used or the complexity of the raw materials present make their study difficult, if not impossible, when approached in a conventional manner.

However, as mentioned, although this area belongs to a new generation, new technologies have found their full and proper use via the combination of innovative methods of applying contemporary and traditional methodologies. This is the winning strategy.

5.3 Oil traceability

The oil matrix is a difficult analysis, therefore the recognition of the raw materials which compose or identify possible frauds is a difficult problem to solve, and require very advanced technologies, which are often costly.

As for other foods then, the traceability in the classical sense, associated with new techniques of molecular traceability, enhances and completes the process, generating more reliable results and solving otherwise impossible problems.

Combining the two approaches make it possible, on the one hand, to identify and recognize the genetic, and, on the other, to characterize the organoleptic, chemical and physical appearance, key for the recognition of good oil from a healthy standpoint.

The oil matrix, even in the field of molecular techniques, as with all products that have undergone major changes in technology, is difficult to figure out, and its study still presents many difficulties. This is true mainly because the DNA of the original matrix (fruit or seed) transferred in the oil after processing is often of poor quality both in terms of quantity and quality. For this reason, an actual take-off molecular approach in this field has been only recent, with the advent of techniques for analysis of specific genetic markers whose investigation is possible even in the presence of genetic material of poor quality.

For example complex processed matrices such as oil, have been well characterized only when specific molecular markers have been employed: the microsatellites or SSR (Simple Sequence Repeats**)** (Tautz *et al*., 1984).

In light of present knowledge, it is evident that molecular biology, both for its versatility and ease of application given its results, has proven to be the new frontier.

5.4 Hazelnut oil traceability

Hazelnut oil, despite being an emerging and growing product, is still a niche product very poorly studied. Although much has been done and is being done on the characterization of the genomic DNA of hazelnuts, which is indeed the foundation for the study of hazelnut oil (Bassil *et al*., 2005, Boccacci *et al*., 2008, Galderisi *et al*., 1999), little has been done directly on hazelnut oil.

6. MOLECULAR TRACEABILITY PRINCIPLES

The groundwork for the molecular approach is the study of the DNA through molecular markers and the identification, through them, of polymorphisms, and therefore the definition of a "fingerprint", a sort of identity card, unique and discriminating for each matrix, invariably along its life.

6.1 DNA based molecular traceability

DNA represents the focal point of molecular investigation. A good recovery of genomic DNA is fundamental for any type of molecular experiment. DNA is a very long and fragile molecule, and it is very sensitive to mechanical stress, therefore it can be damaged (fragmented) during matrix processing or during its extraction.

These issues are further highlighted in complex matrices, such as in oils, where the technological treatments themselves often cause serious damage to DNA even before its extraction process.

6.1.1 The choice of the DNA extraction method

Many different DNA extraction methodology exist for different needs, traditional, commercial and automatic methods having the same aim of extracting DNA. Each one of them has its own advantages and disadvantages, but all of them work through three steps: lysis, DNA separation and DNA wash.

Traditional method with Phenol/chloroform

In the classical method (Doyle and Doyle, 1987) and modifications, cells are lysed in buffers containing a detergent and proteinase K. This pool induces the breaking down of membranes, the aggregation and digestion of proteins, and nucleic acid precipitation. Afterwards, a treatment of organic solvents allows us to separate and eliminate the proteins and thus recover DNA. DNA is then precipitated adding ethanol and salts. At last, a wash of DNA is performed in order to eliminate salts that could inhibit the successive analyses of the sample.

Commercial kit and their main basics treatment

Many kits exist in commerce, and they have to be chosen on the basis of personal needs and used matrices. The main advantage of commercial kits is that the procedure is more rapid and produces cleaner nucleic acids but, on the other hand, they are only able to recover very low quantities of DNA.

As for the traditional method, the first step is aimed at inducing lysis through a buffer containing chaotropic salts. Depending on the type of kit, the successive purification of the sample may then be performed on column or by magnetic beads.

The purification on column is performed through an ion exchange chromatography on silica that determines the absorption of nucleic acid. Different centrifugations and wash buffers are then needed for removing residual debris and contaminants.

Purification may also be performed in magnetic beads where nucleic acids tie to the beads because they are coated with silica. The removal of whatever unknown substance is then performed through a magnet employment.

Automatic systems

Actually many automatic systems exist for purification of nucleic acids. These apparatus apply the same principles as the commercial kit based on column purification, but the device is totally automatic with an enormous advantage in terms of time. On the other hand, they have the disadvantage of being expensive.

6.1.2 The choice of the primers for analysis of DNA quality

The choice of the primer is basic for obtaining good results, as DNA has to be in good condition in terms of both quality and quantity. Three types of primer are commonly considered:

- Primers to amplify specific regions of the selected specie, with different homology to genes of other species. These are needed to set up the PCR conditions and to evaluate the possible presence of exogenous DNA in the sample.
- Primers that amplify regions big enough (more than a microsatellite) in order to evaluate the DNA fragmentation and the subsequent possibility to use them for fingerprint analyses.
- Primers that amplify region belonging to genes of different specie, from that studied, necessary to evaluate the specificity and the contamination of the sample.
- Primers corresponding to conserved region of markers and chosen through already existing information in data banks and useful in the characterization of a genome, through the definition of a fingerprint.

6.2 Molecular markers

Molecular markers allow us to identify polymorphisms and therefore characterize a genome even without knowing its whole genomic sequence (Philips *et al.,* 2001, Varshney *et al.,* 2004). Polymorphisms are due to casual nucleotide substitutions or rearrangements like insertion or deletion.

Molecular markers correspond to specific DNA regions (locus), they are representative of the differences at the genome level and they are inherithed according to Mendelian principle. Markers are, commonly, extremely variable (polymorphics), spreadout along the whole genome, and, above all, their analysis isn't affected by environmental conditions. For these reasons they are used as a reliable method to discriminate different cultivars/individuals. Through markers it is possible to characterize the genomic profile and define the fingerprint or a specific genomic marker map for each analyzed matrix (Williams, *et al*. 1990; Hayden *et al*., 2001; Aitken *et al*., 2005)).

An ideal molecular marker should have the following characteristics: 1) be sufficiently polymorphic, 2) be distributed throughout the genome, 3) provide adequate resolution of genetic differences, 4) be simple, quick and economic to analyze 5) need a small quantity of matrix and DNA, 6) have no need for information about the analyzed genome.

Techniques used for molecular markers study are also different from each other with respect to important features such as genomic abundance, level of polymorphism, locus specificity, reliability, technical requirements and cost. The choice of right molecular markers depends on the specific need because no marker is perfect for any situation.

Many molecular types of markers exist, and they are clustered in different classes according to their specific characteristics or or the technique able to analyze them. These classes are divided in two more categories:

1) a category represented by classes of markers that are analyzed through non-PCR (Polymerase Chain Reaction) technique (Mullis and Faloona, 1987).

2) a category represented by classes of markers studied through PCR-technique.

6.2.1 Non-PCR techniques

The category (non-PCR techniques) include the class of RFLP markers (Restriction Fragement Length Polymorphisms) between the first identified as markers and those used in the past.

RFLP

With RFLP, DNA polymorphism is detected by hybridizing a chemically labelled DNA probe to a Southern blot of DNA digested by restriction enzymes that determine different DNA fragment profiles.

The RFLP markers are polymorphics, codominant, and highly reproducible. The technique is good but it isn't largely used because it takes a long time, it is expensive, requires use of radioactive reagents and needs large quantity of high quality DNA (Botstein *et al*., 1980).

6.2.2 PCR techniques

The newer DNA markers based on PCR technology are more suitable for routine analysis characterization. They require small quantity of DNA, and were detailed essentially for the simplicity and low cost of methodology. In addition, usage of random primer overcome the limitation of prior sequence knowledge for PCR analysis.

In the PCR-techniques category different classes of markers are included, which are slargely used and very useful for different purposes.

RAPD

The RAPD (Random Amplification of Polymorphic DNAs) markers (Williams *et al*., 1991) are analyzed through PCR amplifications that identify rearrangement or deletions at/or between oligonucleotide primer binding sites in the genome using short random oligonucleotide sequences. This technique can be employed across species as universal primers are used. Due to

the speed and simplicity of RAPD analysis, high density genetic mapping in many plants, such as alfa alfa (Kiss *at al*., 1993), have been developed in a very short time,

This methodology is not largely used because it isn't highly reproducible, because of low temperature used for PCR annealing, and, in addition, it doesn't discriminate heterozygous from homozygous individuals.

AFLP

The methodology to study AFLP (Amplified Fragment Length Polymorphisms) (Vos *et al*., 1995) represents a really technological innovation that combines the power of RFLP with the flexibility of PCR-based technology. In order to perform the AFLP study, DNA of interest is restricted and fragments obtained are ligated to primer-recognition sequences (adaptors). After that a selective PCR of the restricted fragments is performed through a specific set of primers. Most of AFLP fragments correspond to unique positions on the genome, therefore they can be exploited as landmarks in genetic and physical mapping. AFLP study can be employed to discriminate closely related individuals at the sub-species level (Althoff *et al*., 2007), and CAN ALSO MAP GENES. Applications for AFLP in plant mapping include establishing lindage groups in crosses. The amplified fragments are analyzed by polyacrilamide gels through an automatic sequencer.

The technique is very reproducible and reliable but its drawback is that it isn't too cheap. In addition these markers are dominants.

SNP

SNPs (Single Nucleotide Polymorphism) (Collins *et al*., 1998) are very common molecular markers in the genome of all organisms, and they are widely distributed throughout genomes even though their distribution is different among different species, 1 SNP per 60-120 bp for maize and 1 SNP per 1,000 bp for humans, (Ching *et al*., 2002; Sachidanandam *et al*., 2001). The number of SNPs is quickly increasing because of the improvement of sequencing technology and availability of a great number of EST sequences that identify genetic variation at DNA level (Bueton *et al*., 1999).

Their fame is associated with high efficiency of genotyping methods such as DNA chips, allele-specific PCR and primer extension (Sobrino *et al*., 2005) through which population analysis, germplasm fingerprint and cultivar identification, high density genetic maps, and genotype/phenotype association have been done in a fast way. In addition, SNPs answer to many biological questions, too. In fact, they are associated with different illnesses such as cardiovascular disease, Alzheimer or thrombosis. In plants, SNPs have been found to be

associated with useful traits, such as fragrance (Bradbury *et al*., 2005) or a starch gelatinization temperature trait.

Despite great potentialities for SNPs, a preliminary knowledge of DNA is required for their analysis, a condition that taxes their use when the study is related to an unknown genome.

SSR

SSR or microsatellites (Tautz *et al*., 1984) is a class of markers largely used for different needs and in particular for foodstuffs analysis.

The microsatellites or SSRs are small regions (100-300 base pairs), consisting of tandem repeats of 1-6 pairs (Hamada *et al.*, 1982). They have the peculiarity of being highly polymorphic, co-dominant, relatively abundant and highly dispersed within prokaryotic and eukaryotic genomes, as well as being discernible in other related species (Field et al., 1998, Toth et al., 2000). Their study requires a very low quantity of starting DNA, and the analysis is fast and simple, as samples are analyzed through a simple PCR, and the results obtained are robust and reproducible.

Because they are able to be analyzed in very small regions, the SSRs are proving to be suitable for the study of very degraded DNA, such as that which is obtained from many highly processed food matrices. SSRs in fact, allow us to study matrices with very few and highly fragmented DNA and, as highly polymorphic, are elected for food traceability studies as much as for forensic and medical purposes or for cultivar characterization. Several studies have demonstrated that SSRs are the best markers for studying complex matrices like oils which contains very low quantity and very highly fragmented DNA too (Powell *et al*., 1996; Pasqualone *et al*, 2004). It is already clear that these microsatellites are characteristically very versatile. In addition they have the advantage that the same markers can be used to study related species (Field *et al*., 1998; Toth *et al*., 2000; Rallo *et al*., 2003). This is a very important aspect because the development of locus-specific SSR oligonuclotide primers is time consuming and expensive. Particularly in the 1990s, EST and genomic libraries were screened for identifying sequences that contained microsatellite motifs in order to develop specific primers. The procedures were expensive and inefficient (Squirrel *et al*., 2003).

Nowadays the availability of large numbers of ESTs and other DNA sequences from a data bank helps to identify new SSR markers according to an efficient and cost effective way in respect to the past.

The high polymorphism that distinguishes the microsatellites (higher than in other genomic regions) is most likely due to a mechanism called “slippage”. This mechanism occurs during the replication of repeated sequences. During the formation of replication, stranded DNA that is

separated from the mold reappear in an inappropriate manner, while still allowing the polymerase to continue its polymerization, and thus create a new mutation or polymorphism. In fact, slippage has generated a new polymorphism as a result of duplication or a deletion of one or more of the base units that constitute the tandem repeats of the microsatellite (Schlotterer and Tautz, 1992).

However, although very highly polymorphic, SSRs are relatively easy to study because they are characterized by very well conserved flanking regions and, therefore, indispensable for the construction of appropriate primers through which their amplification and then their analysis proceed.

Until a few years ago, microsatellite regions were defined by a neutral character, and not subject to selective pressure, therefore had no effect on the functional morphology of the organism in question. Today, many studies show that the expansion of the number of repetitions of some markers may also be associated with some serious human diseases. (Cumming *et al*., 2000; Everett *et al*., 2004).

7. THE MOLECULAR FINGERPRINT

Molecular-fingerprint-coming has been useful for very different purposes for forensic or medical analysis or for germplasm characterization.

The fingerprint represents the DNA polymorphisms of a genome. It is obtained by the analysis of a genome with specific molecular markers. Each matrix, therefore, can be associated with a specific and unmistakable fingerprint. Obviously a fingerprint associated with a particular individual may be different if different SSRs are considered. It follows that it is only possible only if the comparison between fingerprints is generated by the same markers. Commonly, to identify a fingerprint of multiple individuals or species or food samples, it is not sufficient to analyze a single marker but it is needed to consider many markers simultaneously. However, samples can be discriminated through the analysis of only one microsatellite in the extremely rare case in which its molecular weight is different for each analyzed sample.

Very often to analyze matrices by molecular markers it isn't necessary to know their whole genomic sequence. This means that it is possible to characterize or define a fingerprint of whatever matrix even though its total DNA sequence is unknown.

In any case, whatever marker is used, the results related to a specific sample remain identical along its whole life. There is no variability in the analyzed parameters due to environmental or

temporal or technological changes, therefore the results can be considered an authentic identity card.

In particular microsatellites have demonstrated their efficiency on foodstuffs characterization because through them it is possible to analyze matrices containing very few and damaged DNA, typical of food matrices. In addition their study is possible through a simple and inexpensive methodology with reliable and reproducible results.

Ultimately, the genetic profile, due to its uniqueness, not only manages to reconstruct the origin of the material studied, but also identifies possible relationships of species or family without the possibility of uncertainty.

The key point of the fingerprint resides in the fundamental parameters of the quality and quantity of DNA, and the selection of the right molecular markers.

The extraction of DNA, a very delicate and laborious step, is an issue to be tacked with skill, especially when it is profoundly transformed by technological processes, arrays or matrices such as oils. There are many extraction methods, some of which are well represented by both commercial kits from traditional systems. Choosing the right extraction method may require a rigorous selection process, and possibly depends on the type of array or the purpose for which you want to do the extraction (Akkak *et al*., 2008), but you cannot find a solution for all needs.

Choosing the right molecular marker is an extremely delicate and critical step. There are several classes of genetic markers, which differ in intrinsic differences or the technology used in their study. The choice of the class and right markers within the same class is important because an incorrect assessment can completely affect the result. The first choices to be made depend on the purpose for which you intend to study DNA, the economic resources available, the reproducibility and the simplicity of the system to be used.

The fingerprint obtained ultimately requires a statistical analysis to evaluate the efficiency of the system and microsatellites used only for the actual processing of the result for which we undertook the study (in the construction of dendrograms, or characterizations of linkage maps in the case of characterization of genomes).

In light of the foregoing, the characterization of fingerprints actually corresponds to the award of a guaranteed mark in terms of origin, quality and safety, and also to the award of a certificate of traceability, thus adding value to the product studied.

7.1 Oil fingerprint

The use of fingerprint technology in the oil sector has only recently found its optimal and successful implementation. This stems from the fact that only the use of markers such as SSR of

the new generation, which have revolutionized this field, allow the study of raw materials, and thus the identification of fingerprints, even in difficult matrices, very successfully.

In the olive sector, therefore, the markers that represent the first choice for fingerprinting analysis are the SSRs. These are optimal for analysis for the following reasons:

1) Their small size allow you to study genomic DNA, even if very fragmented, as in the case of DNA recovered from oils.
2) Their high polymorphism gives them a high discriminating power and ability, particularly useful in the characterization of blended oils.
3) Their wide distribution and abundance, and the fact that they are widely dispersed in genomes, enables their genome to be analyzed in its entirety, and to be completely characterized.

It follows that for these features, SSRs have become absolutely unique and indispensable, especially in the cases where you want to do molecular traceability in oils. Today, there have been many studies conducted on olive oil, which has shown that the molecular road we are taking is the right one. Many varietal characterization studies that have been performed with blended SSR oils and single cultivar (certified or not) have obtained interesting results ((Pasqualone *et al*., 2004; Testolin *et al*., 2005)).

In contrast, studies of fingerprints on the oil of nuts, yet not prominent in the Italian markets, are still non-existent, and the development of such studies should be pursued because nuts and their oil derivatives are products emerging in many countries of the Mediterranean Basin, and, above all, nut oils are used as a product for virgin olive oil adulteration.

As mentioned in the literature, molecular studies on hazelnut oil are non-existent. However, there are several studies that have been carried on hazelnuts, which led to the identification of many molecular markers RAPD (Galderisi et al., 1999). SSRs, moreover, could potentially be used as markers for characterization studies for hazelnut oils, and for identification of hazelnut oil in virgin olive oil, when added fraudulently (Botta et al., 2005; Boccacci et al., 2005; Gokirmak et al., 2009).

8. MICROSATELLITES ANALYSIS

Microsatellite analysis assumes the following three steps: DNA extraction, amplification, and detection of microsatellite fingerprint.

For years it has been reported in the literature that extraction of genomic DNA has proceeded along long and laborious methodologies. However, for some years there were new products introduced which were able to simplify and optimize the extraction as well as make it more

automated. The commercial proposals are very diverse, taking into account the purposes for which the DNA must be used, and their reduction requirements in terms of timing and estimated costs of such analysis.

In traditional methods, surely the introduction of the use of the detergent cetyltrimethylammonium bromide (CTAB) was critical to optimize the extraction of DNA free of contaminants (carbohydrates, lipids, and phenolic compounds). Such contaminants are potentially responsible for the degradation of DNA or inhibition of the enzymatic activity of the polymerase during amplification (Murray et al., 1980; John et al., 1992). However, the traditional methods have gradually lost importance because of the laborious protocols, and the need for use of harmful substances, such as phenol and chloroform, for the elimination of contaminants, and for the inhibition of the activity of nucleases.

New business methods have been passed to the extraction of DNA through the use of silica (in solution or packed in columns) (Ohmori et al., 2008). In such methods, where the principles of Chromatography are exploited, the DNA binds to the silica through the use of chaotropic salts which, in addition to allowing the recovery of DNA, optimize the removal of contaminants.

In fact, there is no ideal method for all expectations, but methods should be refined and adapted to individual requirements, whether technical-scientific or economic.

In the case of oil matrices, the extraction of DNA is already a delicate step.

DNA which is derived from oil is extremely degraded. This is because the traces of DNA which remain are there due to poor cellular debris remaining after oil extraction. Furthermore, the technological treatments suffered cause substantial DNA damage, and therefore fragmentation. (Spaniolas *et al.,* 2008).

Indeed, only a few methods, both traditional and commercial, offer really good solutions for the oil. In most cases, the methods used are only really suited for the oil matrix, and only after long and elaborate tests of development and comparisons of different methodologies (Proietti *et al*., 2003).

Some methods suggest the recovery of DNA directly from small amounts of oil (≈200 μl) while others in large amounts (≈500 ml), using in the latter case pellet centrifuged oils instead of the oil itself. Finally, there are some who suggest the extraction of oil previously treated with solvent (Proietti *et al*., 2003; Breton *et al*., 2004; Testolin *et al*., 2005)

In some cases, however, the use of protease inhibitors is also suggested in oils, especially if you engage in studies involving the analysis of these over time in order to prevent an excessive degradation of DNA so as to render it inadequate to any analysis.

The study of microsatellites makes the use of PCR techniques rapid and simple, and has the objective of identifying the polymorphisms between individuals, or to observe the differences in molecular weight of the amplicons obtained from the same markers, but in different individuals, and attributable to the different number of units that are repetitive.

A study with microsatellites presupposes knowledge of the sequences of microsatellites to be used, although knowledge of the genome to be analyzed is not required.

Many microsatellites have been isolated and characterized by some authors in different species of agronomic interest, or simply botanical or zoological through screening of genomic libraries (Thomas *et al*., 1993; Yamamto *et al*., 2002; Carriero *et al*., 2002). Sequences and sequence analysis of SSR, once published, are then collected in specific online banks.

Knowing the sequence of an SSR is then a necessary and sufficient condition so that the SSR can be used to characterize a genome. Through its sequence, it is, in fact, possible to draw the appropriate primers corresponding to the conserved regions flanking the microsatellite through which to proceed to its amplification.

To proceed to the study of SSR, however, one must first develop the amplification conditions. When these are carried out, the result is sieved on agarose gel. These early amplifications are used only as a preliminary screening since it is not completely suitable for the identification of all types of polymorphisms. The polymorphisms are characterized by differences of a few repeating units of the microsatellite. In fact, they are not detectable by a simple observation of the agarose gel, hence one has to resort to alternative systems.

Up to few years ago microsatellites analysis were evaluated through a capillary electrophoresis which permits us to determine the specific molecular weight of each microsatellite, to identify possible polimorphisms and to define the specific fingerprint of each genome analyzed. Its high sensitivity, rapidity of execution as well as its separation efficiency makes this methodology an indispensable tool in cases in which it must identify polymorphisms due to differences of even a single base pair. To use this methodology, it is necessary to carry out further amplifications that, though carried out accurately compared to those developed, require the use of primers labeled with fluorochrome in order to be identified by the detection system of laser apparatus for EC.

The statistic elaboration of data obtained through CE allows us to characterize each microsatellite, through understanding the number of alleles (n), the relation between the observed heterozygosity (Ho) and expected one (He) (Nei 1973), the polymorphic information content (PIC), and the power of discrimination (PD) (Jones 1972). This information allows us to evaluate if a specific microsatellite would be useful and informative in a specific work.

Amplification of microsatellites in oils requires basically the same procedures used for other matrices supplemented by small measures.

First, oil in the matrix is frequently necessary to repeat the amplification many times in order to identify the correct quantity of DNA to be used in the reaction. As mentioned previously, the DNA, if not extremely pure, as happens in these matrices, may contain contaminants able to inhibit the amplification reaction, or excessively degraded DNA, which can invalidate the results. In effect, a correlation has been observed between a diminished efficiency of PCR, probably due to an increase of inhibitors, and biochemical changes that occur in the oil during its storage. This suggests that the increase of oxidation products and the decrease of antioxidant substances may in some way be, in time, responsible for the degradation of DNA, and the accumulation of PCR inhibitors (Spaniolas *et al*., 2008).

To overcome this drawback before undertaking a study on a DNA extracted from oil, it is necessary to test its suitability to the analysis of PCR. For this purpose, amplifications of control are made that produce amplicon of at least 500 base pairs in order to assess the state of DNA fragmentation.

In oils, the amount of DNA representing the limiting appearance is frequent. This means that in some cases, it is necessary to undertake double-cycles of amplifications to obtain an amplification visible on agarose gel. In these cases, the obtained amplification with the first round of PCR is used as a template for a second round of amplification, using primers designed a little more internally with respect to those used in the first stage of amplification (nested primers). If the double amplification cycle increases, the amount of amplified also increases the risk of obtaining amplicons. That risk is strongly contingent on the use of nested primers.

In other cases, instead, one is forced to repeat amplifications in order to test the correct quantify of DNA to be used in the amplifications. Without this repetition, this inhibits the relationship or the obstacles for the presence of inhibitors in the genetic material, which is easily present in genetic material extracted in oil.

AIM OF THE WORK

Hazelnut oil can be a very precious product for the health because rich of bioactive molecules but it can be used for nefarious purposes too. Sophistication of olive oil with hazelnut oil is difficult to discover and often it requires sophisticate and expensive procedures to solve the problem, because of similarity of composition among olive and hazelnut.

The aim of this work is to identify a simple and reliable molecular method to characterize hazelnut (*Corylus avellana*) oil not only for assessing its origin but mainly for identifying it when it is used for extra virgin oil adulteration. To do this microsatellite markers will be considered.

Although a few molecular analyses of hazelnut are already available in the literature, the application of the methods to a different and more problematic matrix such as oil is not an easy task.

In order to achieve this aim, the molecular approach will be set up on hazelnut matrix before. First of all five representative Italian and foreigner hazelnut cultivars will be selected and used for the optimization of DNA extraction method. Then the appropriate microsatellite will be chosen and identified the right PCR conditions for cultivar characterization. The optimized methodology will be taken advantage of the characterization of oil matrix. The genomic polymorphisms (fingerprint) identified through microsatellite study will be compared and verified the potentiality and reliability of the molecular approach as system for molecular traceability of the oil.

This PhD thesis is concerned with setting up a simple method of traceability for hazelnut oil through a molecular approach. The objective of the thesis has been met through three steps: 1) identification of the best experimental conditions and appropriate microsatellites; 2) characterization of five hazelnut cultivars; 3) application of this method to hazelnut oil. The

results show that molecular analysis allow us to characterize and recognize hazelnut oil by microsatellites analysis even in filtered oil, although filtration slightly reduces this capability.

MATERIALS AND METHODS

PLANT TISSUE AND OIL SAMPLES

Hazelnut

Hazelnuts from five different cultivars were analyzed. Mortarella (Mo), Tonda Gentile delle Langhe-TGL, Tombul (To), Tonda di Giffoni-TG were collected in the experimental farm of the Lazio's Regional Agency for Experimentation in Agriculture. Tonda Gentile Romana-TGR was bought from a commercial company (Bionocciola).

Plant used as control

The plants, *Olea*, *Marcanthia*, *Nephrolepis* and *Picea*, used as control to check the specificity of primers, were bought in a garden centre.

Oil extraction

Oil was extracted by a cold-press system from two different pools (O1 and O2) of TGR cultivar. Half of each produced oil was filtered through cotton filters (1OF, 2OF). The four samples were then bottled and conserved at 4°C.

When sediment of oil was used, 50 ml of not filtered oil and 500 ml of filtered oil were centrifuged at 3.000 r.p.m at 10°C for 25 min and 40 min respectively. This procedure allowed to obtain, approximately, 100 mg of pellet

DNA EXTRACTION METHODS

DNA extraction from hazelnut pools

For each cultivar, 300 g of hazelnuts were homogenized under liquid nitrogen with a mini mill (IKA).

In addition to the traditional method (Consolandi *et al*., 2008), several kits have been tested (QIAmp DNA stool-Qiagen, Genomic DNA from Nucleospin Food-Macherey-Nagel, Gene Elute Plant-Sigma) on two pools of Tonda Gentile Romana (TGR) hazelnut cultivar to define the most efficient, simple, and economic method for DNA extraction.

As respect to the protocol of each kit, some modification to adapt them to hazelnut matrix have been introduced.

For all the kits, samples were treated with 0,3 μg/μl of RNase A and with 0,05 μg/μl of proteinase K to avoid any interference on either spectrophotometric reading and amplifications.

In the protocol of Sigma (S), the quantity of lysis solutions were increased about 30% (up to 500 μl for solution A (containing caotropic salts), and up to 60 μl for solution B). The other buffers were increased proportionally to the lysis buffer.

For Macherey-Nagel (M-N) kit 100 mg or 200 mg of raw material was used, and a double centrifugation was preformed, after the initial incubation of 30 min at 65°C, to facilitate supernatant recovery.

In Qiagen (Q) protocol, 200 mg of sample were treated in 1,6 ml of buffer ASL. After addition of buffer containing chaotropic salts, the samples were homogenized with a mixer. The elution was made in 100 μl.

Regarding the traditional (T) Consolandi method (Consolandi *et al*., 2008), normally suggested for oily matrixes such as hazelnuts, 200 mg of powder was used for the extraction. After the incubation at 48°C, the centrifugation has been performed thrice at 14,000 rpm for 20 min rather than once at 12,000 rpm for 30 min.

DNA concentration and cleanness were determined spectrophotometrically for micro samples quantification (NanoDrop) analyzing each sample at 260, 280 and 230 nm. DNA was detected at 260 nm, whereas absorbance at 280 nm was an index of proteins contamination and absorbance at 230 nm was used as index of other macromolecules (carbohydrate, phenols, peptides and aromatic molecules) contamination.

DNA extraction from single nuts

From each of 12 nuts 100 mg of tissue (including the embryo) was extracted and homogenized thrice through a tissue lyser (Qiagen) treatment for 60 sec, in presence of a 5 mm stainless steel bead.

DNA extraction from plants

DNA from from *Olea, Marchanthia, Nephrolepis* and *Picea* leaves was extracted by Gene Elute Plant-Sigma kit. Leaves were homogenized as decribed for hazelnut samples and then extracted as described in the specific kit protocol.

DNA extraction from oil

Several oils were firstly treated for 90 sec with a tissue lyser in the presence of a stainless steel bead and of the lyses buffer.

Total DNA from oils was extracted with columns system using Nucleospin Food-Macherey-Nagel (M-N), through magnetic beads with Wizard Magnetic DNA Purification System for food-Promega (P), by solvent aid with DNabsolute-Geneaid (G) and Extract-N-Plant PCR-Sigma (S1) kits, while DNA from sediment oil was extracted with S and S1 kit only.

DNA concentration and quality of each sample was determined through spectrophotometric assay (NanoDrop) at 230, 260 and 280 nm.

Primers

Specific hazelnut primers for DNA quality check were designed at the boundary of the intron/exon junction. LOX, LOX1, LOX3 primers were designed on the lipoxygenase gene, while ADH primers were designed and specific for a hazelnut dehydrogenase gene (Tab. 2). AJ810086 primers amplified a portion of a β-1,3-glucanase olive gene (Table 2). Nine SSR loci, CAC-A014a, CAC-B028, CAC-B029b, CAC-B111 (Bassil et al., 2005), CaT-B107, CaT-B502, CaT-B504, CaT-B507, CaT-B508 (Boccacci et al., 2005), were chosen for fingerprint analysis (Table 3).

PCR conditions

PCR was performed in a total volume of 25 μl containing 1X PCR buffer, 2,5 mM MgCl2, 0,8 mM dNTPs, 100 ng DNA, 2,5μM of each primer and 1U Taq polymerase Gold (Applied Biosystem).

Gene amplification conditions were: initial denaturation cycle of 95°C for 10 min, 35 cycles of denaturation at 95°C for 30 s, appropriate annealing temperature for 30 s and extension at 72°C for 30 s. A final extension cycle at 72°C for 7 min. The amplifications were performed in a Biorad termocycler. PCR products were observed on 2% agarose gel stained with Gel Red (Biotium).

In each experiment, negative control was factored in the reaction mix used for the current amplification without any DNA, while positive control was likewise considered in the same mix, using hazelnut DNA with primers LOX3.

Amplifications products were observed on 1,2 % agarose gel, or on 2,2 % pre cast agarose gel of Flash Gel System (Lonza). The molecular weight of fragment was evaluated through 500 ng of Lonza FlashGel marker, M** (100-4000 bp) or Fermentas markers, M (1Kb) and M* (50bp) .

The definitive fragments size were measured through a capillary electrophoresis (Applied Biosystems, mod. 3730) using forward primers labeled with 6-FAM (6-Carboxyfluorescein).

SSR locus	Primers Forward (5'-3')	Primers Reverse (5'-3')	Ta
LOX	TGGCTGGAGTAAACCCTGTC	TTCAATCACCAATGGCTTCA	55°C
LOX1	CCCAAAGGCTTTCAACCATA	CTTTTTGTTCCCGTCATCGT	55°C
LOX3	CCGTACCTGAGGCGAATAAA	CTGGCGTGAACACTTTGCTA	55°C
ADH	TGAATGTTGCAAAACCGAAA	CAGCAGCCTAATCACAACGA	50°C
AJ810086	GTCCCAGTTCGAATTTGGC	TCCCCTGTTGATGGAGGATAC	60°C

Table 2 Sequence and annealing temperature (Ta) of primers used for DNA quality check.

PCR conditions for microsatellites analysis

PCRs of microsatellites were performed in a total volume of 25 µl containing 1X PCR buffer, 0,3 µM of each primer, 2,5 mM MgCl2, 100 ng DNA, 0,8 mM dNTPs, and 0,5U Taq polymerase Gold (Applied Biosystem).

Microsatellites were analyzed according to an initial cycle at 95°C for 10 min, 28 cycles at 95°C for 30 s, appropriate annealing temperature for 45 s, an elongation step at 72°C for 90 s, and finally a cycle at 72°C for 45 min. The amplifications were first checked by agarose gel but the definitive fragment size on TGR, TGL, Tombul and TG DNAs was carried out by a capillary electrophoresis (Applied Biosystems, mod. 3730) using the same primers used for agarose gel analysis but with the forward primers labeled with 6-FAM (6-Carboxyfluorescein).

Before performing PCRs for microsatellites on DNA samples obtained from filtered oil, it was necessary to carry out a generic amplification of the genome, which was performed through the GenomePlex Complete Whole Genome Amplification (WGA) Kit (Sigma) according to manufacturer's instructions.

SSR locus	Primers Forward (5'-3')/Primers Reverse (5'-3')	Ta
CAC A014a	GGTTTGTTACAGAAATTCAGACG/GCGTGTGGTTAATGTTTTCTTT	55°C
CAC B028	ATGGACGAGGAATATTTCAGC/CCTGTTTCTCTTTGTTTTCGAG	55°C
CAC B029b	CAATTTACACCTCAGGGAAGAG/AAGTTCACCCAAGAAATCCAC	55°C
CAC B111	GAAGGAGAAACAAGGGTAGTCA/AGAAGCGTCGTTCCATAGC	55°C
CAT B107	GTAGGTGCACTTGATGTGCTTTAC/AACACCATATTGAGTCTTTCAAAGC	55°C
CAT B502	CTCATGACTGCCCATTTCTCG/AGGCATGCAGGCTTCACAC	55°C
CAT B504	CGCCATCTCCATTTCCCAAC/CGGAATGGTTTTCTGCTTCAG	55°C
CAT B507	CTAAGCTCACCAAGAGGAAGTTGAT/GCTTCTGGGTCTCCTGCTCA	55°C
CAT B508	GGGTCAAGATTTGATAAAGTGGGA/GCACTCCACTTGTGCGTTTTC	55°C

T**able 3**. Sequence and annealing temperature (Ta) of primers used for SSR analyses.

Statistical elaboration

Statistical elaboration of data has been performed by statistic software R with the main aim of defining the discrimination power (DP) and the allele locus number (n). Other parameters were however also considered: expected and observed heterozygosity (He and Ho respectively), polymorphic information content (PIC) and probability of identity (PI). He and Ho measure the homogeneity of a population. The PIC of a marker is the probability that the marker genotype of the offspring of a heterozygous parent affected with a dominant disease allows one to deduce which marker allele the offspring inherited from the parent. It is a measure of a marker's usefulness for linkage analysis. PI measures the probability of finding 2 identical genotypes, while PD indicates the probability that 2 random genotypes could be discriminated by their SSR profile.

The analysis of the data bank (USDA-ARS National Clonal Germplasm Repository, Corvallis, Oregon) (Gokirmak *et al*., 2009) was performed through a Perl script developed for the specific purpose. The program allowed to compare the results obtained on TGR individual hazelnuts with those existing in a data bank.

RESULTS

1. DNA EXTRACTION

1.1. Identification of the best extraction method on TGR cultivar

As described in the methods section, 4 different kits (QIAmp DNA stool-Qiagen, Genomic DNA from Food-Macherey-Nagel, Gene Elute Plant-Sigma) have been tested together with the traditional method (Consolandi et al., 2008) on two hazelnut pools of Tonda Gentile Romana (TGR) hazelnut cultivar, with the aim of selecting the most efficient, economic and simple methodology.

The different methods and the different tested kits resulted able to quantitatively recover DNAs with a highly variable efficiency (Table 4). The quality of the recovered DNAs resulted also widely different, as indicated by the wide variability in the 260/280 nm ratio (indicating the contamination by proteins;1,8 expected value) and 260/230 ratio (indication the contamination by carbohydrates, phenols and aromatic substances; 2,2 expected value).

The results obtained with Macherey-Nagel kit varied significantly according to the experimental conditions. The best results were obtained by increasing the amount of salt and performing multiple centrifugations (samples 27n and 28n) respect to the unmodified method (samples 5on, 6on). However , also the sole increasing of lysis/caotropic salt made possible to observe a substantial improving of DNA recovery (samples 1n and 2n). Concerning the DNA quality, in general, all samples analyzed by Macherey-Nagel kit resulted satisfactory.

Concerning to Qiagen kit, the recovery in the samples extracted as suggested in the kit recommendations (11o, 12o) resulted very low, with an evident contamination, as indicated by the 260/230 nm ratio. Nevertheless, also in this case, increasing amount of ASL buffer (lysis buffer) lead to a proportional increasing of the recovery (samples 7o, 8o, 9n, 10n) and to an improving of the quality of the recovered DNA.

Gene Elute Sigma kit DNA did not provide satisfactory results (samples 13n, 14n, 15o, 16o) for both the quantity and quality of recovered DNA. The same unsatisfactory results were also obtained even when lysis/chaotropic buffer were increased and when a double centrifugation was performed (15o, 16o).

Sample ID	matrix	ng/ul	260/280nm	260/230nm	method	modification
1n	TGR-pool 1	344,42	2,08	2,09	M-N	highsalt/chao
2n	TGR-pool 2	392,64	2,10	2,17	M-N	highsalt/chao
5on	TGR-pool 1	39,3	1,86	1,75	M-N	standard
6on	TGR-pool 2	91,1	1,99	2,17	M-N	standard
7o	TGR-pool 1	25,4	1,83	1,77	Q	high salt
8o	TGR-pool 2	33,7	1.92	1,16	Q	high salt
9n	TGR-pool 1	160,3	2,11	2,20	Q	high salt
10n	TGR-pool 2	191,7	2,12	2,08	Q	high salt
11o	TGR-pool 1	5,2	1,82	0,24	Q	standard
12o	TGR-pool 2	3,4	2,12	0,22	Q	standard
13n	TGR-pool 1	5,1	1,8	0,22	S	standard
14n	TGR-pool 2	4,1	-6,88	0,01	S	standard
15o	TGR-pool 1	3,5	2,02	0,17	S	high salt/chaot+spin
16o	TGR-pool 2	4,8	1,36	0,18	S	high salt/chaot+spin
17o	TGR-pool 1	0	0	0	T	standard
18o	GR-pool 2	0	0	0	T	standard
19o	TGR-pool 1	131,6	1,24	0,43	T	multiple spin/2
20o	TGR-pool 2	69,6	1	0,23	T	multiple spin/2
21o	TGR-pool 1	376,3	1,1	0,28	T	multiple spin/3
22o	TGR-pool 2	609	0,89	0,19	T	multiple spin/3

Table 4. Spectrophotometric analyses of DNA samples from TGR hazelnut samples. M-N, Q, S, T indicate Macherey-Nagel, Qiagen, Gene Elut Plant Sigma and traditional DNA extraction methods respectively.

Finally, Consolandi method provided very different results. Both the unmodified (17o, 18o) and modified methods (19o, 20o, 21o, 22o) allowed to extract DNA that however resulted highly contaminated by both proteins and other macromolecules.

These assays suggested that the Macherey-Nagel kit is the best suitable method for this oily matrix, for both the recovered DNA, that was satisfactory bot in terms of quantity and quality, and for its simplicity of use.

On the contrary, the Sigma Kit and Consolandi method resulted the least suitable methods for this kind of matrix.

It is however worth to mention that the described spectrophotometric analysis are only indicative of DNA quality, useful for identifying and defining the best suitable methodology for extracting DNAs. To confirm these data a further PCR check of them was necessary and is successively described.

1.2. DNA extraction from TGR, TGL, Mortarella, TG and Tombul cultivars

Table 5 shows the recovery and quality of DNA extracted from various cultivars by Macherey-Nagel. As expected, the good results obtained on the TGR cultivar have been also obtained for all the cultivar. Both the amount and the quality of DNA was satisfactory for all the cultivars.

Sample ID	matrix	ng/ul	260/280nm	260/230nm	method	modification
24n	Tombul	345,2	2,14	2,25	M-N	high salt/chao+spin
25n	TG	347,1	2,12	2,12	M-N	high salt/chao+spin
26n	TGL	386,1	2,11	2,17	M-N	high salt/chao+spin
27n	TGR-pool 1	445,7	2,11	2,18	M-N	high salt/chao+spin
28n	TGR-pool 2	493,1	2,12	2,22	M-N	high salt/chao+spin
29n	Mortarella	383,2	2,09	2,11	M-N	high salt/chao+spin

Table 5. Spectrophotometric analyses of DNA samples extracted from different cultivars. M-N indicates Macherey-Nagel kit.

1.3. DNA extraction from oil

DNA extraction tests were performed on four samples, two not filtered oils (O1 and O2) and two filtered oils (1OF and 2OF). For DNA extraction from oil, M-N and S kits, already used for hazelnut DNA extraction, and G, P and S1 kits were used.

As expected, the quantity of DNAs recovered from the oils was very low, basically because oil contains very low amount of cell residues. However satisfactory results have been obtained.

Sample ID	matrix	ng/uL	260/280	260/230	method	modification
30	hazelnut oil O1	174.5	1.03	0.23	G	300 µl
31	hazelnut oil O2	322.9	0.93	0.51	G	300 µl
32	hazelnut oil O1	128.7	0.81	0.15	G	300 µl+tissue lyser
33	hazelnut oil O2	304.6	0.88	0.49	G	300 µl+tissue lyser
34	hazelnut oil O1	66.1	2.12	2.10	M-N	5ml
35	hazelnut oil O2	123.3	2.13	2.17	M-N	5ml
36	hazelnut oil 1OF	3.6	1.28	0.51	M-N	5ml
37	hazelnut oil 2OF	3.8	1.25	0.73	M-N	5ml
38	hazelnut oil O1	6.1	1.51	0.74	M-N	1ml
39	hazelnut oil O2	88.0	2.10	2.2	M-N	1ml
40	hazelnut oil 1OF	2.3	1.31	0.51	M-N	1ml
41	hazelnut oil 2OF	1.4	1.55	0.36	M-N	1ml
42	hazelnut oil O1	177.9	2.43	0.24	P	160 ml
43	hazelnut oil O2	1016.9	0.89	0.22	P	160 ml
44	hazelnut oil 1OF	50.2	1.91	0.19	P	160 ml
45	hazelnut oil 2OF	155.1	2.31	0.36	P	160 ml
46	**sediment** hazelnut oil O1	2.6	2.87	0.68	S	100 mg
47	**sediment** hazelnut oil O2	2.0	4.03??	9.29??	S	100 mg
48	**sediment** hazelnut oil 1OF	0.5	0?	0.59	S	78 mg
49	**sediment** hazelnut 2OF	4.9	1.69	0.31	S	78 mg
51	**sediment** hazelnut oil O1	2056	1.03	0.24	S1	100 mg
52	hazelnut oil O1	307,9	0,7	0,64	S1	200 µl
53	hazelnut oil 1OF	377,5	0,73	0,95	S1	200 µl
54	hazelnut oil O2	361,3	0,73	0,79	S1	200 µl
55	hazelnut oil 2OF	358,4	0,73	0,81	S1	200 µl

Table 6. Spectrophotometric analyses of DNA extracted from hazelnut oil samples. G, M-N, P, S, and S1 indicate Geneaid, Macherey-Nagel, Promega, Gene Elut plant Sigma and Extract-N-Plant Sigma extraction methodologies respectively.

Table 6 shows the spectrophotometric results of DNA extracted from oil. Geneaid kit (samples 30-33) allowed us to recover a good quantity of DNAs even though the observation of 260/280 and 260/230 nm ratios indicated an unsatisfactory quality due to high contamination. A supplementary treatment test with tissue lyser (samples 32 and 33) didn't improved the yield.

The DNAs obtained with Macherey-Nagel kit showed that 5 ml of not filtered oil were enough for a good DNA recovery (samples 34, 35) while the recovery with 1 ml was variable (samples 38, 39).

The extraction of DNA from filtered oil was more difficult, as the same methodology provided DNA in very low quantity and of bad quality either from 5 ml (samples 36, 37) and 1ml (samples 40, 41).

Promega kit required a great amount of oil as starting material and it was a more time consuming and complex. Moreover, the results weren't satisfactory as the DNA quantity was extremely variable and contaminants were always present.

The results obtained for DNA samples extracted from oil sediment with Gene Elute Sigma were totally unsatisfactory, with an extremely low recovery and anomalous 260/280 and 260/230 ratios values indicative of contaminations (samples 46-49).

Finally, Extract-N-Amp Plant PCR Sigma kit (samples 50-55) allowed to recover a sufficient amount of DNA. The method was simple and rapid, but unfortunately, the kit was not satisfactory for this kind of matrix as the recovered DNA resulted highly contaminated.

2. PCR ANALYSIS

2.1. Analysis of hazelnut through PCR

To evaluate more in detail quality of DNA extractedand therefore the efficiency of the different extraction systems, the recovered DNAs were checked through PCR methodologies after the preliminary spectrophotometric test with NanoDrop. To this purpose five couples of primers, each of them with the specific final task to check quality, were expressly designed.

LOX, LOX1, LOX3 primers, all designed on the lipoxygenase gene, were varyingly homologous (with respect to other different organisms), while ADH primers were designed specifically for a dehydrogenase hazelnut gene. AJ810086 primers, instead, were designed to amplify a portion of an olive glucanase gene (Table 2). Primers were designed at the boundary of the intron/exon junction.

A preliminary amplification of TGR1 and TGR2 DNAs (M-N kit) was performed with ADH primers with the aim to identify the optimal condition for PCR experiments (Fig. 1). Amplifications under standard conditions (100 ng DNA, 0,8 mM dNTPs, 5μM primers) (lane 1,

2) were compared with amplifications in the same mix but containing five times more DNA quantity (lanes 3, 4), or the half quantity of dNTPs (5, 6), or half concentration of primers (2,5 μM). Fig. 1 points out that a reduced concentration of dNTPs improve the result as it reduces non-specific amplicons. However the best performance has been obtained with the mix containing a lower amount of primers (lanes 7, 8). Ostensibly an high concentration of dNTPs and in particular of primers determines a rise of non-specific amplicons.

DNA amplification tests performed with LOX3, highlighted in fig. 2, demonstrated, that all DNA samples could be amplified, independently from the kit used and the modification introduced for the extraction, except DNA samples extracted by traditional method. In this case no amplicons were present (samples 19o, 20o, 21o, 22o). Probably contamination of DNA inhibited the PCR process. Samples 2n and 5on showed to have the best performance.

The results of the PCRs confirmed those obtained spectrophotometrically, suggesting Macherey-Nagel (M-N) kit as the most suitable to be used with this matrix, in terms of quality of the results, low cost and simplicity of use.

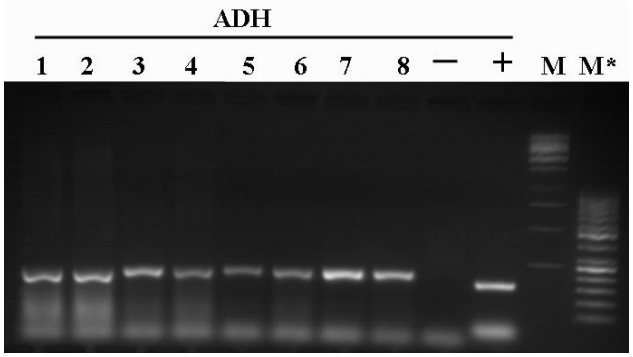

Fig. 1 Electrophoretic agarose gel (1,2%) of the PCR products obtained with ADH primers using TGR1 (lanes 1, 3, 5, 7) and TGR2 (lanes 2, 4, 6, 8) DNAs extracted with M-N kit. DNA amplification under standard conditions (lanes 1, 2); with high concentration of DNA (lanes 3 and 4); with half concentration of dNTPs (lanes 5, 6); with half concentration of primers (lanes 7, 8). (M*) 50bp DNA ladder. (M) 1 Kb DNA ladder.

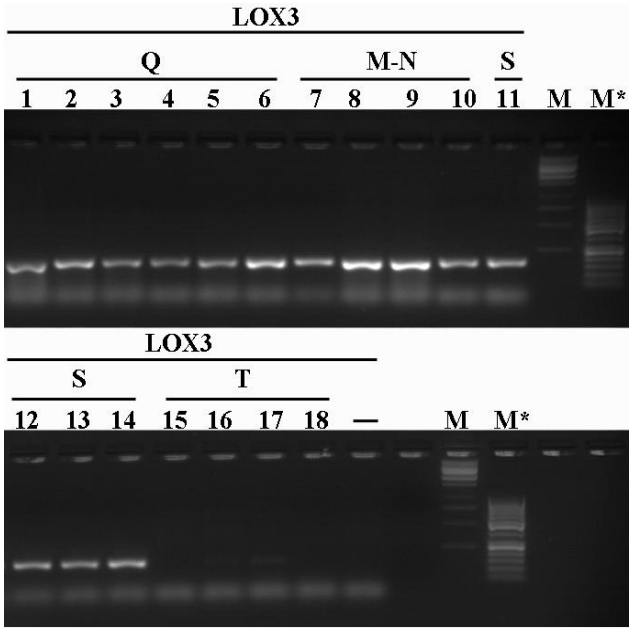

Fig2 Electrophoretic agarose gel (1,2%) of the PCR products obtained using the LOX3 primers. Lanes 1-6 (7o, 8o, 9n, 10n, 11o, 12o DNAs, Table 4). Lanes 7-10 (1n, 2n, 5on, 6on DNAs). Lanes 11-14 (13n, 14n, 15o, 16o DNAs). Lanes 15-18 (17o-22o DNAs). Lane M 1kb DNA ladder. Lane M*, 50 bp DNA ladder.

In order to verify the specificity of the selected LOX3 primers for *Corylus avellana* species, amplifications of DNA samples prepared from *Marcantia*, *Nephrolepis* and *Picea* plants were carried out. The lack of any amplicons in the PCR reactions performed on different plant samples demonstrated that LOX3 was specific for hazelnut species only (Fig. 3, lanes 1-4).
AJ810086 primers (specific for *Olea europaea)* were used for checking possible contaminations of hazelnut DNA preparations, and they did not show any amplification on hazelnut samples, suggesting that the DNA samples were pure (Fig. 4).

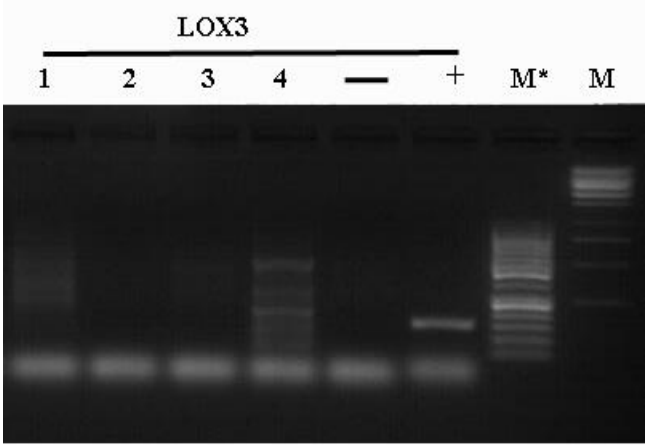

Fig. 3 Electrophoretic agarose gel (1,2%) of the PCR products obtained with LOX3. *Olea* lane 1, *Marcanthia* lane 2, *Nephrolepis* lane 3 and *Picea* lane *4*. Lane * 50bp DNA ladder. Lane M 1 kb ladder.

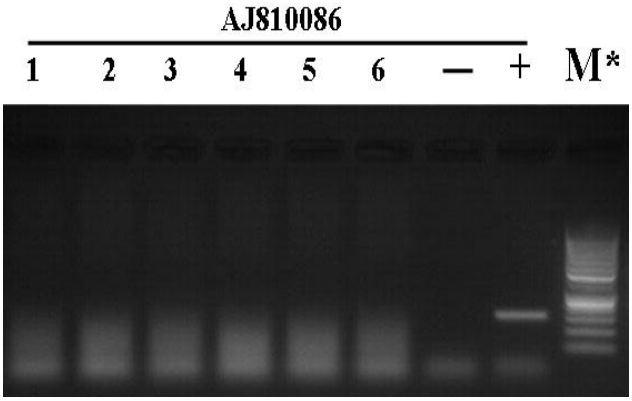

Fig. 4 Electrophoretic agarose gel (1,2%) of the PCR products obtained with AJ810086 primers using DNAs: TGR1 (1), TGR2 (2), TG (3), Mortarella (4), TGL (5), Tombul (6). Lane M and M1, 50bp DNA ladder and 1 Kb DNA ladder respectively.

Concerning the fragmentation level of different DNA preparations, it was evaluated through the use of primers LOX1 and LOX. The amplicons obtained with them are 286bp (LOX1) (Fig. 5) and 478bp (LOX) long (Fig. 6) demonstrating that the DNAs tested present acceptable fragmentation. These experiments confirmed that samples prepared with traditional method were not amplifiable (lanes 17-21).

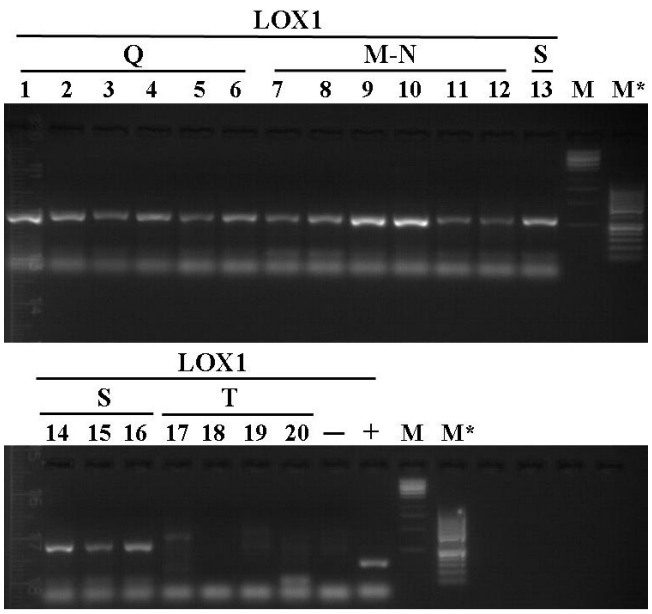

Fig. 5 Electrophoretic agarose gel (1,2%) of the PCR products obtained with LOX1. Lanes 1-6, DNA 7o, 8o, 9n, 10n, 11o, 12o; lanes 7-12 DNA 1n, 1n, 2n, 2n, 5on, 6on; lanes 13-16 DNA 13n, 14n, 15o, 16o and lanes 17-20 DNA 19o to 22o (for DNA details see table 4). Lane M* 50bp DNA ladder; lane M 1 kb ladder.

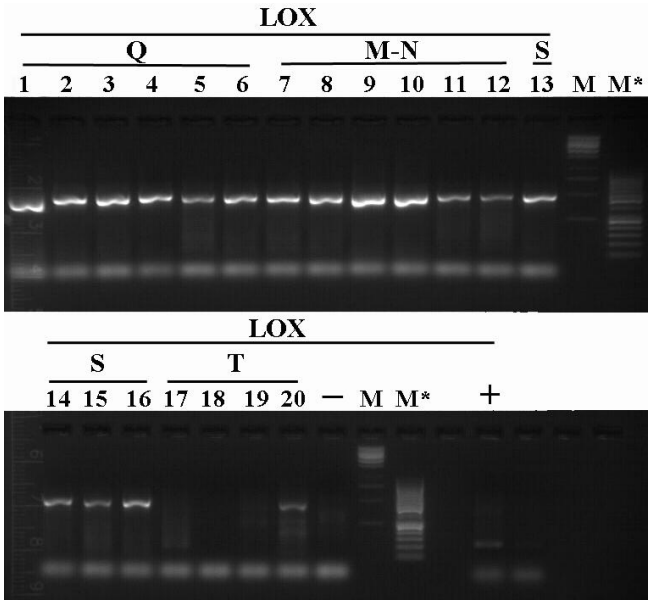

Fig. 6 Electrophoretic agarose gel (1,2%) of the PCR products obtained with LOX using DNAs: 7o, 8o, 9n, 10n, 11o, 12o (lanes 1-6 respectively), 1n, 1n, 2n, 2n, 5on, 6on (lanes 7-12 respectively), 13n, 14n, 15o, 16o (lanes 13-16 respectively), 19o to 22o (lanes 17-20 respectively) (for DNA details see table 4). Lane M and M*, 1 Kb DNA ladder and 50bp DNA ladder respectively.

The PCR condition set up on TGR1 and TGR2 have been successively repeated on the DNAs extracted by M-N Kit from all the cultivars (two pools of TGR, TG, Mortarella (Mo), TGL and Tombul (To)). The amplification resulted highly satisfactory (Fig. 7), the genomic material was not too fragmented (Fig. 8, LOX1 amplification) and it was not contaminated by exogenous DNAs, resulting then suitable for SSR analysis**.**

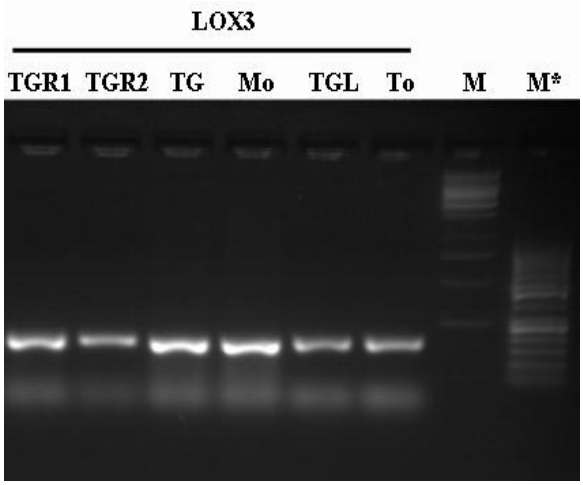

Fig. 7 Electrophoretic agarose gel (1,2%) of the PCR products obtained with LOX3 using DNAs extracted with M-N kit (samples 24n-29n, Table 5). Lane M and M*, 1 Kb DNA ladder and 50bp DNA ladder respectively.

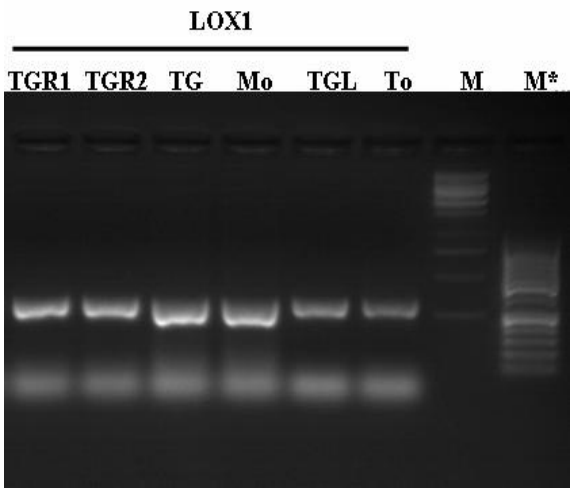

Fig. 8 Electrophoretic agarose gel (1,2%) of the PCR products obtained with LOX1 using DNAs extracted with M-N kit (samples 24n-29n, Table 5). Lane M and M*, 1 Kb DNA ladder and 50bp DNA ladder respectively.

2.1.2. Microsatellites analysis

Microsatellites used for this hazelnut study were chosen taking account of statistical elaboration of same author (Bassil *et al*., 2005; Boccacci *et al*., 2005), and in particular the PD and allelic number.

The PCR conditions for microsatellites analyses have been set up only through CAT B508 microsatellite amplification on TGR cultivar. Results are shown in fig 9 where lane 1 represent the standard conditions (100 ng DNA, 1 μM primers, 1U Taq). The amplification resulted however better by decreasing the amount of primer to 0,5 μM (lane 3), and decreasing the primers to 0,5 μM and the Taq to 0,5U (lane 7).

These PCR conditions have been successively checked on all the microsatellites on TGR-1 DNA (Fig. 10). The good performance of all the amplification confirmed that the selected conditions were suitable for analyzing microsatellites, as no excess of non specific amplicons was observed.

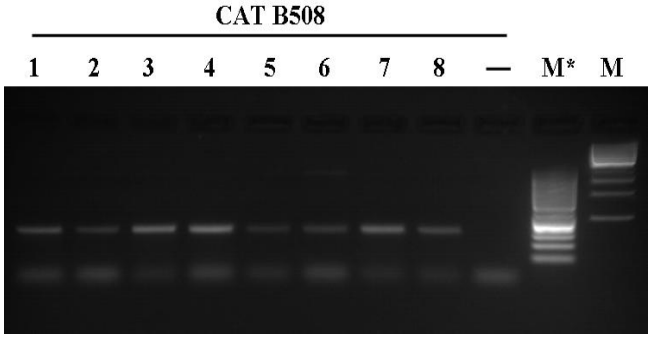

Fig. 9 Electrophoretic agarose gel (1,2%) of the PCR products obtained with CAT B508 microsatellite on TGR1 DNA, extracted by M-N kit (sample 27n). Lane 1 standard PCR conditions, 2-half quantity standard DNA, 3 half standard primers, 4 half standard Taq, 5 half standard primers and DNA, 6 half standard Taq and DNA, 7 half standard primers and Taq, 8 half standard Taq, primers and DNA. Lane 9 negative control . Lane M* 50bp DNA ladder. Lane M 1 Kb DNA ladder.

.

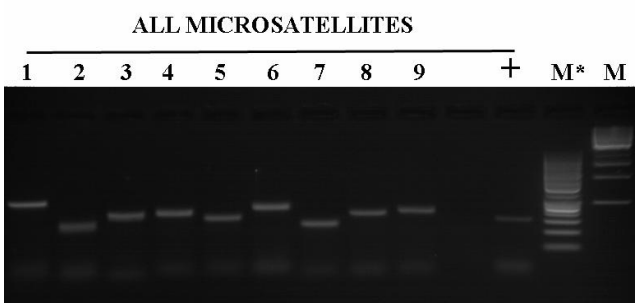

Fig. 10 Electrophoretic agarose gel (1,2%) of the PCR products obtained using TGR1 DNA (extracted by M-N kit, sample 27n) with the following primers: lane 1-CACB028, 2-CaTB107, 3-CaTB504, 4-CaTB507, 5-CaTB508, 6-CACA014, 7-CACB029, 8-CACB111, 9-CaTB502. Lane M* 50bp DNA ladder. Lane M 1 Kb DNA ladder.

The correct performance of PCR was validated on agarose gel, that confirmed the good quality of the results (Figg**.** 11 and 12) and the consequent suitability of the samples for CE analysis.

A preliminary CE analysis demonstrated an high presence of non specific amplicon, so that PCR were repeated with a lower concentration of primers (0,3 μM) and analyzed with CE again.

The molecular weight of the analyzed microsatellites (Table 7) were comparable to those reported in the scientific literature, with the only exception observed in TGR pools where four microsatellites were identified, instead of the expected two. Probably TGR pool samples weren't homogeneous and were contaminated by hazelnuts of other cultivars.

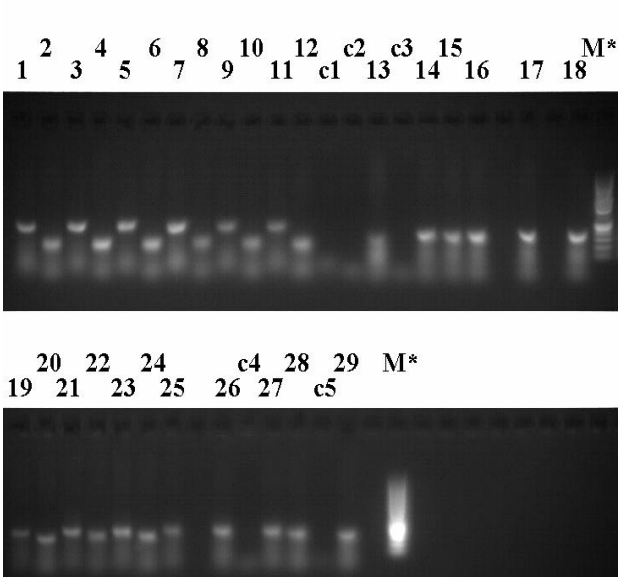

Fig. 11 Electrophoretic agarose gel (1,2%) of the PCR products obtained with CAC B028 (lanes 1, 3, 5, 7, 9, 11), CAT B107 (lanes 2, 4, 6, 8, 10, 12), CAT B504 (13-18), CAT B507 (lanes 19, 21, 23, 25, 26, 27), CAT B508 (lanes 20, 22, 24, 28, 29). DNAs used for the amplification: TGR1 lanes 1, 2, 13, 19, 20; TGR2 lanes 23, 4, 14, 21, 22; TG lanes 5, 6, 15, 23, 24; TGL lanes 7, 8, 16, 25; Tombul lanes 9, 10, 17, 26, 28; Mortarella lanes 11, 12, 18, 27, 29 (for details DNA see table 5). Negative control lanes c1, c2, c3, c4, c5 for TGR, TG, TGL, To, Mo respectively. Lane M* 50bp DNA ladder.

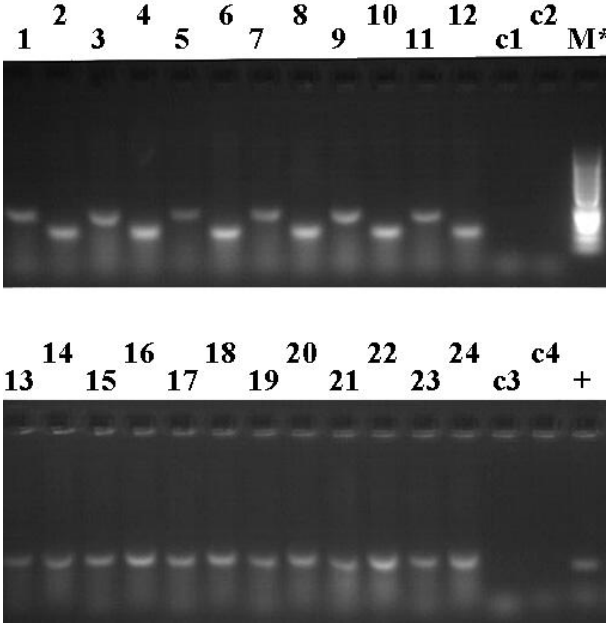

Fig. 12 Electrophoretic agarose gel (1,2%) of the PCR products obtained with CAC A014 (lanes 1, 3, 5, 7, 9, 11), CAC B029 (lanes 2, 4, 6, 8, 10, 12), CAC B111 (13, 15, 17, 19, 21, 23), CaT B502 (lanes 14, 16, 18, 20, 22, 24). The amplifications were obtained with the following DNA: TGR1 (lanes 1, 2, 13, 14), TGR2 (lanes 3, 4, 15, 16), TG (lanes 5, 6, 17, 18), TGL (lanes 7, 8, 19, 20), Tombul (lanes 9, 10, 21, 22), Mortarella (lanes 11, 12, 23, 24) (for details DNA see table 5). Lanes c1, c2, c3 and c4 negative controls for CAC A014, CAC B029, CAC B111 and CaT B502 (respectively). Lane M* 50bp DNA ladder.

SAMPLE ID	SSR								
	CAC B028	**CAT B107**	**CAT B504**	**CAT B507**	**CAT B508**	**CAC A014**	**CAC B029**	**CAC B111**	**CAT B502**
TGR 1	262/268	112 134 122 143	161/183	182/190	157/157	212/216	122/122	180/180	184/192
To	262/262	120/128	169/183	192/198	157/157	212/216	116/131	182/184	188/188
TG	262/278	118/134	159/183	182/192	157/157	212/216	118/122	180/180	184/188
TGL	256/262	134/152	171/183	186/192	147/163	216/222	118/122	181/181	186/190
TGR 2	262/268	112 135 122 143	161/ 183	186/190	157/157	212/216	123/123	180/180	188/192
Mo	262/262	112/134	159/183	182/192	157/166	216/216	118/122	180/180	184/190

Table 7. Molecular weight (base pairs) of microsatellites.

2.1.3. Statistical elaboration data

The performed statistical elaboration (Table 8, see Methods for details) confirmed that the results obtained in our study are quite similar to those existing in the scientific literature. CAT B107 resulted the most polymorphic microsatellite as found by Boccacci *et al*. (2006). However, our results define CAC A014b microsatellite as the least polymorphic which is in contradiction with Bassil *et al*. (2005) who described this microsatellite as the most polymorphic one. Each marker showed an high PD value, similar to those reported in literature (Boccacci *et al*., 2005, 2006).

The results obtained in the statistical data elaboration confirmed that the criterion through which SSRs have been chosen was surely appropriate and sufficient for hazelnut cultivar discrimination.

SSR name	n	He	Ho	PIC	PI	PD
CAC B028	4	0.5	0.67	0.48	0.27	0.72
CAT B107	8	0.8	1.0	0.2	0.02	0.83
CAT B504	5	0.67	1.0	0.29	0.14	0.72
CAT B507	6	0.77	1.0	0.21	0.06	0.78
CAT B508	5	0.51	0.5	0.49	0.24	0.67
CAC A014b	3	0.54	0.83	0.37	0.28	0.49
CAC B029	5	0.71	0.67	0.25	0.1	0.66
CAC B111	4	0.62	0.17	0.32	0.2	0.61
CAT B502	5	0.76	0.83	0.22	0.08	0.83

Table 8. Allelic number (n), expected heterozigosity (He), observed heterozigosity (Ho), polymorphic information content (PIC), power of discrimination (PD) of nine SSR loci.

2.2 Analysis of hazelnut oil

2.2.1. Analysis of hazelnut oil through PCR

Figures 13 and 13a show the results of PCR using LOX3 primers on 1O and 1OF DNAs extracted through the different kits. As it is shown in the figures, the Promega and Genaid kits were ineffectiveness while, among the others, the Macherey-Nagel kit resulted being the most suitable.

The fragmentation of DNA with LOX was observed on all DNA preparations extracted by all the kits for both filtered (1OF, 2OF) and not filtered (1O, 2O) oils. The presence of 500bp amplicons in the amplifications performed with LOX (Figg. 14, 14a) demonstrated that the DNAs were sufficiently undamaged and therefore useful for following fingerprint analysis.

Although the spectrophotometric analysis showed the presence of DNA in each sample (Table 6) some of them couldn't be amplified (DNA extracted from Geneaid, Promega kits or sample extracted from pellet with Extract-N-Plant PCR-Sigma) (Fig. 13, 13a) probably because the extraction through these systems determined a recovery of scarce quality DNA containing inhibitory substances that could inhibit PCR performance.

In conclusion for DNA extraction, as for the nuts also for oils, the M-N kit was surely preferable than the others for the better results provided and because of its simplicity of use and low cost.

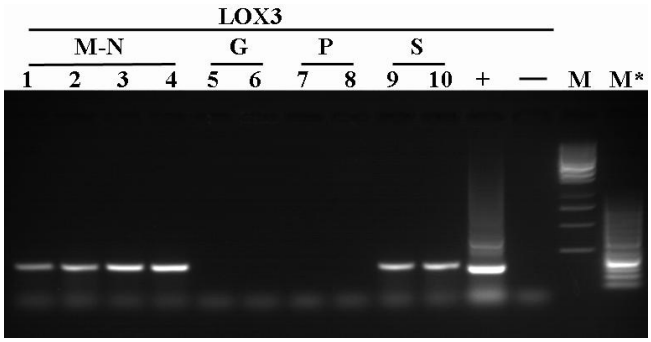

Fig. 13 Electrophoretic agarose gel (1,2%) of the PCR products obtained with LOX 3 primers using O1 (lanes 2, 4, 6, 8, 10), 1OF (1, 3, 5, 7, 9) DNAs extracted by M-N (1- 4 lanes),G (5, 6 lanes), P (7, 8 lanes) and S1 (9, 10 lanes) kits. Lanes M and M* 1 kb and 50 bp DNA ladder (respectively).

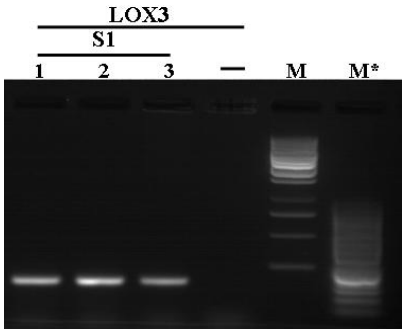

Fig. 13a Electrophoretic agarose gel (1,2%) of the PCR products obtained with LOX3 primers using O1 (lane 1 sample 52), 1OF (lane 2, sample 53) and O2 (lane 3, sample 54) DNAs extracted by S1 (for DNA details see table 6). Lanes M and M* correspond to 1 kb and 50 bp DNA ladder (respectively).

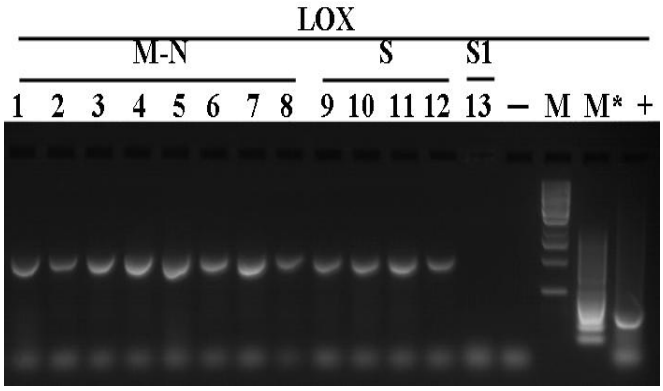

Fig. 14 Electrophoretic agarose gel (1,2%) of the PCR products obtained with LOX. Lanes 1-8 correspond to 34-41 samples, extracted by M-N kit; lanes 9-12 to 46-49 samples, extracted by S kit; lane13, 51 sample, extracted by S1 kit. Lanes M and M* correspond to 1 kb and 50 bp DNA ladder (respectively).

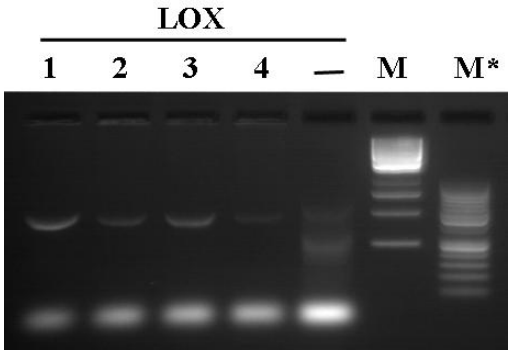

Fig. 14 a Electrophoretic agarose gel (1,2%) of the PCR products obtained with LOX. Lanes 1-4 correspond to 52, 53, 54 and 55 samples extracted by S1 kit. Lanes M and M* correspond to 1 kb and 50 bp DNA ladder (respectively).

2.2.2. Microsatellites analysis on hazelnut oil

Preliminary microsatellite analysis showed that the amplicon signal was very weak in the DNA samples extracted by S and S1 kits and in all the DNAs recovered from filtered oil (Fig. 15). To solve the problem, a whole genome amplification of each unsatisfactory DNA was performed before the definitive SSR amplifications using the kit GenomePlex Complete Whole Genome Amplification (WGA). Results showed that the pre-amplification had been really effective for DNAs exctracted from S kit and from all filtered oils. Amplification of O1 sample (34, Table 6) with all nine microsatellites before and after WGA treatment are shown in figures 16 and 17, respectively.

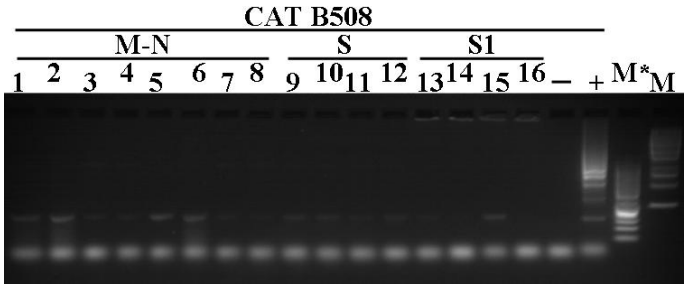

Fig. 15 Electrophoretic agarose gel (1,2%) of the PCR products obtained with CAT B508 using DNA: 34-41 (lanes 1-8), DNA 46-49 (lanes 9-12), DNA 52-55 (lanes 13-16). For DNA details see table 6. Lanes M and M* correspond to 1 kb and 50 bp DNA ladder (respectively).

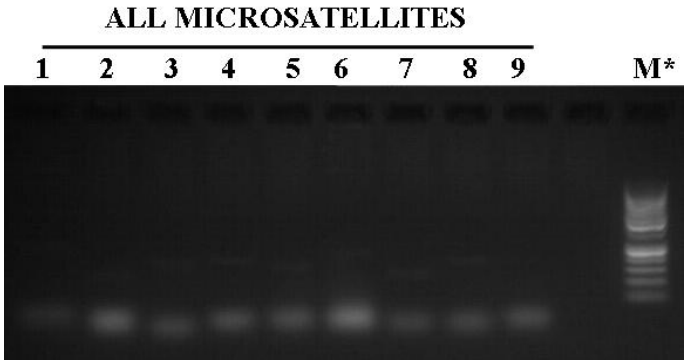

Fig. 16 Electrophoretic agarose gel of (1,2%) the PCR products obtained using DNA 34 (Table 6) with the following primers: (1) CACB028, (2) CaTB107, (3) CaTB504, (4) CaTB507, (5) CaTB508, (6) CACA014, (7) CACB029, (8) CACB111, (9) CaTB502.Lane M* 50bp DNA ladder.

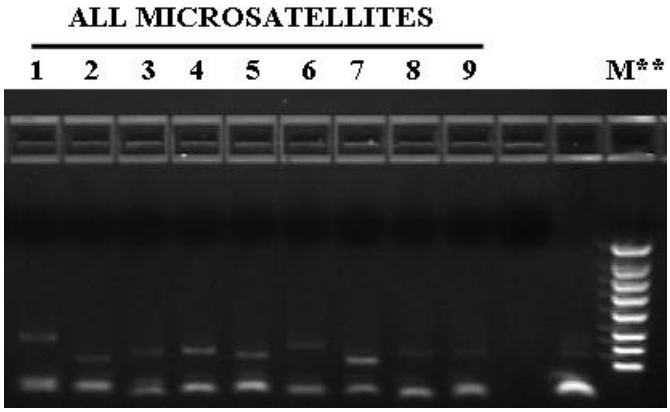

Fig. 17 Electrophoretic agarose gel (2,2%) of the PCR products obtained after using GenomePlex Complete Whole Genome Amplification kit of O1 DNA (34, Table 6) DNA with the following primers: (1) CACB028, (2) CaTB107, (3) CaTB504, (4) CaTB507, (5) CaTB508, (6) CACA014, (7) CACB029, (8) CACB111, (9) CaTB502. Lane M**Lonza Flash Gel ladder.

Concerning to SSR screening, all the amplifications were checked on agarose gel. A preliminary observation of the electrophoretic profile showed that the microsatellites have the expected dimensions for TGR cultivar. However, in order to define more appropriately the

molecular weight of the microsatellites and to verify its correspondence with the data previously obtained on hazelnuts, the amplifications were also performed with labelled primers (6-FAM) and analyzed through capillary electrophoresis.

The microsatellites identified in TGR hazelnut oil (Table 8) corresponded to those distinctive to the hazelnut variety used for oil production and that we have characterized previously (Table 7). Even the anomaly found in hazelnut nuts in microsatellite CaT-B107 was found also in the oil.

SAMPLE ID	**SSR**								
	CAC B028	**CAT B107**	**CAT B504**	**CAT B507**	**CAT B508**	**CAC A014**	**CAC B029**	**CAC B111**	**CAT B502**
TGR HAZELNUT	262/ 268	112 134 122 144	161/183	182/190	157/157	212/216	122/122	180/180	184/192
TGR OIL	254 262 268	112 134 122 144	160/183	182/190	157/157	212/216	123/123	180/180	184/192

Table 8. Molecular weight of microsatellites obtained by capillary electrophoresys.

3. ANALYSIS OF ANOMALOUS ALLELES IN TGR CULTIVAR

3.1 DNA extraction from individual hazelnuts

As the results obtained from TGR microsatellites analysis showed an anomalous number of alleles (four instead of two, Table 7 and 8) a further analysis has been performed to characterize it.

The hypothesis was that TGR1 and TGR2 cultivars weren't homogeneous samples but a mix of cultivars. Extractions from twelve individual hazelnuts were performed to verify the presence of nuts from different cultivars.

The DNA recovery was very low and scarcely clean, with a low value of 260/280 and 260/230 nm ratios (Table 9). This was surely due to the difficulty of extracting DNA from a single nut. In fact, as it was impossible to homogenize it in the mini mill, only a part (100 mg) was taken that could be homogenized in the tissue lyser.

Sample ID	matrix	ng/ul	260/280nm	260/230nm	method	modification
1s	TGR-pool 1	30	1,6	0,83	M-N	high salt+spin
2s	TGR-pool 1	27,3	2,08	1,8	M-N	high salt+spin
3s	TGR-pool 1	33,9	1,79	1,38	M-N	high salt+spin
4s	TGR-pool 1	31,6	1,81	1,34	M-N	high salt+spin
5s	TGR-pool 1	22	1,94	1,3	M-N	high salt+spin
6s	TGR-pool 1	20,6	1,4	0,93	M-N	high salt+spin
7s	TGR-pool 1	28,2	1,84	1,03	M-N	high salt+spin
8s	TGR-pool 1	21,5	1,6	0,94	M-N	high salt+spin
9s	TGR-pool 1	17,5	2,36	1,06	M-N	high salt+spin
10s	TGR-pool 1	27,5	1,82	1,11	M-N	high salt+spin
11s	TGR-pool 1	28,4	1,7	1,33	M-N	high salt+spin
12s	TGR-pool 1	23	1,51	0,84	M-N	high salt+spin

Table 9. Spectrophotometric analyses of DNA samples extracted from individual hazelnuts. M-N indicates Macherey-Nagel extraction method.

3.2. PCR analysis of DNA from individual hazelnuts

In figure 18, LOX1 amplification of all DNAs is shown, demonstrating the good amplification performance and the good status of all DNAs in terms of fragmentation.

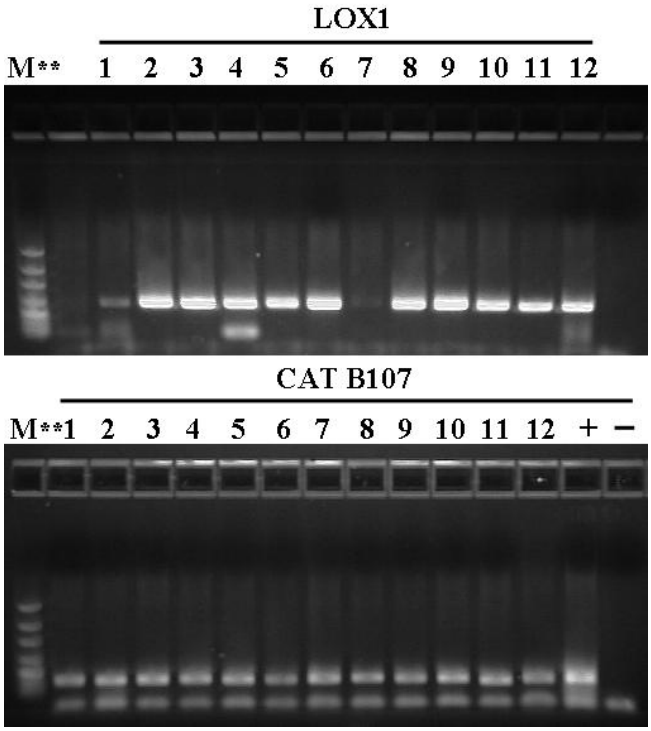

Fig. 18 Electrophoretic agarose gel (2,2%) of the PCR products obtained using 1s to 12s samples (see details in table 9) (lanes 1-12) with LOX1 or CAT B107 primers. DNA ladder of Flash Gel (Lonza) M** lane.

3.3. Microsatellites analysis on individual hazelnut

The amplifications resulted satisfactory. The figures 18, 19, 20, 21, 22 show the electrophoretic profile of all microsatellite PCRs on agarose gel, that provided suitable amplifications, even if several non specific amplicons, previously observed in the analysis with CE, were present contemporary to the main amplicon (microsatellite). However, these non specific amplicons can be considered non influencial and normal (i.e. 40 base pairs (bp) amplicons could be due to primer dimer). In fact, the detection of anomalous bands on agarose gel were probably due to the particular condition used with Flash Gel System (2.2 % agarose gel and longer runs) applied to increase the resolution of the analysis.

Bands of lanes 1 and 7 of fig. 18 were very light because there was a spill over of the sample.

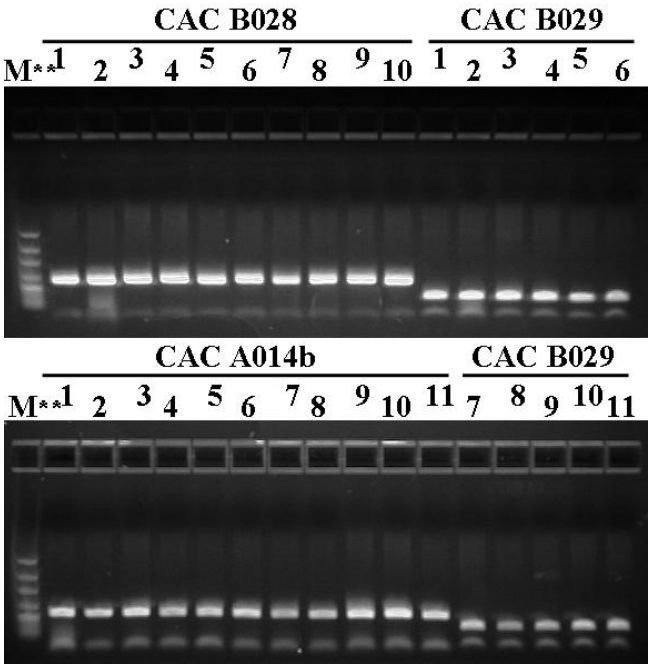

Fig. 19 Electrophoretic agarose gel (2,2%) of the PCR products obtained using 1s-10s samples (see details in table 9) with CAC B028, or 1s-11s samples with CAC B029, or 1s-11s sample with CAC A014b. M** Flash Gel DNA ladder.

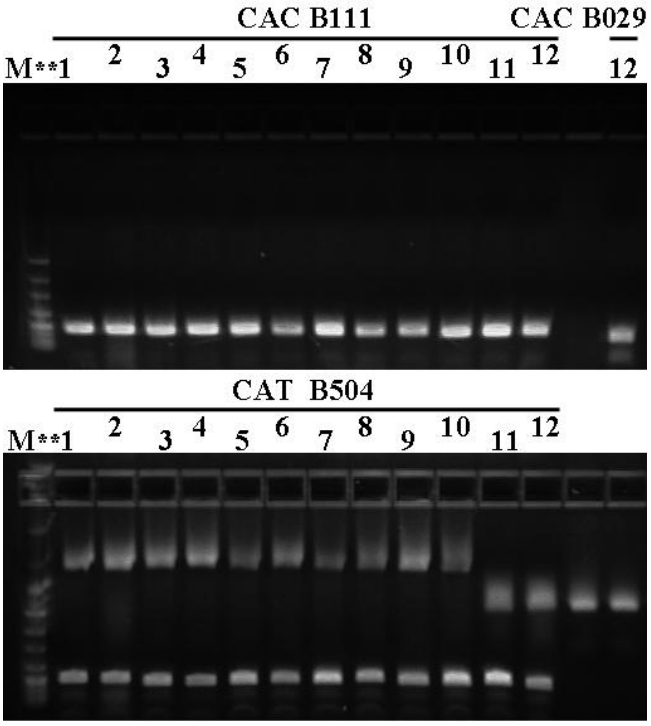

Fig. 20 Electrophoretic agarose gel (2,2%) of the PCR products obtained using 1s-12s samples (see details in table 9) with CAC B111, of samples 1s-12s with CAT B504, of sample 12 of CAC B029. M** Flash Gel DNA ladder.

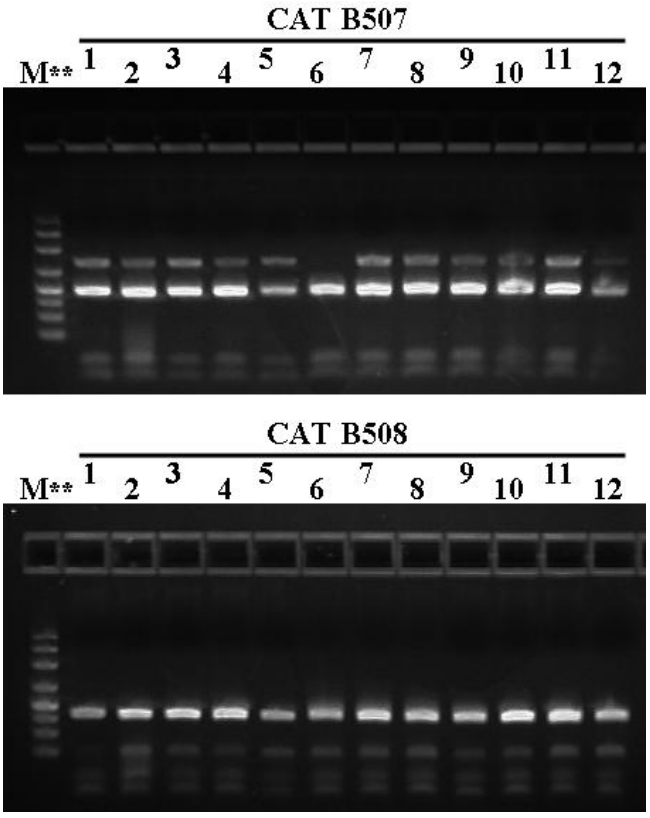

Fig. 21 Electrophoretic agarose gel (2,2%) of the PCR products obtained using 1s-12s samples (see details in table 9) with CAT B507, of samples 1s-12s with CAT B508. M** Flash Gel DNA ladder.

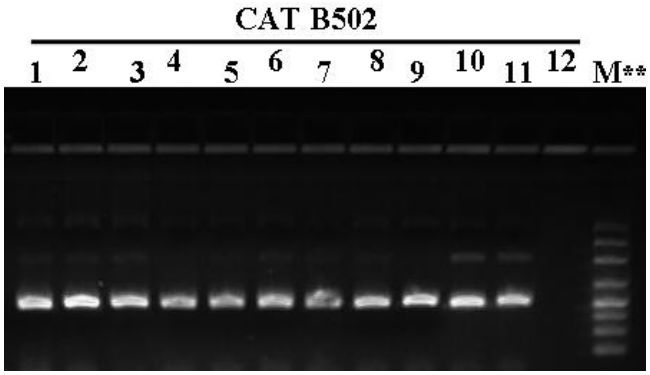

Fig. 22 Electrophoretic agarose gel (2,2%) of the PCR products obtained using 1s-12s samples (see details in table 9) with CAT B502. M** Flash Gel DNA ladder.

The results indicate that it was possible to appropriately amplify microsatellites and the molecular weight of all them was comparable to those that were expected, equal to those obtained in the previous experiments.

.

SAMPLE ID	SSR								
	CAC B028	CAT B107	CAT B504	CAT B507	CAT B508	CAC A014	CAC B029	CAC B111	CAT B502
1s	254/268	144/144	185/185	190/190	155/155	218/218	123/123	181/181	190/190
2s	254/268	136/136	161/185	182/190	157/157	216/216	123/123	181/187	188/188
3	262/268	123/147	161/163	186/192	157/157	218/218	123/123	181/181	192/192
4s	262/268	123/144	155/162	182/190	157/157	212/220	123/123	181/187	192/192
5s	254/262	123/144	155/162	186/192	157/157	212/218	123/123	181/187	192/192
6s	262/278	121/144	162/179	186/186	157/167	212/218	123/123	181/181	184/192
7s	262/262	133/144	162/177	182/190	157/157	214/218	119/123	181/185	184/192
8s	256/268	144/144	179/185	190/190	157/157	212/212	117/123	181/181	192/192
9s	262/266	131/144	162/171	190/190	157/165	212/212	119/123	181/187	188/192
10s	256/262	129/135	162/177	190/195	157/157	216/220	123/123	181/181	192/192
11s		113/121	185/185	192/192	147/157	212/214	119/123	181/181	184/190
12s		119/135	162/171	182/190	147/157		119/123	181/181	188/188

Table 10. Microsatellites analyses of individual hazelnuts.

The subsequent analysis with capillary electrophoresys confirmed the hypothesis that the pools of TGR hazelnut weren't homogeneous in fact, the observation that different individuals present different alleles (Table 10) strongly suggest that the pool contained nuts from different cultivars. In fact, instead of the two expected alleles for each marker, the total number of alleles per locus found in the twelve nuts analyzed individually ranged from three (CAC B029 and CAC B111) to twelve (CAT B107). As expected, the alleles previously identified in TGR (Table 7) were also found and resulted being the main alleles.

DISCUSSION

DNA EXTRACTION

Molecular analysis are refined procedures that require competence and precision and, often, long studies to correctly set up the protocols in order to obtain correct results to be applied in different fields. Molecular traceability, for example, is quickly growing because of the several applications that can be applied on this field and that can not be obtained with other more traditional methodologies.

The focal point of molecular traceability is DNA. It is then obvious that a correct DNA extraction from the matrix is the core of the analyses and, thus, the choice of the most appropriate DNA extraction method represents the groundwork for starting any molecular analysis.

Several type of procedure for DNA extraction exist, and the choice of the correct methodology among them for each specific demand is often a challenge. Matrices can be very different and complex so that a general "correct" methodology does not exist, and it is therefore often necessary to check different methodologies and to set up different experimental conditions for each specific need. This is particularly needed when DNA have to be extracted from complex matrices such as snacks, chocolate, milk, cheese, and oil.

The aim of this study was to set up the most appropriate methodology to characterize, by a molecular approach, oil produced from hazelnut.

In order to reach this objective, different DNA extraction methodologies have been tested, that differ from each other in the principle applied to separate and purify DNA: by columns, by bead and by salting out.

The columns of silica, base their separation on the principles of asorption chromatography. After cell lysis, DNA is separated from the other components (protein and other macromolecules) through its interaction with silica column in presence of caotropic salts that eased DNA-silica interaction and help protein denaturation. Essentially column systems are the best as the passage through the column determine a better removal of any contaminant.

The magnetic beads are coated with silica therefore substantially the systems are similar to those based on columns. The main difference is that, in this procedure, DNA is bound to magnetic beads and is then separated from the other component through a magnet. The main problem that can be found with magnetic beads is that contaminants can bind to DNA attached to beads, and they can be only very difficultly removed through several washing and elution steps.

In selective precipitation based methodologies (salting out), DNA, after lysis, is separated from the other component thanks to the selective precipitation induced with the addition of different salting concentration, depending on the type of kit. With this system, similarly to beads, it is not always easy to remove extraneous molecules. This because the separation of DNA from other substances is based on their precipitation and sometimes the subsequent centrifugation is not able to correctly remove some molecules, particularly the fats.

It is however worth to mention that, whatever methodology is chosen and applied for extracting DNA, very rarely the standard kit protocol can be applied to any matrix. For this reason, in our study, for all the three tested methodologies several modifications to the usual protocol were tested, particularly regarding the amount of chaotropic salt and of lysis buffers and the centrifugation.

A second focal point that have to be mentioned is that the extraction methodology can give satisfactory results only when the sample is correctly homogenized: a sketchy homogenization can greatly reduce DNA recovery whatever is the extraction method used. The homogenization of the matrix in fact determines the cell wall breaking and allows enzymes and/or denaturing substances to lyse cell membranes and, consequently, to release the cellular content and, therefore, the nucleic acids. In our study two types of homogenization procedure were used: a tissue lyser and a mini mill. The latter is theoretically better as frozen matrices can be used, increasing the grinding capacity and, particularly for hazelnuts, avoiding the kneading (and consequent reduction of the recovery) of the matrix that can happen at room temperature and that can therefore happen with the tissue lyser. A combination of the two methodologies has also been tested.

Another trouble that can be encountered in the extraction of DNA from an oily matrix such as hazelnuts regards the elimination of lipid component (about 60% in hazelnuts) that can interact

with DNA, influencing its extraction capacity and, then, the PCR performance (Bally *et al*., 1997; Harvie *et al.*, 1998). In hazelnut the removal of lipids resulted particularly tricky because, after cellular lysis, lipids constituted an almost solid layer which resulted very difficult to be removed. Only multiple centrifugations allowed to appropriately clean the sample.

The conclusions of the several test performed in this study on all the steps of the DNA extraction and analysis indicates the Macherey-Nagel, with the appropriate modification to its protocol of use, is the best extraction for both hazelnuts and oil extracted from hazelnuts.

The Macherey-Nagel kit besides showing the best performance regarding its extractive capacity, proved also to be an economic, fast and a reliable procedure.

The column system M-N kit in fact**,** allowed a good DNA recovery from hazelnut particularly when the lysis/chaotropic buffer have been increased and when particular cares were taken on the procedures aimed at the elimination of fat components of the matrix. An increase of lysis/chaotropic buffer, in fact, (1n and 2n samples, Table 4) determined a substantial improvement of DNA quantity as respect to the standard concentration (samples 5on, 6on, Table 4). That is probably because the increased amount of buffers improved the capacity of eliminating proteins that, besides being contaminants, can also be enzymes capable to destroy DNA (DNase) and/or to prevent DNA amplification. Furthermore, high buffers concentration (lysis substances and chaotropic salts) also partially contributed to disrupt membranes and thus to ease DNA/silica recogniztion and/or to denature a greater quantity of proteins in a oily matrix, as hazelnuts are (60% total fats).

However, besides the amount of buffers, better results have also be obtained performing multiple centrifugations (24-29, Table 5) in order to better remove the thick fat layer over supernatants and, thus, to improve the DNA recovery capacity. The removal of all possible debris from the cellular extract before the purification on the silica column is in fact a key point in order to avoid obstructions and/or bad performance of the column.

The quality of the recovered DNA was worst in the extractions performed on single nuts (samples 1s-12s Table 9), surely due to the impossibility to appropriately homogenize the low quantity of sample (100 mg) instead of 300 g used for the homogenization of the pools, making impossible to use the mill under liquid nitrogen, and obliged us to use a tissue layer, working at room temperature. However, although the DNA quality wasn't ideal, it was sufficient to perform a correct DNA analysis.

The same satisfactory results obtained in nuts have been also obtained in hazelnut oil (samples 34-41, Table 6), using the same extraction conditions. As expected, DNA recovered from non-filtered oil was noticeably higher (Table 6 samples 36, 37, 40 and 41) than in filtered oils, as in

this case. DNA is fundamentally recovered from a very scarce amount of cellular residuals after filtration. However, as for the extraction performed in the individual nuts, although the DNA quality wasn't perfect (contaminants were still present perhaps attached to the residual lipidic fraction that was not possible to be removed), it was sufficient to allow the DNA analyses. All the other tested methodologies gave variable results that however weren't as satisfactory as those obtained with M-N kit.

Gene Elute Sigma, although using the same principle as that of M-N kit, was not similarly efficient, even though modification in the amount of the lysis/chaotropic salts were tested.

The DNA extracted through this kit was totally unsatisfactory both in terms of quantity and quality, either from nuts and from oils (13n, 14n, 15o, 16o, Table 4) (46-49, Table 6). Also the increase of lysis/chaotropic salt and an additional centrifugations (samples 15o and 16o, Table 4) weren't capable to improve the recovery. On this regards, it is reasonable to hypothesize that pellets, although almost fat free, concentrate higher number of cells, and thus of genomic material, but similarly concentrate an equally higher amount of contaminants other than fats as well.

The third kit column that have been tested (QIAmp DNA stool of Qiagen) provided satisfactory results when increased buffer amounts were used(samples 7o, 8o, 9n, 10n, 11o, 12o, Table 4), with significant improvement of quality/quantity of recoverd DNA as compared to that obtained with the standard protocol (samples 11o, 12o and 7o to 10o respectively, Table 4). The M-N kit has been preferred to the QIAmp basically because the former is easier and less time consuming to be used as the latter needs an additional step after lysis, namely a tablet have to be added to the lysate to adsorb DNA-damaging substances and PCR inhibitors.

DNA extracted from oil samples with Promega kit (magnetic beads) resulted not satisfactory in both filtered and non-filtered oil. With this approach contaminants resulted very hard to be removed, as when they are attached to DNA bound to the silica beads and/or remain among beads. Moreover, results were very variable (Table 6 samples 42 to 45), probably due to the losses of several magnetic beads during DNA purification, during washing and elution steps.

Concerning selective precipitation strategy, three methods were tested: traditional, Dnabsolute (Geneaid) and Extract-N-Amp Plant (Sigma).

The traditional approach proved to be the least successful, as no DNA was recovered (17o, 18o Table 4). The lack of DNA recovery was probably due to the high amount of contaminants still present after the contrifugation post lysis. Only after multiple centrifugations it was possible to recover some DNA even though it was highly contaminated (19o-22o, Table 4).

On the contrary, Dnabsolute (Geneaid) and Extract-N-Amp Plant (Sigma) kits permitted to recover DNA (sample 30-31 and samples 51-55, Table 6) but, as for the traditional method, even after multiple centrifugations its bad quality did not allow the successive analyses of the genomic extract.

Even an additional treatment with tissue lyser (samples 32 and 33, Table 6) wasn't able to improve the final results, suggesting that the standard treatment was sufficient to break residual cell walls in the sample, but the procedure didn't improve DNA quantity recovery.

According to all the performed tests, salting out methodology didn't result to be an efficient strategy. It is probably due to the absence of any apparatus (filters, columns or beads) absolutely needed to eliminate contaminants from a oily matrix .

PCR AMPLIFICATIONS

PCR amplification were performed on all the samples extracted, except for samples 17o and 18o (Table 4) obtained through traditional method that did not provide any DNA. The amplifications were satisfactory for many samples, even for some of those whose quality was previously defined as poor due to anomalous 260/280 and 260/230 nm ratio values. This mean that DNAs also in those samples weren't contaminated or damaged enough, as appeared in the spectrophotometric analyses, to prevent a correct amplification.

The preliminary analysis with ADH primers, performed on TGR pools, permitted us to establish the best PCR conditions for proceeding with the other PCRs (lane 7 Fig. 1) . The analysis of the results indicated that a reduction of primers, in respect to the standard protocol, produced a substantial reduction of non specific amplification products and a better performance of the priority amplicon (Fig. 1 lanes 7 and 8).

An amplification was performed with *Nepholepsis, Picea, Marchantia* and *Olea* plants (Fig. 3), belonging to different tassonomic division from *Corylus*, with the aim to verify the specificity of primers LOX3 for hazelnut. The lack of any amplicon suggested that primers were specific for hazelnut.

Finally, hazelnut DNA amplification of with AJ10086, specific for *Olea*, demonstrated that samples were pure because no amplicons were present (Fig. 4).

LOX3 experiments demonstrated that all samples were amplifiable except for DNAs obtained from traditional (Fig. 2) or G kit (Fig. 13). It is worth to mention that the results obtained by PCR analyses not always overlap those obtained spectrophotometrically. This is particularly evident by comparing samples from S1 and G kit, that provided very similar unsatisfactory result

in the spectrophotometric analyses, but whereas the former was easily amplifiable, it was not possible to amplify the latter. This could be due to the specific composition of the solutions utilized in the different kits, as they could influence NanoDrop analysis or/and can maintain the inhibitors inactive avoiding DNA damages or inibition of PCR performance.

The amplification with LOX performed on DNAs that had resulted positive to LOX3 analysis also demonstrated that fragmentation was not excessive, as it has been also possible to amplify fragment big enough (478 bp and 286 bp) to be used for microsatellites screening (Figg. 14 and 14 a). This was not possible for DNA sample 51 (obtained from sediment oil) (ob (S1 kit) (Fig. 14) that was not amplifiable probably for being highly contaminated.

MICROSATELLITE ANALYSIS

The capillary electrophoresys performed on DNA extracted from oils evidenced the presence of microsatellites with the same molecular weight as those found in the nuts (Table 8), demonstrating that this methodology is capable to recognize the genotype and, thus, the cultivar, used for the production of the oil.

This conclusion is reinforced by the analysis performed on TGR cultivar, as the same anomaly on CAC B107 microsatellite (four alleles instead the expected two) found in nuts has been found also in the oil.

An exception was observed in CAC B028 microsatellite as three alleles have been found in the oil whereas only two alleles was detected in the nuts, probably because the greater pool of nuts used to produce the oil was more contaminated from non-TGR nuts that the smaller pool of nuts directly used in the molecular analyses. However, both the results obtained in CAT B107 and CAC B028 microsatellites strengthened the hypothesis that the "TGR pool" was actually composed by nuts belonging to different cultivars.

The statistical elaboration of capillary electrophoresys demonstrated that the studied microsatellites were sufficient for discriminating the cultivars, having a suitable enough good allelic number (n) and discriminating power (PD).

Within these positive results, some differences have been found as respect to data existing in scientific literature. CAT B107 resulted the most polymorphic microsatellite as found by Boccacci *et al*. (2006), but our results define CAC A014b microsatellite as the least polymorphic which is however in contradiction with Bassil *et al*. (2005) who described this microsatellite as the most polymorphic one. Each marker showed an high PD value, similar to those reported in literature (Boccacci *et al*., 2005, 2006), excepting for CAC A014b that showed the PD lower

than expected. It is worth to mention that some differences between our data as compared to those from literature statistical elaboration can be expected, as a different number/type of samples was examined.

As microsatellites analysis had provoked some perplexity about the composition of TGR hazelnut pool, twelve nuts from TGR1 pool were selected and individually analyzed. DNAs obtained from these nuts were checked with LOX 3 and CAT B107 (Fig. 18) microsatellite demonstrating that all DNAs were perfectly amplifiable. The same good results were obtained with the other microsatellites.

The analysis of the nuts showed that each individual enclose two alleles for each marker, but the total number of alleles in the twelve nuts were ranged from 3 to 12 (Table10), confirming that the pool was constituted of a mixed population.

The Perl script used for the analysis of microsatellites data suggested that no one of the twelve nuts had an high homology with the genomic profile of TGR hazelnuts. The highest homology (50%), found in the samples 10s and 11s, was with Trene Barrettona e Rosetta cultivars. Only samples 2s and 6s showed a light homology with TGR (30-40%), clearly not sufficient to define these nuts as belonging to this cultivar.

CONCLUSION AND FUTURE PERSPECTIVES

This work permitted us to demonstrate that it is possible to characterize hazelnut oil through a simple, economic molecular approach that could be used for identifying the origin of the oil, and also for discovering hazelnut oil when added fraudulently to olive oil.

It is clear that at the beginning the set up of the method can requires more time but subsequently it could be used routinely for screening of oils for different purposes.

However this study has demonstrated that through molecular analysis it is possible to identify the origin of a matrix even though it is transformed and even though the results are uncertain because of its non homogeneity. The identification of the right alleles that justified the affiliation, of the matrix, to a specific cultivar and the anomalous allele numbers that suggested the non homogeneous of the matrix, demonstrated the big potentiality of the approach. Nevertheless the good performance of the approach also to characterize filtered oils, demonstrated its big potentiality for discovering frauds where chemically extracted hazelnut oil, very similar to filtered oil, are used.

Obviously, further study will be needed to properly set up the methodology.

First of all it should be interesting to analyze individual nuts from several varieties obtained from different experimental farm in order to verify the homogeneity of samples, taken from different trees or/and different areas, and to establish which role could have the pollinators for allele variability.

On the other hand it could be analyzed hazelnut oil added to different concentrations of olive oil with the aim evaluating the limits of the system and therefore to evaluate its employment for discovering more common frauds and to observe the potentiality of the system in the presence of oils extracted by chemical practice, probably similar to filtered oils. In fact, oil extracted by solvent, even though representing a less healthy and valuable product than oil extracted by mechanical processes, remains a highly commercialized product.

REFERENCES

Dossier/ Il Nocciolo a cura di CRPV, Cesena, 2008

Tabelle di composizione degli alimenti a cura dell'INRAN, 2000

Agiomyrgianaki A., Petrakis Panos V., Dais P. (2010) "Detection of refined olive oil adulteration with refined hazelnut oil by employing NMR spectroscopy and multivariate statistical analysis" *Talanta* 80 (5), 2165-2171

Aitken K. S., Jackson P. A., McIntyre C. L. (2005) "A combination of AFLP and SSR markers provides extensive map coverage and identification of homo(eo)logous linkage groups in a sugarcane cultivar" Theor. Appl. Gen. 110 (5), 789-801

Akkak A., Boccacci P., Botta R. (2008) "An efficient DNA-extraction for nut seeds" J. Food Qual. 31, 549-557

Alasalvar C., Amaral J. S., Shahidi F. (2006) "Functional lipid characteristics of Turkish Tombul hazelnut (*Corylus avellana* L.)" J. Agric. Food Chem. 54, 10177-10183

Alasalvar C., Shahidi F., Ohshima T., Wanasundara U., Yurttas H. C., Liyanapathirana C. M., Rodrigues F. B. (2003) "Turkish Tombul hazelnut (*Corylus avellana* L.). 2. Lipid characteristics and oxidative stability" J. Agric. Food Chem. 51, 3797-3805

Alphan E., Pala M., Ackurt F., Yilmaz T. (1997) "Nutritional composition of hazelnut and its effects on glucose and lipid metabolism" Acta Horticulturae 445, 305-310

Althoff D. M., Gitzgendanner M. A., Segraves K. A (2007) "The utility of amplified fragment length polymorphisms in phylogenetics: a comparison of homology within and between genomes" Syst. Biol. 56, 477-484

Aparicio R., Garcia-Gonzalez D. L., Viera-Macias M., Aparicio-Ruiz R., Morales M. T. (2007) "Validation of a method based on triglycerides for the detection of low percentages of hazelnut oil in olive oil by column liquid chromatography" Journal of AOAC International 90 (5), 1346-1353

Awad A. B., Williams H., Fink C. S. (2001) "Phytosterols reduce *in vitro* metastatic ability of MDA-MB-231 human breast cancer cells" Nutr. Cancer 40, 157-164

Ayed R. B., Grati-Kamoun N., Moreau F., Rebai A. (2009) "Comparative study of microsatellites profiles of DNA from oil and leaves of two Tunisian olive cultivars" Eur. Food Technol. 229, 757-762

Baeten V., Pierna J. A. F., Dardenne P., Meurens M., Garcia-Gonzalez D. L., Aparicio-Ruiz R. (2005) "Detection of the presence of hazelnut oil in olive by FT-Raman and FT-MIR spectroscopy" J. Agric. Food Chem. 53 (16), 6201-6206

Bally M. B., Zhang Y. P., Wong F. M. P., Kong S., Wasan E., Reimer D. L. (1997) "Lipid/DNA complexes as an intermediate in the preparation of particles for gene transfer: an alternative to cationic liposome/DNA aggregates" Ad. Drug Delivery Rev. 24, 275-290

Bassil N. V., Botta R., Mehlenbacher S. A. (2005) "Microsatellite markers in hazelnut: isolation characterization, and cross-species amplification" J. Amer. Soc. Hort. Sci. 130 (4), 543-549

Boccacci P., Akkak A., Bassil N. V., Mehlenbacher S. A., Botta R. (2005) "Characterization and evaluation of microsatellite loci in European hazelnut (*Corylus avellana* L.) and their transferability to other *Corylus* species" Mol. Ecol. Notes 5 (4), 934-937

Boccacci P., Botta R., Rovira M. (2008) "Genetic diversity of hazelnut (*Corylus avellana L.*) germplasm in Northeastern Spain" Hort. Sci. 43 (3), 667-672

Botstein D., White R. L., Skolnick M., Davis R. W. (1980) "Construction of a linkage map in man using restriction fragment length polymorphisms" Am. J. Hum. Genet. 32, 314-333

Botta R., Akkak A., Boccacci P. (2005) "DNA-typing of hazelnut: a universal methodology for describing cultivars and evaluating genetic relatedness" Proc. VIth Int. Congress on Hazelnut, Eds: Tous J. & Romero A., ISHS Acta Hort. 686,

Bouic P. J. (2001) "The role of phytosterol and phytosterolins in immune modulation: a review of the past 10 years" Curr. Opin. Clin. Metab. Care 4, 471-475

Bradbury L. M. T., Henry R. J., Jin Q., Reinke R. F., Waters D. L. E., (2005) "A perfect marker for fragrance genotyping in rice" Mol. Breeding 16, 278-283

Breton C., Claux D., Metton I., Skorski G., Bervillè A. (2004) "Comparative study of methods for DNA preparation from olive oil samples to identify cultivar SSR alleles in commercial oil samples: possible forensic application" J. Agric. Food Chem. 52, 531-537

Bueton K. H.,Edmonson M. N., Cassidy A. B. (1999) "Reliable identification of large numbers of candidate SNPs from public EST data" Nat. Genet. 21, 323-332

Bullò M., Lamuela-Raventos R., Salas-Salvadò J. (2011) "Mediterranean diet and oxidation: Nuts and olive oil as important sources of fat and antioxidants" Curr. Topics Med. Chem. 11 (14), 1797-1810

Carriero F., Fontanazza G., Cellini F., Giorio G. (2002) "Identification of simple sequence repeats (SSRs) in olive (*Olea europaea* L.)" Theor. Appl. Genet. 104, 301-307

Cappellehi G. M., Nicoletti G. M. (2007) "Quantitative and qualitative analysis of the "Dauno" P.D.O. (protected designation of origin) extra virgin olive oil" Rivista delle Sostanze Grasse 84 (1), 15-24

Chen O. C-Y, Blumberg J. B. (2008) "Phytochemical composition of nuts" Asia Pac. J. Clin. Nutr. 17 (SI), 329-332

Chen H., Angiul M., Ferrari C., Tombari E., Salvetti G., Bramanti E. (2011) "Tocopherol speciation as first screening for the assessment of extra virgin olive oil quality by reversed-phase high-performance liquid chromatography/fluorescence detector" Food Chem. 125 (4), 1423-1429

Ching A. D. A., Caldwell K. S., Jung M., Dolan M., Smith O. S., Tingey S., Morgante M., Rafalski A. (2002) "SNP frequency, haplotype structure and linkage disequilibrium in elite maize inbred lines" BMC Genet. 3, 19

Collins F. S., Brooks L. D., Chakravarti A. (1998) "A DNA polymorphism discovery resource for research on human genetic variation" Genome Res. 8, 1129-1231

Contini M., Cecchini M., Massantini R., Monarca D., Moscetti R. (2010) "Proprietà nutrizionali-salutistiche e conservazione della frutta secca in guscio" Italus Hortus 17 (4), 13-27

Crews C., Hough P., Godward J., Brereton P., Lees M., Guiet S., Winkelmann W. (2005) "Study of the main constituents of some authentic hazelnut oils" J. of Agric. Food Chem. 53, 4843 4852

Cumming C. J., Zoghbi H. Y. (2000) "Trinucleotide repeats: Mechanisms and pathophysiology" Ann. Rev. Genomics and Human Genet. 1, 281-328

D'Angelo F., Santillo A., Sevi A., Albenzio M. (2007) "*Technical Note:* A simple salting-out method for DNA extraction from milk somatic cells: Investigation into the goat *CSN1S1* gene" J. of Dairy Sci. 90 (7), 3550-3552

Doyle J., Doyle J. L. (1987) "A rapid isolation procedure for small quantities of fresh leaf tissue" Phytochem. Bull. 19, 11-15

Dreher M. L., Maher C. V., Kearney P. (1996) "The traditional and emerging role of nuts in healthful diets" Nutr. Rev. 54, 241-245

Durak I., Kocsal I., Kacmaz M., Buyukkocak S., Cimen B., B. M. Y., Ozturk, H. S. (1999) "Hazelnut supplementation enhances plasma antioxidant potential and lowers plasma cholesterol levels" Clin. Chim. Acta 284, 113-115

Elvevoll E. O., Moen P., Olsen R. L., Brox J. (1990) "Some possible effects of dietary monounsaturated fatty acids on cardiovascular disease" Atherosclerosis 81, 71-74

Everett C. M., Wood N. W. (2004) "Trinucleotide repeats and neurovegetative disease" Brain 127, 2385-2405

Field D., Wills, C. (1998) "Long polymorphic microsatellites in simple organisms" Proc. Royal Soc. London, Series B: Biological Sciences 265, 209-215

Galderisi U., Cipollaro M., Di Bernardo G., De Masi L., Galano G., Cascino A. (1999) "Identification of hazelnut (*Corylus avellana*) cultivars by RAPD analysis" Plant Cell Rep. 18, 652-655

Garcia-Gonzalez D. L., Viera M., Tena N., Aparicio R. (2007) "Evaluation of the methods based on triglycerides and sterols for detection of hazelnut oil in olive oil" Grasas y Aceites, 58 (4), 344-350

Gokirmak T., Mehlenbacher S. A., Bassil N. V. (2009) " Characterization of European hazelnut (*Corylus avellana* L.) cultivar using SSR markers" Genet. Resour. Crop Evol. 56, 147-172

Gurdeniz G., Ozen B. (2009) "Detection of adulteration of extra-virgin olive oil by chemometric analysis of mid-infrared spectral data" Food Chem. 116, 519-525

Hamada H., Petrini M. G., Kakunaga T. (1982) "A novel repeated element with Z-DNA-forming potential is widely found in evolutionary diverse eukaryotic genomes" Proc. Natl. Acad. Sci. USA 79, 6465-6469

Harvie P., Wong F. M. P., Bally M. B. (1998) "Characterization of lipid DNA interactions. Destabilization of bound lipids and DNA dissociation" Biophysical J. 75, 1040-1051

Hayden M. J., Sharp P. J. (2001) "Targeted development of informative microsatellite (SSR) markers" Nucl. Acid Res. 29 (8), E44-44

Ibanez M., Gonzalez-Penas E., Lizarraga E., Lopez de Cerain A. (2011) "Co-occurence of aflatoxins, ochratoxin A and zearalenone in barley from a northern region of Spain" Food Chem. 132, 35-42

John M. E. (1992) "An efficient method for isolation of RNA and DNA from plants containing polyphenolics" Nucl. Acids Res. 20, 2381

Jones D. A. (1972) "Blood samples: probability of discrimination" *Sci. Soc.* 12, 355-359

Kiss G. B., Csanadi G., Kalman K., Kalo P., Okresz L. (1993) "Construction of a basic linkage map for alfalfa using RFLP, RAPD, isozyme and morphological markers" Mol. Gen. Genet. 238, 129-137

Kris-Etherton P. M., You-Poth S., Sabatè J., Ratcliffe H. E., Zhao G., Etherton T. D., (1999) "Nuts and their bioactive constituents: effects on serum lipids and other factors that affect disease risk" Am. J. Clin. Nutr. 70, 504-511

Lerma-Garcia M. J., Herrero-Martinez J. M., Ramis-Ramon G., Simo-Alfonso E. F. (2008) "Prediction of the genetic variety of Spanish extra virgin olive oils using fatty acid and phenolic compound profiles established by direct infusion mass spectrometry" Food Chem. 108, 1142-1148

Maguire L. S., O'Sullivan S. M., Galvin K., O'Connor T. P., O'Brien N. M. (2004) "Fatty acid profile, tocopherol, squalane, and phytosterol content of walnuts, almonds, peanuts, hazelnuts, and macadia nuts" Int. J. Food Sci Nutr. 3, 171-178

Martin-Lopes P., Gomes S., Santos E., Guedes-Pinto H. (2007) "DNA markers for Portuguese olive oil fingerprinting" J. Agric. Food. Chem. 56 (24), 11786-11791

Mercanligil S. M., Arslan P., Alasalvar C., Okut E.,Akgul E., Pinar A., et al. (2007) "Effects of hazelnut-enriched diet on plasma cholesterol and lipoprotein profiles in hypercholesterolemic adult men" Eur. J. Clin. Nutrition 61, 212-220

Mildner-Szkudlarz S., Jelen H. H. (2008) "The potential of different techniques for volatile compounds analysis coupled with PCA for the detection of the adulteration of olive oil with hazelnut oil" Food Chem. 110, 751-761

Mullis K. B., Faloona F. (1987) "Specific synthesis of DNA in vitro via Polymerase Chain Reaction" Methods Enzymol. 155, 350-355

Murray M. G., Thompson W. F. "Rapid isolation of high molecular weight plant DNA" Nucl. Acids Res. 1980 (8), 4321-4325

Nei M. (1973) "Analysis of diversity in subdivided population" Proc. Nat. Acad. Sci. USA 70, 3321-3323

Ohmori K., Tsuchiya K., Watanabe T., Akiyama H., Maitani T., Yamada T., Hirayama K., Satoh S. (2008) "A DNA extraction method using a silica-base resin type kit for the detection of genetically modified papaya" J. Food Hyg. Soc. Jpn 49 (2), 63-69

Pasqualone A., Montemurro C., Caponio F., Blanco A. (2004) "Identification of virgin olive oil from different cultivars by analysis of DNA microsatellites" J. Agric. Food Chem. 52, 1068-1071

Pasqualone A., Montemurro C., Summo C., Sabetta W., Caponio F., Blanco A. (2007) "Effectiveness of microsatellites DNA markers in checking the identity of protected designation of origin extra virgin olive oil" J. Agric. Food Chem. 55, 3857-3862

Pasqualone A., Di Rienzo V., Bianco A., Summo C., Caponio F., Montemurro C. (2011) "Characterization of virgin olive oil from *Leucocarpa* cultivar by chemical and DNA analysis" *In press*

Philips, R.L. and Vasil, I.K. eds (2001) "DNA-based markers in plants", Kluwer Academic Publishers

Peres B., Barlet N., Loiseau G., Montet D. (2007) "Review of the current methods of analytical traceability allowing determination of the origin of foodstuffs" Food Control 18 (3), 228-235

Plat J., Melsink R. P. (2001) "Effects of plant sterols and stanols on lipid metabolism and cardiovascular risk" Nutr. Cardiovasc. 11, 31-40

Powell W., Machray G.C., Provan J. (1996) "Polymorphism revealed by simple sequence repeats" Trends Plant Sci. 1**,** 215-222

Proietti P., Pilli M. (2003) Atti del Convegno Nazionale "Germoplasma olivicolo e tipicità dell'olio" 05 dicembre 2003

Rajaram S., Burke K., Connell B., Myint T., Sabatè J. (2001) "A monounsaturated fatty acid-rich pecan-enriched diet favourably alters the serum lipid profile of healthy men and women" J. Nutr. 131, 2275-2279

Rallo P., Tenzer I., Gessler C., Baldoni L., Dorado G., Martin A. (2003) "Transferability of olive micro satellite loci across the genus *Olea*" Theor. Appl. Genet. 107, 940-946

Sachidanandam R., Weissman D., Schmidt S. C., Kakol J. M., Stein L. D., Marth G., Sherry S., Mullikin J. C., Mortimore B. J., Willey D. L. (2001) "A map of human genome sequence variation containing 1.42 million single nucleotide polymorphisms" Nature 409, 928-933

Sayago A., Garcia-Gonzalez D. L., Morales M. T., Aparicio R. (2007) "Detection of the presence of refined hazelnut oil in refined olive oil by fluorescence spectroscopy" J. Agric. Food Chem., 55 (6), 2068-2071

Schlotterer C., Tautz D. (1992) "Slippage synthesis of simple sequence DNA" Nucl. Acids Res. 20, 2211-2215

Shephard G. S. (2008) "Impact of mycotoxins on human health in developing countries" Food Additives and Contaminants 25 (2), 146-151

Spaniolas S., Bazakos C., Ntourou T., Bihmidine S., Georgousakis A., Kalaitzis P. (2008) "Use oh lambda DNA as a marker to assess DNA stability in olive oil during storage" Eur. Food Res. Technol. 227, 175-179

Sobrino B., Brion M., Carracedo A. (2005) "SNPs in forensic genetics: a review on SNP typing methodologies" Forensic Sci. Int. 154, 181-194

Squirrel J., Hollingsworth P. M., Woodhead M., Russel J., Lowe A. J., Gibby M., Powell W. (2003) "How much effort is required to isolate nuclear microsatellites from plants?" Mol. Ecol. 12, 1339-1348

Tautz D., Renz M. (1984) "Simple Sequences are ubiquitous repetitive components of eukaryotic genomes" Nucl. Acids Res. 12, 4127-4138

Testolin R., Lan O. (2005) "DNA extraction from olive oil and PCR amplification of microsatellites markers" J. Food Sci. 70 (1), 108-112

Thomas M. R., Matsumoto S., Cain P., Scott N. S. (1993) “Reptitive DNA of grapevine: classes present and sequences suitable for cultivar identification” Theor. Appl. Genet. 86, 173-180

Toth G., Gaspari Z., Jurka J. (2000) “Microsatellites in different eukaryotic genomes: Survey and analysis” Genome Res. 10, 967-981

Varshney R.K. *et al*. (2004) “Molecular maps in cereals: methodology and progress” In cereal Genomics (Gupta, P.K. and Varshney, R.K. eds), pp35-82, Kluwer Academic Publishers

Vlahov G. (2006) “13C NMR determination of olive oil adulteration by hazelnut oil” Handbook of Environmental Chemistry, vol 5: Water pollution 17 (8), 535-536

Vos P., Hogers R., Bleeke, M., Zabeau M. (1995) “AFLP: a new technique for DNA fingerprinting” Nucl. Acids Res. 23, 4407-4414

Williams J. G. K., Kubelik A. R., Livak K. J., Rafalski J. A., Tingey S. V. (1990) “DNA polymorphisms amplified by arbitrary primers are useful as genetic markers” Nucl. Acids Res. 18 (22), 6531-6535

Williams J. G. K., Kubelik A. R., Livak K. J., Rafalski J. A., Tingey S. V. (1991) “DNA polymorphisms amplified by arbitrary primers are useful as genetic markers” Nucl. Acids Res. 18, 6531-6535

Yamamoto T., Kimura T., Soda M., Ban Y., Hayashi T., Matsuta N. (2002) “Development of microsatellite markers in the Japanese pear (*Pyrus pyrifolia* Nakai)” Mol. Ecol. Notes 2, 14-16

GLOSSARY

ADH: primers designed for amplifying region (175 bp) of alcohol dehydrogenase *Corylus avellana* gene.

Amplification/PCR (polymerase chain reaction): molecular technique used with the aim to create multiple DNA copies of a specific DNA region. To do this, DNA is denature, at high temperature, in presence of specific primers and the Taq polymerase enzyme. The cooling down determine a preferential annealing among DNA and the primers. At this point the Taq starts to polymerize a complementary copy of template DNA using as starter point the primers bound to the DNA.

AJ810086: primers designed for amplifying 151 bp region of β-1,3-glucanase *Olea europaea* gene.

Capillary electrophoresys: electrophoretic technique that permit to identify the specific DNA fragment size. The DNA fragments separation is determined from a passage through a capillary. The passage through capillary is revealed by an UV detector. To perform this analysis, in fact, fragments have to be labeled with a fluorophore

DNA: deoxyribonucleic acid. Molecule constituted of two long complementary coiled polymers of nucleotides. DNA contains information for development and function of organisms.

LOX: primers designed for amplifying 478 bp region of lipoxygenase *Corylus avellana* gene.

LOX1: primers designed for amplifying 286 bp region of lipoxygenase *Corylus avellana* gene.

LOX3: primers designed for amplifying 163 bp region of lipoxygenase *Corylus avellana* gene.

Taq polymerase: is a thermostable enzyme, purified from *Thermus aquaticus* bacterium, and used in the amplification reaction to copy DNA from a template.

RNase: ribonuclease enzyme specific for degradation of the RNA molecule.

Proteinase K: peptidase enzyme specific for proteins degradation.

Primer: small fragment of DNA complementary to a specific DNA/RNA region and used as starter for DNA replication, by Taq polymerase enzyme.

Molecular markers: represent specific hypervariable genomic region recognizing with different molecular approach (PCR, specific digestion) and that permit us to identify polymorphisms and to characterize the analyzed matrix (fingerprint).

Microsatellite/SSR (simple sequence repeat): specific molecular markers recognizable through PCR technique. They correspond to small regions consisting of tandem repeats of 1-6 pairs. They are highly polymorphic, co-dominant, relatively abundant and highly dispersed within prokaryotic and eukaryotic genomes, as well as being discernible in other related species. Their recognizing is possible amplifying the markers with specific primers corresponding to the non variable region of them.

SNP (Single Nucleotide Polymorphism): single nucleotide variation, used as molecular marker to distinguish individual or specie. The identification of polymorphisms is possible through sequence analysis.

AFLP (Amplified Fragment Length Polymorphisms): single/multiple nucleotide variation (polymorphism) that determinate a change in a restriction site. The identification of polymorphisms is possible through the restriction of genomic DNA.

RAPD (Random Amplification of Polymorphic DNAs): this markers are analyzed through PCR amplifications that identify casual rearrangement or deletions at/or between oligonucleotide primer used for the analysis.

DNA polymorphisms: different allelic version of a specific locus detected through molecular markers analysis.

Fingerprint: definition of a DNA profiling of polymorphisms detected through molecular markers study. To define a fingerprint multiple markers have to be considered. Each matrix has an own specific fingerprint.

ABBREVIATIONS

ADH	primer alcohol dehydrogenase
AJ810086	primer for β-1,3-glucanase
CE	Capillary electrophoresys
CAC A014a	microsatellite
CAC B028	microsatellite
CAC B029b	microsatellite
CAC B111	microsatellite
CaT B107	microsatellite
CaT B502	microsatellite
CaT B504	microsatellite
CaT B507	microsatellite
CaT B508	microsatellite
G	DNabsolute Geneaid kit
LOX	primer for lipoxygenase
LOX1	primer for lipoxygenase
LOX3	primer for lipoxygenase
M**	FlashGel DNA ladder
M*	50 bp DNA ladder
M	1 kb DNA ladder
M-N	Nucleospin Food-Macherey-Nagel kit
Mo	Mortarella
P	Wizard Magnetic System Promega kit
PCR	Polymerase chain reaction
Q	QIAmp DNA stool Qiagen kit
S	Gene Elute Plant Sigma kit
S1	Extract-N-Plant PCR Sigma kit
T	traditional extraction method
TG	Tonda di Giffoni
TGR	Tonda Gentile Romana
To	Tombul

APPENDIX

1 14th Workshop *on the Development in the Italian PhD Research on Food Science Technology and Biotechnology,* Sassari, 16-18 Settembre 2009

2 15th Workshop on *Developments in the Italian PhD Research on Food Science Technology and Biotechnology,* Portici (NA), 15-17 settembre 2010.

-3 16th Workshop on *Developments in the Italian PhD Research on Food Science, Technology and Biotechnology,* Lodi 21-23 settembre 2011.

RINGRAZIAMENTI

Innanzitutto desidero ringraziare Gianni Pastore, insuperabile responsabile di laboratorio e ottimo compagno di lavoro, per il sostegno e la comprensione dimostratami in questi anni di dottorato e soprattutto per la consulenza tecnico scientifica datami per lo svolgimento della tesi di dottorato.

Un grazie particolare va anche alla mia amica e collega di lavoro Simona Baima sempre a me vicina e grande supporter scientifico.

Un sentito ringraziamento anche a Guido Leoni e Serena Arima per l'elaborazione dei dati statistici e al grande collega Marco Possenti per il supporto grafico.

Ringrazio anche i miei giovani colleghi di dottorato, in particolare Silvia Ricci e Guglielmo Santi, per aver reso piacevole, insieme ai professori, il percorso del dottorato e le trasferte durante gli Workshop. Un sincero ringraziamento va anche a tutti i professori del dottorato per l'ottima organizzazione del corso.

Infine desidero ringraziare Pino, mio inseparabile compagno di vita, per avermi incoraggiata e sostenuta ad affrontare l'ennesima piccola sfida nel mio percorso di vita.