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Genetic Characterization of Olive Germplasm by Molecular Markers

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ABSTRACT

The olive tree is a member of the *Oleaceae* family, which contains the genera *Fraxinus*, *Forsythia*, *Forestiera*, *Ligustrum* and *Syringa*, as well as the genus *Olea*. Commercial olives are products of the *Olea europaea* L. species. There are roughly 20 species of *Olea* found throughout tropical and subtropical regions of the world, but only *O. europaea* L. produces edible fruit. The fruit of olive trees can either be processed to make table olives or milled to produce olive oil. Of the many different varieties of olives, some olives are cultivated specifically for table consumption while the majority are used for oil extraction. The origin of *O. europaea* in the Mediterranean basin is not clear. Since olive cultivation has been practiced in all Mediterranean basin countries for many millenia, the presence of a large number of synonymic and homonymic species is very probable. The species' poorly-defined genetic natures give rise to several problems, both for olive nurseries and for correctly estimating the platforms needed to properly classify and exploit olive products like canned olives and oil. From a commercial perspective, the Mediterranean basin grows many varieties of olive trees, and this region alone produces 99% and consumes 87% of the world's olive oil. Thus, a solution to this problem is highly desirable. A formidable effort has been made to characterize olive germplasm using different types of biochemical and molecular markers. This review highlights the importance of studying the degree and distribution of genetic diversity for better exploitation of olive resources and for the design of plant breeding programmes.

Keywords: fingerprinting, genetic relationships, *Oleaceae*, *Olea europaea*

Abbreviations: AFLP, Amplified Fragment Length Polymorphism; **ANN**, Artificial Neural Network; **cpDNA**, chloroplast DNA; **FAO**, Food and Agriculture Organization; **ISSR**, Inter Simple Sequence Repeat; **ITS**, Internal transcribed spacer; **mtDNA**, mitochondrial DNA; **PGI**, Protected Geographical Indication; **PDO**, Protected Designation of Origin; **QTL**, Quantitative trait locus; **RAPD**, Random Amplified Polymorphic DNA; **RFLP**, Restriction Fragment Length Polymorphism; **SCARS**, Sequence Characterized Amplified Regions; **SNP**, Single Nucleotide Polymorphism; **TSG**, traditional specialty guaranteed

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INTRODUCTION

Cultivation of olives

The olive tree is one of the oldest cultivated plants, and its fruit has been used for nourishment for more than 5,000 years in the Mediterranean regions where it originated. Over the last few centuries, cultivation of the olive tree has spread to North and South America, as well as Japan, South Africa, and Australia. Due to the tree's need for a warm but not excessively hot climate, it can be cultivated in both the northern and southern hemispheres between 30 and 45 degrees latitude, with the exception of some equatorial regions where olive trees are grown at high altitude. Nowadays, olives are produced in more than 40 countries spread across all six inhabited continents, and even in exotic places like Hawaii.

The exact origin of *Olea europaea* within the Mediterranean basin is not clear, but multi-local domestication of its cultivated forms likely contributed to its development. Most studies agree that after the initial spreading of a few ancestral olive varieties along the Mediterranean basin, a majority of modern cultivars were derived either from the inter-crossing of these ancient cultivars, or from interbreeding with wild plants, followed by local selection (Angiolillo *et al.* 1999; Besnard and Berville 2000; Besnard *et al.* 2001; Rotondi *et al.* 2003). Since the cultivation of olives has proceeded for many millennia in all Mediterranean basin countries, in all probability a large number of synonymic and homonymic species exist today. The true nature of olive germplasm and the extent of its genetic variability are still undefined. This undefined genetic nature gives rise to several problems, both for olive nurseries and for the correct estimation of the platforms needed to classify and properly

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Invited Review

Table 1 World production of olives in 2003. On a world scale the importance of olive production can be summed with these data.

Size of world olive production	
total area	7 000 000 ha
trees in production	600 000 000
olives produced	8 400 000 tons
oil produced	1 600 000 tons
Data: 2003 FAO Stat	
World olive oil production in 2005. These seven countries alone account for 90% of world production.	
Spain	36%
Italy	25%
Greece	18%
Tunisia	8%
Turkey	5%
Syria	4%
Morocco	3%
Portugal	1%
Data: 2005 International Olive Oil Council	

exploit olive products like canned olives and olive oil.

The current scale of world-wide olive production is depicted in **Table 1**. Although olive production is distributed over five major continents, the Mediterranean Basin, which accounts for 98% of all olive production area and 97% of all olive production, dominates the industry. By themselves, the four countries of Spain, Greece, Italy and Tunisia represent: 65% of the total production area; 76% of the total trees in production; and 74% of world-wide olive production.

Presently, Spain is the world's single largest producer of olives, having displaced Italy sometime in the early 1990's. That surge in production was mainly due to a change in Spanish methods of olive cultivation. More than in other regions, Spanish farmers have used modern, intensive, high-yielding methods to displace traditional, extensive techniques. In addition, Spain's olive-producing acreage has increased by more than 15% in the last decade. Thanks to government efforts, the olive sector has grown into one of Spain's most important agricultural and industrial branches.

Five countries of the European Union (EU) actively produce olives. In addition to Spain, Italy, and Greece, both Portugal and France contribute to the olive-producing Community (**Table 1**). With three-quarters of the world market and annual production levels exceeding two million tonnes, the EU is by far the leader in the olive business.

Nonetheless, Tunisia, Turkey (an EU), Syria, and Morocco are also important growers; all together, they produced more than 500,000 tonnes of olives during the 2000-2001 growing season. This amount is equivalent to approximately 25% of EU and 20% of total world production levels.

Based on estimates by the FAO Plant Production and Protection Division Olive Germplasm, the world's olive germplasm contains more than 1,200 different cultivars and over 3,600 synonyms (Bartolini and Petruccelli 2002; Fiorino *et al.* 2005), with many local varieties and ecotypes contributing to this richness. The olive germplasm therefore represents an important reserve of genetic diversity for the Mediterranean basin ecosystem.

BOTANICAL CLASSIFICATION AND DESCRIPTION

The olive tree is a member of the *Oleaceae* family, which contains the genera *Fraxinus*, *Forsythia*, *Forestiera*, *Ligustrum*, and *Syringa*, in addition to the genus *Olea* (**Table 2**).

The genus *Olea* of the sub-family *Oleideae*, includes two sub-genera, *Olea* and *Paniculatae.* According to recent revisions of the *O. europaea* taxonomy (Green and Wickens 1989; Green 2002), this species is divided into the following six sub-species based on morphology and geographical distribution:

1) subsp. *cerasiformis*, found on the island of Madeira;
2) subsp. *cuspidata*, distributed from Iraq to China;

Table 2 Botanical classification.

Scientific classification	
Kingdom	Plantae
Division	Magnoliophyta
Class	Magnoliopsida
Order	Lamiales
Family	Oleaceae
Genus	*Olea*
Species	*Olea europaea*

3) subsp. *europaea*, divided into the two botanical varieties *europaea* (cultivated olive) and *sylvestris* (wild olive), and widely-distributed throughout the Mediterranean Basin;
4) subsp. *guanchica*, found on the Canary Islands.
5) subsp. *laperrinei*, localized to the Sahara region;
6) subsp. *maroccana*, found in Morocco;

Commercial olives are products of *Olea europaea* subsp. *europaea*, as only this species produces edible fruit. The olive tree (variety *europaea*) can reach heights ranging from just a few meters to 20 meters. The trunk is irregular, and the branches bear evergreen, elliptical and/or lanceolate leaves whose upper and lower surfaces are green and silvery, respectively. Blooming occurs between April and June and pollination is prevalently anemophilous. Populations of wild olive trees (variety *sylvestris*) are restricted to a few isolated areas of the native Mediterranean forest, where it is possible that wind and birds are responsible for the distribution of pollen and seeds (Lumaret *et al.* 2004). Molecular analysis using both nuclear and cytoplasmic markers has shown that the eastern and western Mediterranean populations of wild olive trees are strongly differentiated from each other (Besnard *et al.* 2001a, 2001b, 2002b; Lumaret *et al.* 2004). In contrast, cultivated olives do not exhibit such geographical differences, even though individual variability can be quite high. Empirical data gathered from olive growers associated with naturally crossbred genotypes have repeatedly shown evidence for the multi-local selection of most cultivars (Besnard *et al.* 2001b; Rotondi *et al.* 2003).

The olive tree is a long-lived evergreen (**Fig. 1**), and some specimens have been reported to live for nearly 2,000 years. Its wood can resist decay, and when mechanical damage or environmental extremes kill the top of the tree, new growth arises from the root system. When propagated by either seed or cuttings, the root system generally is shallow, spreading to only 0.9-1.2 m even in deep soils. The above-ground portion of the olive tree is recognizable by its dense assembly of limbs, short internodes, and the compact nature of the foliage. Light does not readily penetrate into the interior of an olive tree unless the tree is pruned to create light channels. If left unkempt, olive trees develop multiple branches with cascading limbs. The branches are able to bear large quantities of fruit on their terminal twigs, which are pendulous, flexible, and sway with the slightest breeze.

Olive leaves are thick, leathery, and oppositely arranged. Each leaf grows over a two-year period. Leaves have stomata on their lower surfaces only. Stomata are nestled in peltate trichomes that restrict water loss and make the olive tree relatively resistant to drought. Some multi-cellular hairs are present on the leaf surfaces. Olive leaves usually abscise in the spring after they are 2 or 3 years old. As with other evergreens, however, leaves older than 3 years are often present.

Flower bud inflorescences are borne on each leaf's axil (**Fig. 2A**). The bud is usually formed during one season, at which point it can remain dormant for more than a year before beginning visible growth during the subsequent season. After the buds become viable inflorescences, flowers bloom a season later than expected. Each inflorescence contains between 15 and 30 flowers, depending on the cultivar and on the extent of that year's development. During the time when each leaf axil maintains a developing inflorescence, there are hundreds of flowers per twig.

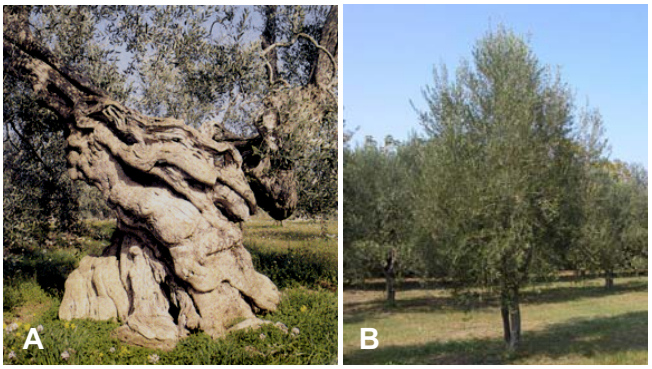

Fig. 1 Olive trees. (**A**) Monumental olive tree in Apulia Region – Southern Italy. (**B**) A young olive tree in CRA-OLI collection Calabria Region - Southern Italy.

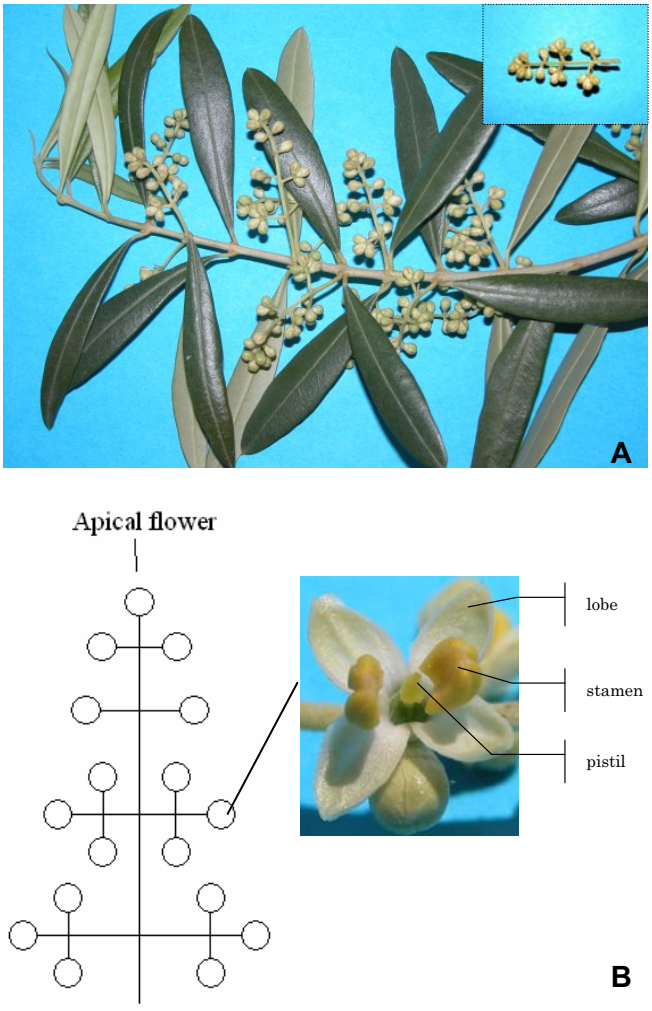

Fig. 2 (**A**) olive branch laden with inflorescence (photo by G. Godino). (**B**) Inflorescence structure of olive. Shaded circles represent the position of the flowers (on the left). Flower structure of olive (on the right) (photo by G. Godino).

Olive is andromonoecious, i.e. individual trees bear both hermaphrodite and staminate flowers (Besnard *et al.* 2000). Hermaphrodite flowers consist of a small, greenish calyx, four white petals, two stamens with large anthers, and a pistil composed of a bilobulate stigma, a short style and a bilocular ovary with four ovules. Staminate flowers result from pistil abortion at varying stages of gynoecium differentiation and possess a non-functional, rudimentary pistil. Flowers are wind-pollinated. Although self-fertilization is not totally precluded, cross-pollination results in earlier and greater levels of fertilization (Cuevas and Polito 2004).

The flowers are small (**Fig. 2B**), yellow-white, and inconspicuous. Each contains a short, four-segmented calyx and a short-tubed corolla with four lobes. The two stamens are on opposite sides of the two-loculed ovary, which bears a short style and a capitate stigma. Two types of flowers are present each season: perfect flowers, which contain stamen and pistil; and staminate flowers, which contain aborted pistils but functional stamens. The proportion of perfect to staminate flowers varies with inflorescence, cultivar, and year. Large commercial olive crops occur when 1 or 2 perfect flowers are present among the 15 to 30 flowers per inflorescence. As a rule, more staminate flowers than perfect flowers are present.

A perfect flower is identified by its large pistil, which nearly fills the space within the floral tube. The pistil is green when immature and deep green when open at full bloom. Staminate flower pistils are tiny and barely rise above the floral tube base. Their style is small and brown, greenish-white, or white, and their stigma is as large and plumose as a functional pistil.

The olive fruit is a drupe (**Fig. 3**), which is botanically similar to the almond, apricot, cherry, nectarine, peach, and plum. The olive fruit consists of carpel, and the wall of the ovary has both fleshy and dry portions. The skin (exocarp, 1.5-3.5% of the total fruit) is free of hairs and contains stomata. The flesh (mesocarp, 70-80% of the total fruit) is the tissue that is eaten, and the pit (endocarp, 13-24% of the total fruit) encloses the seed (2-4% of the total fruit). Fruit shape, fruit size, pit size, and surface morphology all vary greatly among cultivars.

Quantitatively, the largest constituents of the drupe are water (40-70%) and oil (6-25%). The composition of this fruit is variable because it depends on olive variety, soil, climate, and cultivation.

Olive fruit pulp naturally possesses a bitter taste due to the presence of the glycoside oleuropein, and has very high oil content (De Nino *et al.* 2005). The fruit is typically subjected to fermentation or cured with lye or brine to make it more palatable. Both green olives and black olives are washed thoroughly with water to remove any oleuropein. Sometimes they are also soaked in a solution of sodium hydroxide in order to accelerate the fermentation process. Epimeric derivatives of oleuropein have been detected in olive fruits (Bianco *et al.* 1999). These hydrolytic metabolites, obtained by enzymatic catalysis, can be molecular microcomponents, present in Mediterranean food, table olives, and olive oil, responsible for complex sensorial attributes and for pathogen natural defence.

The histological location of phenolic compounds change during olive fruit maturation (Bitonti *et al.* 2000). Namely, in green drupes vacuolar phenolic inclusions are homogenously present in the different tissues, while in full-ripened drupes the presence of phenolic vacuolar inclusions strongly diminish in the inner tissues, while the epicarp layer, as well

Fig. 3 Olive branch laden with fruit. (photo by G. Godino).

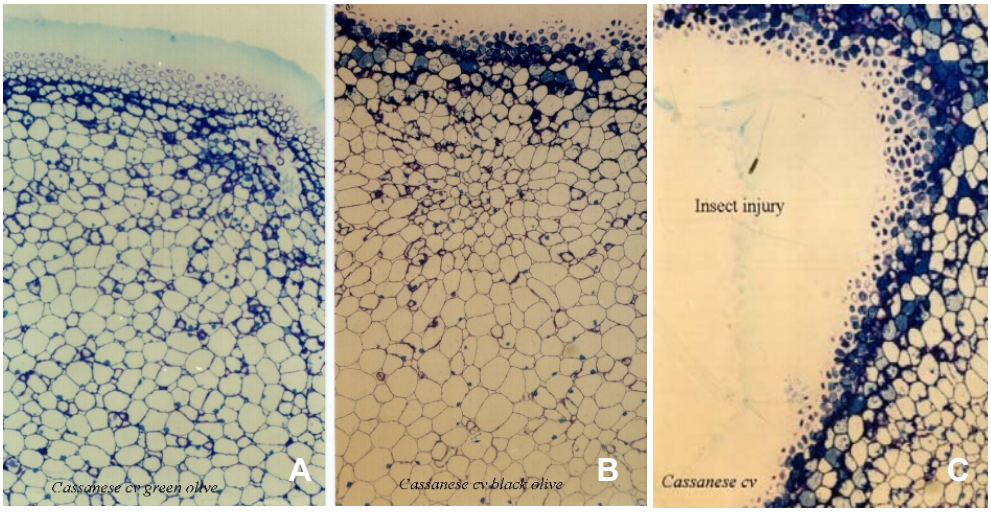

Fig. 4 Levels of phenolic compounds in fruit at different developmental stages. (**A**) Green and (**B**) dark fruits. Levels of phenolic compound in fruit sectors severely injured by *Bactrocera oleae* pathogen (**C**) dark fruits). (Photos by Bitonti *et al*. 2000)

as the first two external layers of mesocarp, show a large increase of phenolic content (**Fig. 4**). This preferential accumulation of phenolic compounds in the external zones of fruit, which occurs during maturation, appears well related to the defence role of phenolic compounds against abiotic and biotic stress factors. In this context it is noteworthy that the preferential location of phenolic compounds is detected around the insect injury. In facts, the phenolic compound (oleuropein and derivatives) were present more abundant in infected fruits by *Bactrocera olea* as compared to wealth fruits used as control (**Fig. 4C**). The fly directly attacks the fruit mesocarp, and can have serious consequences on production, by inducing early fruit fall or causing total disruption of the pulp, for this representing the most dangerous pathogen of olive plants.

Traditionally, the olive tree was grown mainly in the Mediterranean area, but as the human health benefits of olive products became widely recognized, growth of the plant spread throughout the world. Tocopherols are antioxidant compounds that confer nutritional value to many drupes. Tocopherol biosynthesis takes place on the inner membrane of chloroplasts and chromoplasts. The four tocopherols, α, β, γ and δ, are distinguished from one another by the number and position of methyl groups located on the phenolic part of the chromane ring. The multifunctional biochemical roles of tocopherols are related to their ability to protect against damage from reactive oxygen species. In the past, α-tocopherol was considered to be the isomer exhibiting the greatest biological activity. Recent studies suggest, however, that the other vitamin E isoforms also play important roles in human biology. For example, γ-tocopherol is thought to prevent cancer and to be a potent and effective agent for preventing cerebral infarction resulting from middle cerebral artery occlusion. Several studies demonstrate that during the development of the olive fruit, the tocopherol profiles are variable, and the extent of this variability frequently depends on the cultivar and the stage of fruit ripening (Cunha *et al.* 2006; Muzzalupo *et al.* 2007a).

The fruit's oil component is contained in both the pulp (mesocarp) and the stone (endocarp and kernel), and so olive oil is generally extracted from the whole fruit.

In general, most types of fruit have a rather low fat content. In contrast, olive fruits have a relatively high fat content which increases with maturation. Mature fruit will always possess a higher fat content than less mature green fruit. The fat composition also changes with maturation. The proportions of palmitic, linoleic and linolenic acids diminish as the maturation index increases, while the levels of both stearic and oleic acids increase during this period (Perri *et al.* 2002; Lombardo *et al.* 2003, 2004). The concentration of reducing sugars present in olives is also lower than in most other fruits, and their levels decrease further as maturation progresses. Reducing sugars are very important for the fermentation process of all table olives, where they serve as the main carbon source for microbial growth. Fresh olives also have a high concentration of dietary fibre, which mainly consists of cellulose, hemicellulose, and lignin. Olives' protein content may range from 0.3 to 0.6%. The mineral content in fresh fruits is relatively low, with potassium being the most abundant ash element. Recent studies have shown that olives contain an abundance of antioxidants (up to 16 g/kg), including acteosides, hydroxytyrosol, tyrosol, and phenilpropionic acids (Perri *et al.* 2002; Lombardo *et al.* 2003, 2004). Additionally, fresh olives contain significant amounts of organic acids (malic, oxalic, citric, etc.).

The mature olive seed consists of a thin coat covering the starch-filled endosperm. The endosperm surrounds the tapering, flat, leaf-like cotyledons, the short radicle (root), and the plumule (stem). Seed shape and absolute size vary greatly with cultivar.

ECONOMIC IMPORTANCE

The olive tree has great economic importance in the Mediterranean basin, as about 80% of worldwide olive production is concentrated there. Moreover, the olive is the most extensively cultivated fruit crop in the world (FAO 2004). Cultivation areas dedicated to its production have tripled in the past 44 years, increasing from 2.6 to 8.5 million of hectares.

The two commercial products that are obtained from olives are olive oil and table olives. Some varieties of olives are cultivated specifically for table consumption, but the majority are used for oil extraction.

Virgin olive oil is overwhelmingly composed of triglycerides (>98%), along with traces of other compounds. The dominant triglyceride fatty acid species are the oleic acids (57-78%), palmitic, stearic, linoleic and linolenic (Salas *et al.* 2000; Caravita *et al.* 2007). The other minor constituents (alcohols, polyphenols, chlorophyll, carotenoids, sterols, tocopherols and flavonoids) contribute to the olive's organoleptic qualities, taste, flavour, and nutritional value (Perri *et al.* 2002; Servili and Montedoro 2002; Benincasa *et al.* 2003; Garcia-Gonzalez *et al.* 2004) and may also serve to distinguish olive oils originating from different regions. Olive oil, especially extra-virgin oil, contains not only small amounts of hydroxytyrosol and tyrosol, but also contains secoiridoids, lignans (Bianco *et al.* 1999; De Nino *et al.* 2000; Bianco *et al.* 2001; De Nino *et al.* 2005), and other compounds thought to possess anticancer properties (e.g., squalene and terpenoids) (Fabiani *et al.* 2002; Owen *et al.* 2004).

Extracting olive oil from the pulp of the drupe alone yields higher-quality oil. Indeed, separating the stone from the olive pulp, if performed without any violent crushing of the drupes, minimizes rancidification of the product oil because fewer mechanical and thermal actions are required

to process the olive paste. Eliminating the fruits' ligneous parts results in oil with a more delicate taste and a more fruity relish. Furthermore, oils extracted from de-stoned paste demonstrate higher resistance to forced oxidation (Rancimat's test) and better organoleptic characteristics. Experiments comparing the oil resulting from different extraction processes confirmed that by using de-stoned olives, one can obtain olive oil with a high phenolic content, higher oleuropein content, good stability, and a low concentration of peroxides (De Nino *et al.* 2008).

Removing the stone from the pulp reduces the volume of paste by 20-25%, depending on the types of olives used and their unique pulp/stone ratios. The residual oil quantity retained within the stone is about 2%. Assuming the intact fruit initially contains roughly 20% oil, discarding the stone therefore corresponds to a loss of at least 0.4 kg of product oil for every 100 kg of olives pressed. In practice, however, the humidity of the de-stoned paste is usually higher than that of a stone-pulp mixture. As a result, even under ideal conditions, losses of ~0.5 kg per 100 kg of olives are expected. From this analysis, however, it becomes clear that the quantity of total oil retained in the stone is very small. Moreover, the stone's oil has characteristics which differ from those of extra-virgin olive oil. Like many seed oils, olive stone oil has a high polyunsaturated triglyceride content. It also contains relatively large quantities of squalene as well as traces of oleuropein (de Nino *et al.* 2008). Thus, oil extracted from de-stoned paste is superior in terms of both flavor and resistance to oxidation. Oil extracted from stones can be further separated into its constituent components, which are valuable for their commercial applications in beauty products and pharmaceuticals. In addition, the ligneous part of the stone can be used as an alternative combustible fuel, and the olive husks can be used either as zootechnic fodder or to produce qualitative compounds (Amirante *et al.* 2006).

OLIVE GERMPLASM CHARACTERIZATION

The genetic patrimony of the Mediterranean Basin's olive trees is very rich and is characterised by and abundance of varieties. The 2005 web-based edition of the olive germplasm database (Bartolini and Petruccelli 2002) contains information extracted from 1,256 publications on 1,208 cultivars reported in 52 different countries and conserved in 94 separate collections. These figures most likely represent an underestimate of the true diversity, because they do not include many of the minor local varieties that are specific to certain olive growing areas.

The extent of this diversity has important implications for both the adaptation of cultivars to their local environment and for the optimization of these cultivars' agronomical performance under a given set of environmental conditions. For example, every initiative promoting olive cultivation should consider the potential repercussions of such action on any local olive varieties. Every region should preserve its own plant material in order to safeguard both the adaptation and productivity of the species and the unique characteristics of the region's olive oil. The problem of characterizing the olive tree germplasm is complicated not only by the richness of its genetic patrimony, but also by the absence of reference standards and a well-defined system of nomenclature that is free from homonymy and synonymy (Bartolini and Petruccelli 2002).

The preliminary work performed in olive tree genomics is currently very far from producing results that are useful for selecting new cultivars using molecular tools. This combined with the general lack of prior knowledge regarding the cultivated and wild olive germplasms, has focused attention mainly on the evaluation of the germplasm. This paper demonstrates the importance of studying olive tree genetic diversity for better exploitation of olive genetic resources and the design of plant breeding programmes.

There is a strong need for a means of reliably identifying different olive tree varieties, partly because so many of these varieties are propagated solely via vegetative methods. This would also be of substantial benefit to nurserymen and growers, because the cost of plants represents the major investment in establishing new orchards. At the same time, it is also important to improve the *ex-situ* plant germplasm collection in order to characterize adequately all accessions, and to develop future breeding programs.

Several Mediterranean regions have promoted international olive germplasm collections, including Cordoba (Spain), Porquerolles (France) and Marrakech (Morocco), which host most of the Mediterranean cultivars. The systematic collection of Italian olive cultivars for deposit into specific catalogue fields began in Italy in the 1980s. A similar international collection was begun in 1997 by Consiglio per la Ricerca e la sperimentazione in Agricoltura – Centro di ricerca per l'OLivicoltura e l'Industria olearia (CRA-OLI) of Rende in Cosenza, Italy. Collection entailed the following steps: a survey of the territory, individuation, basic characterization, and introduction into the gene bank field. Material identified by other Italian scientific institutions (EU Project RESGEN) was also included. To date, roughly 500 accessions have been introduced into the CRA-OLI collection, and this list has been published (Bartolini and Petruccelli 2002; Fiorino *et al.* 2005) along with a description of the pertinent CRA-OLI aims and methods (Lombardo 2006).

A useful olive germplasm collection also requires an organizational system devoid of mislabelling, homonymy and synonymy, so that a reliable characterization of all accessions can be achieved without unnecessary confusion. Recent research has focused on using morphology and biochemical and molecular markers to characterise and identify olive cultivars. The identification of cultivars and accessions using molecular markers is a crucial aim of modern horticulture, because such a technique would greatly facilitate breeding programmes and germplasm collection management.

Morphological and biological characteristics are widely used for descriptive purposes and are commonly used to distinguish olive cultivars (Barranco and Rallo 1985; Barranco *et al.* 2000; Lombardo *et al.* 2003, 2004). Agronomic characterization has also aided in the classification of different olive cultivars (Del Rio and Caballero 1994; Barranco and Rallo 2000; Lombardo *et al.* 2006). Morphological characterisation of olive cultivars is potentially unreliable, because environmental factors strongly influence the plants' morphology. Despite this drawback, the age of trees, their training systems, and the phenological stage of the plants continues to be a key preliminary step in the description and classification of the olive tree germplasm (Lombardo *et al.* 2003; Fiorino *et al.* 2005). At the same time, improving *ex-situ* olive plant germplasm collections remains an important objective, which will ultimately prove useful for characterising all accessions and for developing future breeding programs.

Recently, a variety of molecular markers as been used to characterize and distinguish between olive cultivars. In light of these efforts, some combination of enzymatic markers with distinct morphological, physiological, and agronomic characteristics may ultimately provide a method for the reliable and systematic classification of olive tree varieties (Ouazzani *et al.* 1995).

Assessments of microsatellite markers, RAPD profiles, AFLPs, and RFLPs provide direct genotypic information, which has numerous, valuable applications in genetic studies. The main advantages of generating RAPD profiles are the technique's simplicity and low cost (Bogani *et al.* 1994; Fabbri *et al.* 1995; Powell *et al.* 1996, Wiesman *et al.* 1998; Besnard *et al.* 2001; Belaj *et al.* 2002; Muzzalupo *et al.* 2007b). Nevertheless, RAPD experiments demonstrate poor reproducibility, which hampers comparison between individual studies. Experiments assessing an organism's AFLP markers are more technically demanding than RAPD but are highly effective in detecting DNA polymorphisms (Angiolillo *et al.* 1999; Baldoni *et al.* 2000; Jakše *et al.* 2001;

Montemurro *et al.* 2005; Owen *et al.* 2005). In contrast to a plant species' chloroplast DNA (cpDNA), which occasionally can be insufficiently variable for intra-species comparison (Wolfe *et al.* 1987; Yamagishi *et al.* 1997; Amane *et al.* 1999; Besnard *et al.* 2000; Lumaret *et al.* 2000), mitochondrial DNA (mtDNA) within a given species varies enormously in terms of organization, size, structure, and gene arrangement (Brennicke *et al.* 1996). As a result, intra-species mtDNA variation is common in plants, especially in naturally occurring populations (Besnard and Berville 2000; Budar *et al.* 2001; Cavallotti *et al.* 2003). Taken together, these distinctive features make mtDNA sequencing a powerful tool for analysing a given plant population's genetic structure and phylogenetic relationships (Budar *et al.* 2001). Microsatellite markers are ubiquitous, abundant, and highly dispersed in eukaryotic genomes, but are costly to assess experimentally. Once these markers have been ascertained, the data can be readily shared among laboratories. Since not all microsatellites are identical (Rallo *et al.* 2000; Sefc *et al.* 2000; Macaulay *et al.* 2001; Carriero *et al.* 2002; Cipriani *et al.* 2002; Muzzalupo *et al.* 2006, 2008a, 2008b), however, successful utilisation of known microsatellite markers requires prior information regarding the characteristics of a particular genetic locus.

Internal transcribed spacer 1 (ITS-1) sequences, RAPD profiles, and inter-SSR (ISSR) markers have been employed to evaluate the colonization history of *O. europaea* (Hess *et al.* 2000; Pasqualone *et al.* 2001; Vargas and Kadereit 2001). A number of *O. europaea* retroelements have also been identified (Hernandez *et al.* 2001), and their copy number has been estimated (Stergiou *et al.* 2002). Using previously established RAPD profiles (Hernandez *et al.* 2001), Mekuria *et al.* (2001) developed SCAR markers linked to leaf peacock spot tolerance.

Another method to distinguish inter-cultivar variability and to characterise clonal variants using single nucleotide polymorphisms (SNPs) in the olive tree genome is also currently under development (Reale *et al.* 2006).

All the aforementioned genetic techniques provide useful information regarding the level of olive tree polymorphism and diversity, which demonstrates their utility for the characterisation of germplasm accessions (Belaj *et al.* 2003). Although these characterisation methods are effective, they are resource- and labor-intensive, and they require skilled technical staff to be performed correctly. We therefore assessed the application of artificial neural networks (ANNs) as a possible alternative method for olive tree cultivar classification and identification (Mancuso and Nicese 1999).

ANNs are processing devices that are loosely modelled after the neuronal structure of the brain. An ANN consists of a pool of simple processing units which communicate by sending signals over a large number of statistically weighted connections. Each unit performs a relatively simple job. It receives an input signal from either neighbouring units or external sources, and uses this signal to compute an output signal which is then propagated to other units in the network. The system is inherently parallel, because many units carry out their computations simultaneously. Most neural networks are programmed with a set of learning rules, through which the weights of inter-neuronal connections are adjusted on the basis of output feedback. In other words, neural networks learn by example, and so they must be taught using a training data set in which both the initial inputs and the known solutions are supplied. Mancuso *et al.* (1998) conducted a study in which ANNs were employed to identify grape (*Vitis vinifera* L.) cultivars. Mancuso and Nicese (1999) similarly applied ANNs with great success to distinguish among olive cultivars. In these experiments, quantitative analysis of leaf morphology served as the input parameters to the ANN.

One potential application for olive cultivar fingerprinting is the varietal certification of plants for the production of olive oils that are typical of specific geographical areas (PDO, PGI and TSG). In recent years, increasing demands for food safety have raised interest in methods for determining a product's origin and authenticity (Muzzalupo and Perri 2002; Perri *et al.* 2002; Busconi *et al.* 2003; Breton *et al.* 2004; Pasqualone *et al.* 2004; Pafundo *et al.* 2005; Testolin and Lain 2005; Muzzalupo *et al.* 2007c). Being able to trace an olive oil's origins has become instrumental to consumer protection strategies, because the quality of the oil depends upon both the cultivars responsible for its production and the environmental conditions surrounding olive growth. Moreover, oil traceability is indispensable for avoiding marketplace fraud in the form of mixing high-quality products with lower quality oils. Sadly, many olive oils on the market today are tainted with oil from anonymous cultivars and/or by mixture with cheaper alternatives, such as sunflower, peanut, and corn oils.

Traceability also allows for verification of a particular cultivar's contribution to a given PDO oil. The new European Council Regulation, EEC/2081/1992, has defined the relative contribution of specific cultivars for every commercial oil designation. For this reason, it is necessary to develop procedures for cultivar identification in order to be able to directly demonstrate that the legally mandated cultivar composition has been respected. One promising method for verification of oil composition would be through DNA analysis, since it enables cultivar fingerprinting (Muzzalupo and Perri 2002; Perri *et al.* 2002; Busconi *et al.* 2003; Breton *et al.* 2004; Pasqualone *et al.* 2004; Pafundo *et al.* 2005; Testolin and Lain 2005; Muzzalupo *et al.* 2007c).

BREEDING FOR OLIVE GERMPLASM IMPROVEMENT

To date, modern molecular technologies in plant breeding have not been applied extensively in olive, but using biotechnology may provide profitable results. As has been demonstrated in other crops, biotechnological methods can improve the efficiency and increase the speed of breeding.

Plant propagation is generally by cutting or grafting onto seedling rootstocks. Cultivars are mostly diploid ($2n = 2x = 46$) (Minelli *et al.* 2000), but tetraploid plants have been reported (Rugini *et al.* 1996). The DNA content is 2.2 pg per 1C nucleus (Rugini *et al.* 1996), equivalent to a genome size of 2.2 Gbp (De la Rosa *et al.* 2003).

In trees, with a long reproductive cycle, high levels of heterozygosity and sometimes self-incompatibility, methods for obtaining homozygous plants are of strong interest, as their production through conventional methods requires several generations which is difficult to realize in woody plants.

Due to poor knowledge of olive genome and because of species biological features (i.e., long juvenile phase and prevailing self-incompatibility), strategies for olive crop breeding are still fairly limited.

The most important successful in olive breeding are directed towards overcoming current limiting factors for production. These include: increasing fruit size and number; increasing oil content; quality improvement; shortening the juvenile stage; stabilising yield; manipulations of tree architecture to facilitate mechanical harvesting and improving resistance to pests and diseases (*Bactrocera oleae*, *Verticillium dahliae*, *Pseudomonas savastanoi*). Other important objectives were as: to relate in cold tolerance, to the promotion of self-fertility and to the promotion of dwarfing. Even the rootstock selection was focused on the ability to control scion vigour, and to improve the level of resistance to biotic and abiotic stresses.

It is possible to greatly reduce the length of the juvenile phase by using forcing protocols, but the evaluation of the agronomic performance of mature plants still requires at least five years of experimentation (Santos Antunes *et al.* 1999). Furthermore, the genetic control of the major traits is unknown (De la Rosa *et al.* 2003). Vigour, leaf size and fruit shape seem controlled by major genes showing dominance (Bellini 1993), while the inheritance of other characters, such as fruit size, flowering intensity, fruit set, ripening time and yield remains uncertain (Bellini 1993; Parlati *et al.*

1994).

The use of *in vitro* techniques, even if still in a preliminary phase, could be promising (Rugini *et al.* 1996, 2000; Rugini and Baldoni 2004; Briccoli-Bati *et al.* 2006). As a general rule, the *in vitro* culture of *Olea europaea* species is widely dependent on the medium composition (Roussos and Pontikis 2002; Brhadda *et al.* 2007; Maalej *et al.* 2006). In particular a specific medium for recalcitrant olive varieties has been defined by undertaking an analysis of mineral elements in embryos or young shoots (Rugini *et al.* 1996; Maalej *et al.* 2002, 2006). The level and carbon source strongly influences shoot proliferation and growth rates (Ruggini *et al*. 2000; Briccoli-Bati *et al.* 2006). In spite of these extensive studies, the *in vitro* propagation of *Olea europaea* species is still limited due to poor growth, poor lateral bud outgrowth and variable rooting ability of the explants (Rugini *et al.* 2000; Roussos and Pontikis 2002; Rugini and Baldoni 2004). The problem is compounded by intraspecific variation in tissue culture responses between different cultivars. The literature clearly shows that medium and intraspecific genotypic variations strongly influence tissue culture response of *Olea europaea*. Indeed differences have been detected in growth pattern, leaf differentiation and rooting ability of micropropagated shoots when comparing different cultivars and media.

Most selection programs have so far relied on clonal selection, on the assumption that in a long-living plant such as olive, natural mutations generating any positive alteration in a trait of agronomic interest, can be maintained by vegetative propagation (Belaj *et al.* 2004). Exploration of phenotypic variability in agronomic characters has led to the identification of valuable clones within numerous olive cultivars (Bartolini and Petruccelli 2002; Lombardo *et al.* 2003, 2004, 2006). The evaluation of minor local cultivars, present in every cultivation area, has recently been exploited to identify individuals highly adaptive to extreme environmental conditions (Lombardo *et al.* 2003, 2004; Muzzalupo *et al.* 2006). Clonal rootstocks have shown ability to control scion vigour and resistance to frost injury (Bitonti *et al.* 2000; Pannelli *et al.* 2002).

Experiments of genetic transformation are in progress with the aim to select disease resistant cultivars or to introduce key genes involved in important metabolic pathways (Rugini *et al.* 2000; Rugini and Baldoni 2004).

Very few cultivars have been emerged from formal breeding programmes. Three new olive cultivars ('Arno', 'Basento' and 'Tevere') were released from the progeny of the cross 'Picholine' × 'Manzanilla' (Bellini *et al.* 2002) and their performance is still under evaluation (Seinolta Project, CRA-OLI).

GENETIC MAPS AND GENE MAPPING

Two linkage maps of the olive genome have been constructed. The first was assembled by De la Rosa *et al.* (2003) based on RAPD profiles, AFLPs, RFLPs, and SSRs exploiting the progeny derived from two highly heterozygous cultivars, 'Leccino' and 'Dolce Agogia'. The second was assembled by Wu *et al.* (2004), using RAPD profiles, SCARs, and SSRs from plants resulting from a cross between the 'Frantoio' and 'Kalamata' cultivars. At present, no additional olive genome mapping data are available, no QTLs have been detected, and genome organization remains largely a mystery.

A gene encoding the geranylgeranyl hydrogenase enzyme (CHLP) has been characterised in the Italian olive cultivar 'Carolea'. This enzyme reduces free geranylgeranyl diphosphate to phytil diphosphate, which provides the side chain for tocopherols, plastoquinones, and chlorophylls. The 1,395 bp-long open reading frame, which is most similar to a gene present in *Nicotiana tabacum*, encodes a deduced protein (*Oe*CHLP) that is 464 amino acids in length with a predicted molecular weight of 51.2 kDa. In order to relate gene activity to tocopherol synthesis, *Oe*CHLP expression levels in the fruits of five olive cultivars with different tocopherol contents have been evaluated by Q-PCR (Bruno *et al.* 2006; Chiappetta *et al.* 2007).

Mapping of olive tree gene sequences has focused on orthologous genes previously characterised in other species (GenBank web site: http://www.ncbi.nlm.nih.gov/Genbank). Particular attention has been paid to the genes encoding the key enzymes involved in fatty acid biosynthesis, fatty acid modification, triacylglycerol synthesis, and fat storage (Haralampidis *et al.* 1998; Poghosyan *et al.* 1999; Giannoulia *et al.* 2000; Hatzopoulos *et al.* 2002; De la Rosa *et al.* 2003; Banilas *et al*. 2005).

CONCLUSIONS AND FUTURE RESEARCH

The cultivated olive germplasm contains at least 1,200 main cultivars. National and international collections maintain about 4,200 genotypes, and more than 5,300 cultivar names are recognized (Bartolini and Petruccelli 2002). The unknown genetic origin of most cultivars and their patchy distribution across large geographic regions often contribute to the confusion surrounding their identities.

Different groups working independently to characterise and identify olive cultivars have produced interesting information with respect to partial genotypes. Nevertheless, comparison of classification data among research groups still remains a cumbersome obstacle to progress. Up until now, lack of agreement regarding the standardisation of genetic and molecular fingerprinting techniques has made it impossible to gain a complete and accurate picture of olive tree cultivar variety and distribution.

Establishment of a common protocol for olive tree molecular analysis should be useful for population genetic studies, to enable discriminating among closely related cultivars (Belaj *et al.* 2003; Muzzalupo *et al.* 2006, 2008a, 2008b), and for association mapping. The use of the selected SSRs and the application of a common strategy for data comparison will finally allow for data convergence, which is a necessary precondition for the creation of an open-source molecular database for storing olive tree genetic resources.

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