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Amelioration des productions végétales et ressources génétiques

Caracterisation de quelques cultivars algeriens d'oliviers (olea europea L.) par l'utilisation de marqueurs moleculaires (SSR) (Simple Sequence Repeats)

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Dédicace

Alla memoria di mia madre, Alla memorie di mio padre, & Alla mia famiglia Sihem

Summary

Olive **(*olea europea* L.)** is a very important tree crop, long cultivated around the Mediterranean. Distinguishing cultivars is complicated by the frequency of homonyms and synonyms. In this work thirty four **(34)** accessions of olive from the Algerian germplasm collection of the ITAF-Institut technique de l'Arboriculture Fruitiére were studied.

This work presents the results of molecular characterization and identification of thirty four **(34)** Algerian olive varieties using five **(05)** SSR markers. Five SSR markers were genotyped in 34 Algerian varieties using PCR. This study showed that the use of molecular markers like SSRs is very useful to build a data base available for variety analysis and for olive germplasm collection management.

Key words: *Olea europea* L., Algerian cultivars, Molecular characterization, SSR markers and genetic variability.

Résumé

Olivier **(*Olea europea* L.)** est une culture très important, cultivé depuis longtemps autour de la Méditerranée. La distinction des cultivar est compliquée par la fréquence des homonymes et synonymes.

Dans ce travail, trente-quatre **(34)** accessions d'olive de la collection du germoplasme Algérien de l'Institut technique de l'Arboriculture Fruitière (ITAF) ont été étudiés.

Ce travail présente les résultats de la caractérisation moléculaire et identification de ces trente quatre **(34)** variétés Algérienne d'olivier l'aide de cinq (05) marqueurs moléculaires SSR.

Cette étude a montré que l'utilisation de marqueurs moléculaires SSR est très utile pour construire une base de données disponibles pour l'analyse des variétés et pour la gestion du matériel génétique de la collection d'olivier.

Mots clés : Olivier (*Olea europea* L.), Cultivars Algériens, Caractérisation moléculaire, SSR, et variabilité génétique.

ملخص

الزيتون هو محصول مهم جدا ، المزروعة طويلة حول البحر الأبيض المتوسط. وتعقدت تمييز من الصنف من توائر هومونيم والمرادفات.
في هذا العمل ، تم دراسة (34) نوع من الزيتون المادة الوراثية للمعهد القي الجزائري جمع من أشجار الفاكهة (ITAF).
هذا العمل يقدم نتائج التوصيف الجزيئي وتحديد هذه أربعة وثلاثين (34) أصناف الزيتون الجزائري باستخدام خمس RSS(05)
وأظهرت هذه الدراسة أن SSR مفيدة جدا لبناء قاعدة البيانات المتوفرة لتحليل وإدارة أصناف من جمع المادة الوراثية للزيتون.
كلمات البحث : الزيتون , أصناف الجزائري ، التوصيف الجزيئي ، RSS، والتنوع الجيني.

Introduction

Olive **(*olea europea* L.)** (n=23) is one of the oldest cultivated plants and is an important oil producing crop in the Mediterranean Basin. Interest in olive is increasing due to the adoption of olive oil in diets as a healthy source of fats and other chemicals compared to other oils.

The olive tree has been part of Mediterranean civilization since before recorded history **(Bandelj *et al* , 2004);** it is one of the most ancient cultivated fruit trees **(Virginie *et al* .2002).** The specie ***Olea europea*** includes several subspecies with different morphological traits and different geographical origins **(Ergulen *et al* .2002).** All could have contributed to the evolution of the cultivated olive, of which precise origin is still unclear **(De La Rosa *et al* .2002., Perri *et al* .2002., Besnard *et al* .2001., Grigg. 2001)**.

Cultivar intercrossing and crosses between wild and cultivated forms, along with local selection of outstanding and subsequent olive cloning, could have led to a large number of varieties around their possible original areas of cultivation **(Barranco,1997).** Cultivar fingerprinting could be used in variety control, quality certification of plants for production of typical olive oils of determined geographical areas (determination of origin),quality certification against illicit sale of propagated materials, inter-cultivar discrimination and synonym identification **(Bassi *et al.* 2002.,Perri *et al* .2002. Resta *et al* .2002).**

Europe, with an estimated tree population of 400-500 millions, is the major growing area of olive accounting for 80% of world production (Spain 42%, Italy 24% and Greece12%).

However, olives are now cultivated throughout the world, from South Africa to Latin America, California, New Zealand and Australia, where one million trees are planted every year. The olive genetic patrimony is very rich. The long –living characters of the tree and its low breeding pressure have contributed to the preservation of variability within the species. Nevertheless, although cultivars diversity is very high, these cultivars are mainly local and old, having a limited diffusion area.

Algeria, like many countries within the Mediterranean basin, offers very favorable ecologic conditions for olive tree cultivation. The climate and soil, particularly along the coast and surrounded areas, are ideal for this tree to thrive.

The olive **(*Olea europea* L.)** is , among cultivated fruit trees, the richest in genetic diversity. The longevity of this species and the low breeding pressure it has undergone has contributed to the conservation of its variability, and the reduced extent of genetic erosion within its germplasm has allowed the persistence of most of olive diversity **(Rallo *et al* , 2000).**

This poses a series of problems concerning germplasm characterization, management and presentation. In addition, there is the problem arising from the existence of homonyms and synonyms. This makes cultivars identification very difficult and coplex. The development of markers as tool for olive germplasm characterization and early progeny selection absolutely necessary to enasble an effective breeding program for the species. In the recent years, different kinds of markers have been successfully used in olive species. **Ouzzani *et al.* (1993)** and **Trujillo *et al* . (1995)** found a high level of isoenzyme polymorphism for cultivar identification.

Random amplified polymorphic DNA (RAPD) has also been performed (**Bogani *et al* .1994**;**Fabbri *et al.* 1995).**More recently, the relation among cultivated olive, wild forms and related species have been explored through amplified fragment lengh polymorphism (AFLP) (**Angliolillo *et al* .1999**; **Grati-Kammoun *et al* .2006).** Nevertheless, the dominant character or the lack of reproducibility of some of these markers represents serious limitations when applied for breeding purposes.

Simple sequences repeats (SSR) or microsatellites are short tandem repeats of DNA. Microsatellite polymorphism is based on the different numbers of a short repeated motif at a given locus. SSRs are becoming the markers of choice in many plant breeding programs because they are transferable, multiallelic co-dominant markers, PCR based, easily reproducible, randomly and widely distributed along the genome.

Cultivar identification is a primary concern for olive growers, breeders and scientists.This study was aimed at evaluating the potential of several microsatellite markers, **(5),** to reveal polymorphism of thirty four **(34)** Algerians variety in order to characterize the olive accessions that the morphological Characterization was already done. With this work also, we intended to construct a molecular data-base for Algerian olive cultivars using SSR markers for ***Olea europea*** **L**.

Bibliographical Synthesis

I- Description of olive tree (*olea europea* L.)

I-1- Brief History of the Crop

The tree species olive **(*Olea europaea* L.)** is among the most ancient of crops of the Mediterranean region **(Zohary and Spiegel-Roy., 1975) (Figures 1 and 2).** In the archaeological records, woods of cultivated olive from Eastern Spain and Southern France have been dated up to the Neolithic age **(Terral., 2000).** Before its domestication, wild olive was endemic across the Mediterranean region, but particularly in the Middle East.

Wild olive grows abundantly in thick forest, and is believed to be indigenous to the Mediterranean Basin **(Green., 2002).**The domestication process is thought to have involved the selection of trees of large fruit size and/or high oil content, and their vegetative propagation, either directly planted via cuttings or grafted onto indigenous oleasters.

There is evidence for contemporaneous starting of olive domestication at both ends of the Mediterranean. In the Near East it occurred in the Early Bronze Age (second half of the 5th millennium BCE), as has been demonstrated both by the discovery of olive oil presses and by the presence of pollen grains, stones and wood remains **(Zohary and Spiegel-Roy., 1975; Liphschitz *et al* . 1991);** while analysis of archaeological charcoal and olive stones have dated domestication to the end of the Bronze Age in the north-western Mediterranean area **(Terral., 2000; Terral *et al.* 2004).**

From the 6th century BC, cultivated olive spread throughout the Mediterranean, reaching Tunisia and Sicily, and later Northern Italy. With the European settlement of America after theXV century, olives arrived in the New World, but only in recent times has its cultivation extended significantly beyond the Mediterranean area. Today, it is grown commercially in Australia, South America (Argentina and Chile) and South Africa.

Figure 1 : *The tree species olive (Olea europaea L.).*

I-2- Botanical Description

Olive belongs to the Oleaceae family, sub-family Oleideae. The family includes about 30 genera **(Johnson., 1957),** accounting ornamental shrub species such as jasmine (Jasminum fruticans L.), lilac (Syringa vulgaris L.) and forsythia (Forsythia×intermedia Zabel); and tree species, such as ash (Fraxinus excelsior L. and F. angustifolia Vahl.), privet (Ligustrum vulgare L.) and phyllirea (Phyllirea angustifolia L., P. media L. and P. latifolia L.).

The genus Olea, sub-family Oleideae, includes two sub-genera: Olea and Paniculatae. The former is divided in two sections: Olea, which contain only O. europaea (including both cultivated and wild forms),and Ligustroides. According to recent revisions of O. europaea **taxonomy (Green and Wickens., 1989; Green., 2002)**, this species is divided into six sub-species, based on morphology and geographical distribution:

1) subsp. europaea, with the two botanical varieties europaea (cultivated olive) and sylvestris (wild olive),widely distributed throughout the Mediterranean Basin;

2) subsp. cuspidata, distributed from SE Asia to SW China, as well as from the Arabian peninsula through East and South Africa;

3) subsp. laperrinei, restricted to the Sahara region;

4) subsp. maroccana, restricted to Morocco;

5) subsp. cerasiformis, restricted to the island of Madeira;

6) subsp.guanchica, restricted to the Canary Islands.

Wild olive fruits are smaller in size and have lower mesocarp oil content than do cultivars **(Terral and Arnold-Simard., 1996).**Populations of wild olive are restricted to a few isolated areas of native Mediterranean forest, where pollen/stones may be wind/bird distributed **(Lumaret *et al* . 2004).**

Figure 2 : *Thefruits of olive tree (Olea europaea L.).*

Molecular analysis, using both nuclear and cytoplasmic markers, has shown that the eastern and western Mediterranean populations are strongly differentiated from one another **(Besnard *et al* . 2001(a), 2001(b), 2002(b); Lumaret *et al* . 2004).** On the contrary, cultivated olives do not show such geographical structure, even though their variability is quite high. It has been repeatedly shown evidence for the multi local selection of most cultivars **(Besnard *et al* . 2001(b); Rotondi *et al.* 2003),** empirically undertaken by olive growers from naturally crossbred genotypes. At least 1275 cultivars have been described **(Bartolini *et al* . 1998**), but many other local varieties and ecotypes contribute to the richness of the olive germplasm. Few cultivars are dispersed over a widespread area; rather, the majority is highly localized.

The olive is a long-living evergreen tree, which can attain a mature height of up to 15m and a spread of 9m; its life span is typically more than 500 years, but trees older than 2,000 years have been recorded. Mature leaves are elliptic and characteristically gray green in color, as a result of the presence of star-hairs. Flowers are wind pollinated, and although most cultivars are self-incompatible, some are self-compatible. The flowers are generally hermaphroditic, but certain cultivars are male-sterile **(Besnard *et al* . 2000),** while others are purely staminate. The fruit is a drupe, with a thick, fleshy oil-accumulating mesocarp. When pulped, the mesocarp is made up of oil (22%), water (50%), proteins (1.6%), carbohydrates (19.1%), cellulose (5.8%) and minerals (1.5%) ***(Figure 3)*** .

Figure 3 : *Theleaves and flowersof olive tree* ***(Olea europaea L.).***

Green olives destined for canning are usually harvested when the fruit is completely developed and the skin color starts to change from green to reddish, while olives used either as a source of oil, or for processing as black olives, are picked later in the ripening process, when oil accumulation is completed and the skin has become black. The characteristic compound oleuropein, which confers a strong bitter taste to the fruit, makes the fruit unpalatable, so that pretreatment is necessary before table consumption.

Olive trees grow in semi-arid to temperate climates, on almost any well-drained soil with a pH below 8.5, and are reasonably tolerant of mild soil salinity.

They show cold winter hardiness, tolerating temperatures as low as −12 ◦C. Even though olive has the ability to initiate vegetative shoots from the base of the trunk, productivity may be compromised for several years following episodes of severe cold-induced die-back.

Significant pests and diseases include the olive specific pathogens Spilocaea oleagina Cast., causing olive leaf spot, and the olive fruit fly (Bactrocera oleae).

Major non-specific pathogens cause Verticillium wilt (Verticillium dahliae Kleb.) and olive knot (Pseudomonas syringae subsp. savastanoi). B. oleae directly attacks the fruit mesocarp, and can have serious consequences on production, by inducing early fruit fall or causing total disruption of the pulp. Plant propagation is generally by cutting or grafting onto seedling rootstocks. Cultivars are mostly diploid (2n = 2x = 46) **(Falistocco and Tosti 1996; 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).**

I-3- Economic Importance

Olive is one of the most important crops as a source of oil, and for table consumption. Olive oil has favorable nutritional properties, and as a result, its consumption, traditionally restricted to the Mediterranean area (77% of the world production area), is increasing worldwide (mainly United States, Canada, Australia and Japan). Some varieties are cultivated specifically for table consumption, but the majority is used for oil extraction.

Virgin olive oil is mechanically extracted from pressed or centrifuged pulped fruit. In the commonest process (the continuous extraction system), two centrifugations generate three

fractions: oil, pomace and vegetable water. Olive production is concentrated in Southern Europe, mainly Spain and Italy, followed by Greece, Portugal and France, which together account for about the 85% of world production. Turkey, Syria, Lybia, Morocco, Algeria and Tunisia are also important producers.

Over the last ten years olive cultivation has extended around the world, from South Africa to Latin America (Argentina and Chile), California, New Zealand and Australia and, since the late 1990s, there has been a strongly rising production trend in these countries; nevertheless, the major producers remain in Europe. Olive oil production in Europe in 2003 was 2.3 Mt, while competitor oil-producing crops such as rapeseed and sunflower generated, respectively, 4.2 Mt and 4.9 Mt (data from FAOSTAT database).

Olive oil is a relatively expensive vegetable oil due to its high cultivation costs and limited production. Fruit production can start 3–5 years after planting, if properly cultivated, but generally optimal yields are not attained before trees are 10 years old. Mean production per tree (15–50 kg fruit) and per unit area (about 2 t/ha) are low in comparison with other oil crops, and extractability rarely exceed 24% of fresh weight (depending on variety, agro-climatic conditions and extraction method). Yield is unpredictable from year to year, but the source of much of this variation remains unclear. Oil accumulates in the fruit mesocarp, and to a lesser extent, also in the seed **(Harwood and Sanchez 2000).**

Virgin olive oil is overwhelmingly made up of triglycerides (98 – 99%), along with a small proportion of other compounds. The dominant triglyceride fatty acid species are the mono-unsaturated acids oleic (18:1) (57 – 78%), palmitic (16:0), and stearic (18:0), and the poly-unsaturated acids linoleic (18:2) (7 – 19%) and linolenic (18:3) (0.6 – 0.8%) **(Salas *et al* . 2000).**

The minor compounds (alcohols, polyphenolic compounds, chlorophyll, carotenoids, sterols, tocopherols and flavonoids) contribute to the organoleptic qualities, taste, flavor, and nutritional value **(Servili and Montedoro 2002; Garcia-Gonzalez et al. 2004),** whichmay distinguish olive oils originating from different production regions. Recent studies have shown that olives contain antioxidants in abundance (up to 16 g/kg), represented by acteosides, hydroxytyrosol, tyrosol and phenil propionic acids. Olive oil, especially extra virgin, contains smaller amounts of hydroxytyrosol and tyrosol, but also contains secoiridoids and lignans, as well as other compounds deemed to be anticancer agents (e.g. squalene and terpenoids) **(Fabiani *et al* . 2002; Owen *et al* . 2004).**

The European Union has developed a PDO (Protected Designation of Origin) assignation to olive oils with important regional traditional origins. Oil quality is strongly cultivar-dependent, but is also affected by agro-climatic factors and agronomic practices. Various categories of olive oil have been defined **(Reg. CEE 1513/01):**

- ***virgin oil*** : oil produced by mechanical or other Physical means under conditions (e.g. temperature) that do not lead to any chemical alteration in the oil, and which has not undergone any treatment other than washing, decantation, centrifugation and filtration. Within this category is included the 'extra virgin olive oil': virgin olive oil which has a free acidity, expressed as oleic acid, of not more than 0.8 grams per 100 grams, and the other characteristics of which correspond to those fixed for this category in this standard;
- ***refined oil:*** oil obtained from virgin oil by refining methods which do not lead to an alteration in the initial glyceric structure;
- ***olive oil:*** oil consisting of a blend of refined and virgin olive oil;

- ***olive-pomace oil*** : oil obtained by treating olive pomace with solvents, to the exclusion of oil obtained by re-esterification processes and of any mixture with oils of other kinds.

Two types of adulteration have been identified: blending of virgin olive oils with olive oils of lower grade, and mixing olive oils with other vegetable oils. Mislabeling of olive oils is of considerable concern, as this results in the product not being of the claimed grade **(Lai *et al* . 1994; Yoke *et al.* 1994; Spangenberg and Ogrinc., 2001).** The International Olive Oil Council (1993) and the Codex Alimentarius Commission (1993) have therefore produced standards for virgin, refined and olive-pomace oils. Instruments such as Protected Designation of Origin (PDO), Protected Geographical Indication (PGI) and Traditional Specialty Guaranteed (TSG) are all important for quality control in this context.

I-4- Breeding Objectives

Primary goals in olive breeding are directed towards overcoming current limiting factors for production. These include: shortening the juvenile stage; increasing fruit number and size; increasing oil content and quality (fatty acid composition, polyphenol content, etc.); stabilising yield; dwarfing, and other manipulations of tree architecture to facilitate mechanical pruning and harvesting; improving resistance to pests (in particular olive fruit fly, Bactrocera oleae) and diseases (leaf peacock spot, caused by Spilocaea oleagina; Verticillium wilt, Verticillium dahliae; and olive knot, Pseudomonas savastanoi).

Other important objectives relate to improvement in cold tolerance (to allow cultivation in more northerly areas) and to the promotion of self-fertility (to reduce reliance on pollinators). Tree architecture and vigor are particularly important because the height of the tree prevents mechanical harvesting and pruning, thereby increasing the costs of cultivation. Although the olive is generally considered to be a drought-tolerant species, its productivity is strongly reduced under drought conditions, and thus there is interest in the possibility of tolerant cultivars, as well as those that can thrive on saline and heavy soils. Rootstock selection is focused on the ability to control scion vigor, and to improve the level of resistance to biotic and abiotic stresses.

I-5- Breeding Achievements

In spite of its economic importance to all Mediterranean countries, there has been little directed olive breeding to date, despite the pressing need to improve productivity and agronomic performance. 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 **(Rallo 1995; Belaj *et al* . 2004).**

Exploration of phenotypic variability in agronomic characters has led to the identification of valuable clones within numerous olive cultivars **(Suárez *et al* . 1990; Lavee *et al.* 1995; Bartolini *et al* . 2002; Grati-Kammoun *et al* . 2002).** However, in spite of the significant efforts made towards clonal selection, very few clones have outstanding performance **(Loussert and Berrichi 1995; Tous *et al* . 1998).**

Similarly, induced mutagenesis has not been encouraging, and so far has succeeded in producing only a compact mutant of the cv. Ascolana Tenera **(Roselli and Donini., 1982).** The evaluation of minor local cultivars, present in every cultivation area, has recently

been exploited to identify individuals highly adaptive to extreme environmental conditions **(Pannelli *et al* . 2003; Rotondi *et al* . 2003).**

Clonal rootstocks with high rooting ability have been identified from crossbred populations **(Baldoni and Fontanazza., 1990),** and other selected rootstocks have shown ability to control scion vigor and resistance to frost injury **(Pannelli *et al* . 2002).** The use of the cultivars. Souri, Muhasan and Barnea as rootstocks under dry conditions, after 10 years from planting, did not show any significant effect on tree vigor, shape and fruit production **(Lavee and Schachtel., 1999).**

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).**

The long generation time has severely hindered both classical breeding and genetic studies **(De La Rosa *et al* . 2003).**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).**Vigor, 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).** Very few cultivars have been emerged from formal breeding programmes.

A new cultivar (Maalot) resistant to Spilocea oleagina has been selected from the selfed F1 progeny of a semi resistant seedling probably of Chemlali **(Lavee *et al* . 1999).** From seedling populations obtained by unknown parents two other cultivars were selected: 'Barnea', with vigorous and upright growth, and 'Kadesh', as a table olive **(Lavee 1978; Lavee *et al* . 1986).**

Three new olive cultivars (Arno, Tevere and Basento) were released from the progeny of the cross Picholine×Manzanilla' **(Bellini *et al* . 2002)** and their performance is still under evaluation.

The University of Adelaide has recently established a selection program utilizing the plant olives locally reproduced from cultivars previously introduced in Australia and well adapted to that environment. The aim of the project is the identification of new improved olive cultivars showing superior morphological and oil characteristics **(Sedgley., 2000).**

I-6- Olive Situation

I-6-1- Olive World Situation

The olive tree **(*Olea europea* L.)** is an evergreen plant and long-lived, lives in a corridor on the 30th and **45** th parallel north and south, probably originating from Asia Minor, was in fact widespread in Syria and in Palestine and then reach due to migration and trade in human beings (the Phoenicians, Greeks and Romans) in all the Mediterranean countries and recently has found widespread use in many areas of the world (Australia, Chile, South Africa, Argentina ...).

The world's land planted with olive trees amounted to about **10.8** million hectares, almost **97%** of this located in the Mediterranean countries. In these countries, olive oil is a very significant proportion of locally-produced vegetable oils and a percentage for determining which countries reaches **100%** and **47%** of all the countries that will officiate in

the Mediterranean basin. **30%** of the world's productive assets is a specialized type, while the remainder is represented by olive groves in the area of hills or mountains (traditional). The average planting density of olive trees is **120** trees per hectare, with extremes in Tunisia (and average of **32** plants per hectare) and Cyprus (**320** plants per hectare).

The average annual world production of olives in the period **1996** to **2004** was more than **15** million tons, of which approximately **14** million were brought into use in oil, and over **1.3** million direct consumption **(Cappelletti *et al* . 2005).**

The European community, Syria, Tunisia and Turkey are the main world producers of oil with shares of **64.13%, 5.30%** and **4.37%.** Within Europe the main producing countries are Spain **(43%),** Italy **(31.02%)** and Greece **(29.78%) (COI., 2005).**

I - 6 - 2 - Algerian olive Situation: history, production, olive growing areas and prospects

In Algeria, the olive has an ancient history. The word "Azemmour" language Tamazight (Berber) by failing Semitic root, does in fact suggest that the cultivation of olives has been carried out before the arrival of the Phoenicians. In this country, the Olive has had its greatest period of expansion during the Roman domination. From that period the olive tree and its products have become one of the essential bases of economic activities of the rural population **(Alloum D., 1975).**

The archaeological data confirm that the country's most important olive-growing region was the eastern part. From the tenth century, the plains are occupied by nomadic people and goes on the decline began under the rule of olive Vandals and Byzantines. The olive-groves Algeria remains especially in mountainous areas of Kabylia and dell'Aures and assumes the configuration that still characterizes it today **(Chabour M., 2003).** In Algeria, the cultivation of olive trees covering an area of **274,629** hectares **(COI, 2003).**The olive-groves consists of **26** million and **803** thousand plants, **22** million of which are specialized olive groves. **17** million of these assets are in production.

During the **2005/2006** marketing year, the Algerian olive production was **85,703** tons of table olives and **37,900** tons of oil. The average yield of oil was **15.4%** and the average production per tree is **18.7** kg.

The statistical data on the performance of surfaces olive trees ***(Figure 4)*** in the last ten years **(1990-1999)** show a steady decline between **1990** and **1995**. With the restructuring of the agricultural sector started in **1997**, the olive-growing areas are again increasing **(Kerboua M., 2003).**

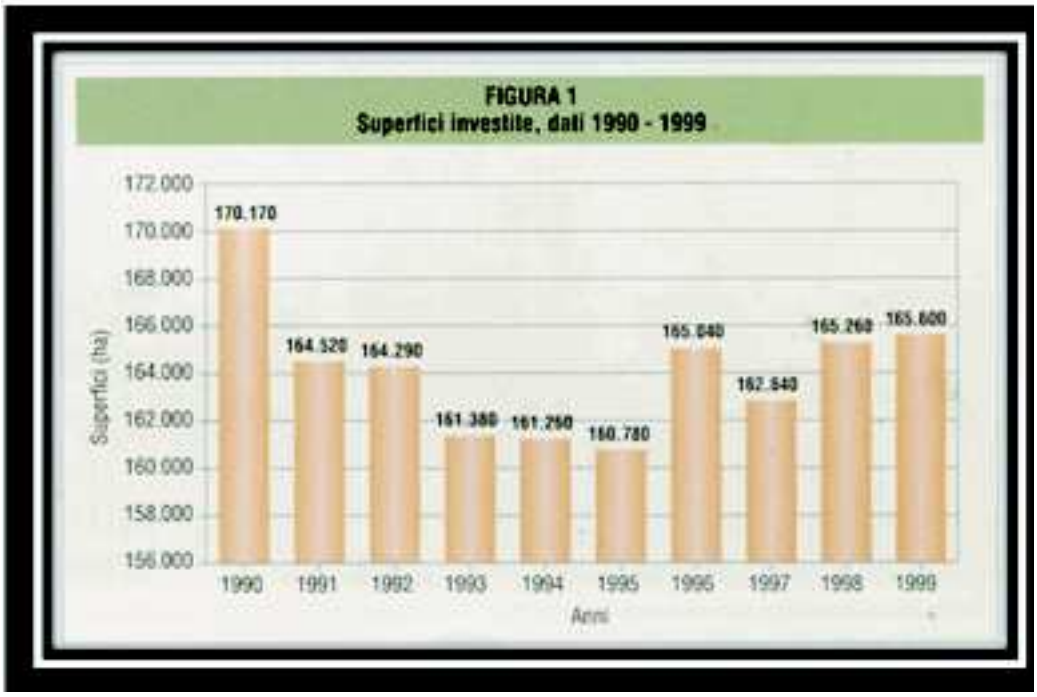

Figure 4 : *The areas of olive production in Algeria (1990-1999).*

Reference source : (Kerboua M., 2003).

The revival of the national plan of agricultural development **(PNDA)** in **2000** and funds granted to industry in the context of the national fund restructuring and rural development (FNDRA) enabled reinforce this upward trend, and so between **2000-2006** new plantations an area of **87,000** hectares have been created, representing a rate of **46.69%. (COI, 2003).**

As for the oil industry and its modernization, until December **2006**, the **PNDA** has allowed the entry into operation of **211** plants and **106** continuous extraction plants for processing of table olives **(MADR, 2006).**

The Algerian olive map ***(Figure 5)*** shows the distribution of olive plantations in three main areas: East, West and Central, and these regions divide the country in **20** olive-growing areas **(ITAF, Algeria). 83%** of this heritage is located in mountainous areas on rough terrain, marginal and poorly fertile with average precipitation of **400-900** mm annually. These groves are dedicated to the production of olive oil consumption and the majority are located in the center and east of the country **(Kerboua M., 2003).**

Modern olive groves represent **17%** of the total area and are located in the plains in the western part of the country where the average annual rainfall reaches **300-400mm** per year accompanied with irrigation rescue. These groves are mainly dedicated to the production of table olives using them, the majority of the cultivars Sigoise (**Sadoudi, 1996; Kefs and Guerri, 2004).**

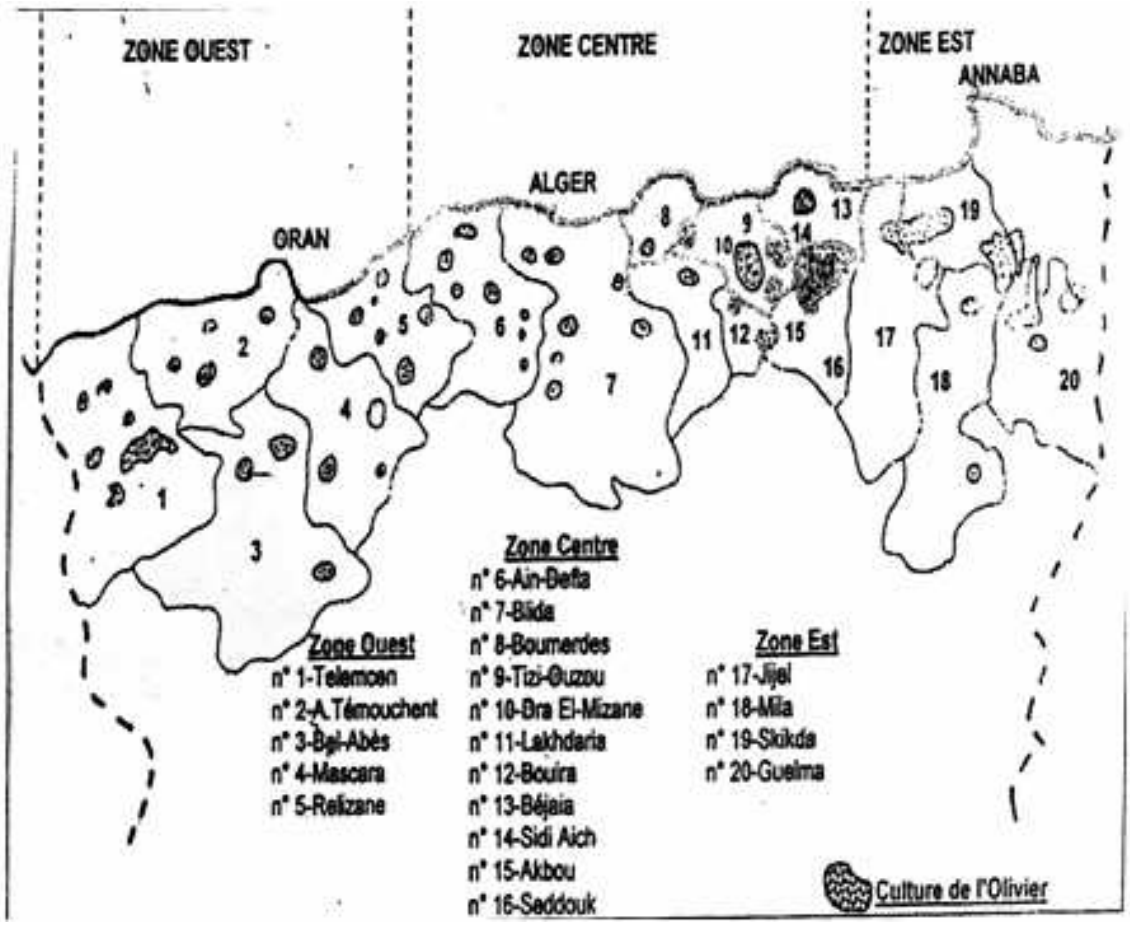

Figure 5 : *The distribution of olive plantations in three main areas: East, West and Central in Algeria.*

Reference source : (Kerboua M., 2003).

II- Olive germplasm and varietal classification

The olive tree belongs to the botanical family Oleaceae, which includes about 30 kinds, including Franxinus, Jasminus, Ligustrum, Phillyrea, Olea and Syringa. In the genus includes 35 species Olea **(Fontanazza G. *et al.,* 1996),** for which it was further divided into sub-species of sylvestris and sativa. The first also called olive and wild olive. The Olea europaea L. sativa sub-species is the only species of the family Oleacee with edible fruit **(Alfei Be Panels G., 2003).**

II-1- Characteristics of olive germplasm

The term "germplasm" of a crop species is the set of possibilities (and related genomes) explored man spatially and temporally during the domestication of the species and through the adaptive pressures put in place to improve performance **(Fiorino P. *et al* ., 2002).**

The olive tree, a plant cultivated from ancient times, has many varieties that have a significant phenotypic and genotypic diversity. The species has a wide plasticity to adapt to very different climatic and soil conditions, guaranteed by the evolution and differentiation of hundreds of local varieties and ecotypes, which have been subjected to natural and anthropogenic selection pressure during the ancient history of cultivation in micro- areal extremes. It can be argued, in fact, that few crops such as olives can boast such a rich variety **(Fontanazza G. *et al* ., 1996)** and currently research is affecting hundreds of varieties in the main olive-growing countries of the Mediterranean **(Loussert and Brousse, 1978).**

The identification and classification of varieties of this species are complicated by the presence of many homonyms and synonyms ***(Annex 1),*** for which different varieties are listed with the same name (omonomie) or the same cultivar, in the same room or in different environments, is called with different names (synonyms).

The olive tree, about **1200** cultivars distributed worldwide, has about **3,000** synonyms **(Bartolini *et al* ., 1998; Ambrosino *et al* ., 2002).**In recent years we have added a number of other accession to local spread, with an estimated total of about **4,000** genotypes **(Tombesi.A, 2003)**. The high number of synonyms is probably a direct result of a high variability of the expression of genes controlling morphological characters due to different climatic conditions existing in different growing areas. In addition, many synonyms are derived from error handed down over the years by the farmers themselves. Many varieties are also considered as "population range" composed of several "types" or "clones" with distinct genetic and phenotypic traits. **(Fontanazza G., 1993; Essadki *et al* ., 2003).**

In Algeria, the native olive germplasm was recently the subject of considerable descriptive study of the **COI** under the project entitled "Conservation, characterization, collection and utilization of genetic resources of the olive" and began in **1995**.

This study realized by **Mendil M. and H. Sebai** from ITAF institute led to morphological and agronomic variety of **36** Algerian.

According **Daoud (1989),** the main olive varieties are grown are:

- ***"Chemlal of Kabylie"*** variety is the most widespread in Algeria, which represents 40% of the national heritage. It is grown mainly in large Kabylie which represents 90% of the olive groves of this region. It is grown for oil.
- ***"Sigoise"*** means varieties of the West of Algeria, which represents 20% of the olive-groves and is mainly grown for table olives.
- ***"Limli"*** accounts for 8% of Algerian heritage olive oil, for oil production.
- ***"Azeradj"*** dual purpose varieties located in Kabylie, occupies 5% of the olive capital of Algeria.

The location of all the varieties of Algerian olive germplasm are represented in the following map ***(Figure 6).***

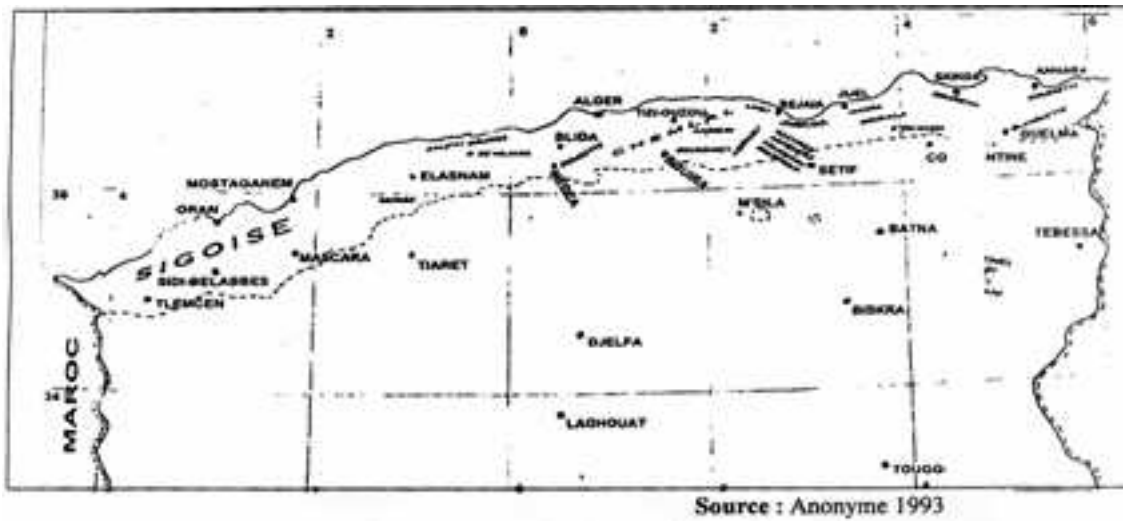

Figure 6 : *Distribution map of variety on the Algerian territory.*

Reference source : (Kerboua M., 2003)

II-2- Identification of cultivars

The olive tree is a species characterized by a large number of cultivars. The presence of synonyms and homonyms caused a great confusion about their identity. Varietal characterization would thus not only the preservation and maintenance of biodiversity and germplasm of the species itself, but would allow a system of certification of the plant material at the nursery level, thus ensuring the entire production**(Ambrosini *et al* ., 2002).**

The germplasm presents a source of variability of fundamental importance for plant breeding, is in fact the only capital capable of providing plant genotypes and / or clones that have agronomic characteristics particular to avoid confusion at the time to build new plants **(COI, 2003).**

The first attempts of identification of olive cultivars date from the early twentieth century: the classification of the varieties was traditionally based on morphological discrimination of the various organs of the tree **(Essadki *et al* ., 2003).**

The need to overcome the problems encountered in this classification has led researchers to undertake new studies based on DNA analysis (molecular identification).

II-2-1-.Morphological identification

Allows the characterization of varieties based on morphological and agronomic **(Montemuro *et al* .,2003).** The initial work of identifying and classifying the varieties of olive trees date back to the nineteenth century, however, **Ruby (1917)** was the first to use the different organs of olive trees (leaves, fruit and endocarp) to characterize the varieties of this species.

Currently, the method adopted for the International Olive Oil Council **(COI),** includes 32 morphological characters used. These were chosen in light of the consistency between individuals in the same genotype, the ability to guarantee a reliable discrimination. Fifteen characters on thirty-two (one for the leaf, four for the fruit, and ten for the endocarp) were found to be highly discriminated against. **(COI, 2003)**.

In general, the relevant aspects of selecting, describing and assessing the varieties of olives proposed by Alfei and Panels, **2004**, include:

- Biometric indicators: with which they are made available measurements on leaves, the inflorescences, the fruit and the endocarp.
- Aspects of vegetation: with which describes physiological and morphological traits related to the plant, such as strength, posture, volume, density and branching of the crown, bearing branches.
- Aspects of production: with which describes fertility, early entry into production and production.
- Model of fruit ripening: locate the optimal time for collecting quantitative and qualitative production.
- Characteristics of the oil composition: in addition to compliance with the main chemical parameters of quality assessment, used to assess the oil of each variety in relation to the type and sensory characteristics.

The difficulty of this classification is complicated by environmental and cultural factors that may act to modify certain morphological and biological characters of each cultivar**(Vergari.G *et al* ., 1996)**.

At present, the "official" method of comparing the morphological characters provides only a crude instrument with limited room for certainty **(Fiorino. P *et al* ., 2002).** Moreover, it often happened that the same name served to designate different varieties, which had in common a certain characteristic often only on the shape or size of fruit or some other salient aspect **(COI, 2003).**

II-2-2- Genetic identification

These molecular techniques have been developed in recent years, are based on the use of several molecular markers for the exploration of genetic resources of a plant species. It can differentiate varieties or clones that are difficult to identify by morphologic or biometric methods **(Ziliotto *et al* ., 2002).**

As for the olive tree, unfortunately, often the observed phenotype is not an indicator of genetic modification **(Rugini E., 2001).** The adoption of techniques "fringerprinting of DNA resulted in identification of the genetic relationships and to test the levels of diversity / genetic similar between species and between varieties of Olea generates widespread **(Zliotto *et al* ., 2002).**

The use of molecular techniques used to establish a molecular profile for each variety, based on the analysis of sites or loci of the genome, which allows you to identify the variety with security. The molecular profile obtained with regard to a certain name, is based on material from various sources and is considered the reference genotype of the variety **(Khadari, 2001).**

II-2-2-1- Molecular markers

For the identification and characterization of olive varieties are now available for molecular analysis systems based on polymorphism of DNA sequences.Among the most used in the last decade have been the RAPD (Random Amplified Polymorphic DNA), the AFLP (Amplified Fragment Length Polymorphism) and microsatellites or SSR (Simple Sequence Repeats).

II-2-2-1-1- Random Amplified Polymorphic DNA *(RAPD)*

Random Amplified Polymorphic DNA(RAPD ***),*** they were the first markers used to highlight polymorphism among cultivars **(Gemas *et al* ., 2000, Rugini *et al* ., 2003; Muzzalupo *et al.,* 2002),** and groped the identification of facilities **(Besnard *et al* ., 2001(a))** or establish the source **(Besnard *et al* ., 2001(b)).**We have analyzed dozens of cultivars of the World Bank Olive Germplasm in Cordoba (Spain), highlighting a high degree of variability among cultivars **(Belaj *et al* ., 1999; Belaj *et al* ., 2001; Belaj *et al.* , 2002).** All cultivars examined were discriminated by the combination of a few primers.

II-2-2-1.2- Amplified Fragment Length Polymorphism *(AFLP)*

Amplified Fragment Length Polymorphism **(**AFLP), they were used for the analysis of cultivar more representative of the various olive-growing countries **(Angiolillo *et al* ., 1999)** in order to resolve cases of synonymy and homonymy and understand the genetic relationships between them. There are data on several cultivars of Umbria **(Marchionni *et al* ., 1999),** Molise **(Pilla *et al* ., 1999),** Veneto **(Ziliotto *et al* ., 2002),** Marche **(Baldoni *et al* ., 2001).** By AFLP analysis it was established that some popular varieties in different environments and with different names are actually the same genotype **(Baldoni *et al* ., 2002)** and that the morphological differences observed between some varieties are actually due to the presence of eye peacock rather than genetic differences **(Sebastiani *et al* ., 2003) .** The AFLP were used for genetic mapping **(De La Rosa *et al* ., 2003).**

II-2-2-1-3- Microsatellites (Simple Sequence Repeats) *(SSR)*

Microsatellites or SSR, they consist of fragments of the genome sequences of **2-3** nucleotides repeated several times. You can put in evidence by PCR amplification using primer sequences flanking. Require a preliminary phase, rather complex for the identification of the sequence of primers flanking regions through the use of libraries enriched with microsatellites. They can be used both for varietal discrimination for the selection of markers associated with characters in selection **(Sarri *et al.,* 2006).**

Several groups have selected microsatellite (SSR) specific olive with a high degree of polymorphism and a high number of alleles **(Rallo *et al* ., 2000; Sefc *et al* ., 2000, Cipriani *et al* ., 2002; Carriero *et al* ., 2002).** They were tested for comparison between different molecular systems **(Belaj et al., 2002)** and, at the moment, experiments are in progress between different institutions to select a set of polymorphic SSR loci, and particularly effective for varietal discrimination **(Baldoni, 2003).**

II-3- Construction of Genetic Maps

The first linkage map of the olive genome was based on RAPD (random amplified polymorphic DNA) and AFLP (amplified fragment length polymorphism) dominant markers, along with a small number of codominant RFLPs (restriction fragment length polymorphisms) and SSRs (simple sequence repeats) **(De La Rosa *et al* . 2003).**

The mapping population consisted of a progeny derived from two highly heterozygous cultivars, Leccino and Dolce Agogia. The Leccino map covered 2,765 cM and comprised 249 markers, falling into 22 major and 17 minor linkage groups (the latter each involving less than four markers). The Dolce Agogia map was of similar length (2,445 cM) and comprised 236 markers arranged in 27 major and three minor linkage groups. Mean inter-marker distances were similar in both maps (13.2 cM in Leccino and 11.9 cM in Dolce Agogia). AFLP and

RAPD markers were homogeneously distributed across all of the linkage groups. Based on the olive genomic size, estimated around 3,000 cM **(Wu *et al* . 2004),** the Leccino ×Dolce Agogia map is thought to have covered about 80% of the genome.

A second linkage map was constructed **byWu *et al* . (2004)**, based on RAPDs, SCARs and SSRs exploiting the progeny of a cross between the cultivars Frantoio and Kalamata. The greater use of codominant markers allowed the integration of the two parental maps to generate 15 linkage groups, covering 101 loci and 879 cM with a mean inter-marker distance of 10.2 cM. In situ hybridization using tandem repeated sequences has allowed most of the olive chromosomes to be distinguished, and has also revealed structural heterozygosity in three chromosome pairs **(Minelli *et al* . 2000).** At present, no further olive genome mapping data are available, and as yet, no QTL have been detected, neither is there any detailed analysis on genome organization.

II-4- Gene Mapping

Mapping of gene sequences has concentrated on orthologous genes characterised in other species ***(Table 1).*** Particular attention has focused on genes encoding key enzymes involved in fatty acid biosynthesis, modification, triacylglycerol synthesis and storage. These include enoyl-ACP reductase (ear), stearoyl-ACP desaturase, omega 6 plastidial desaturase (fad6), omega 3 plastidial desaturase (fad7), cytochrome b5 (cyt b5), omega 6 cytoplasmic desaturase (fad2), omega 3 cytoplasmic desaturase (fad3), acyl-CoA:diacylglycerol acyltranferase (DGAT) and oleosin **(Hatzopoulos *et al* . 2002).**

The temporal and transient expression of stearoyl-ACP desaturase (a key enzyme for the conversion of 18:0 stearic acid to 18:1 oleic acid, the main component of olive oil) has been studied during fruit development **(Haralampidis *et al* . 1998).** Expression of a cDNA encoding an ω-3 fatty acid desaturase has been studied in leaves, anthers and embryos **(Poghosyan *et al* . 1999),** and two cytochrome b5 genes and their spatial and temporal patterns of expression have been characterized during flower and fruit development **(Martsinkovskaya *et al.* 1999).** The differential expression of other genes such as diacyl glycerol acyltransferase (DGAT) and oleate desaturase has been evaluated in various tissues **(Giannoulia *et al.* 2000; Banilas *et al* . 2005).** Finally, a candidate stearoyl-ACP desaturase was mapped on linkage group 4 of cv. Leccino **(De La Rosa *et al* . 2003).**

II-5- Marker-Assisted Breeding

The very preliminary works performed on olive genomics are far before producing effective results toward the selection of new cultivars by the use of molecular tools.

For that reason and considering the lack of knowledge on the real useful variability already present in the cultivated and wild olive germplasm, attention has been focused in the last ten years mainly on the evaluation of such germplasm. The large number of cultivars and wild populations, in fact, positions olive as a crop species with a very extensive germplasm. The geographic distribution of variability within the Olea genus and the genetic relationships among the different species have been studied using various molecular methods, including cpDNA profiles **(Lumaret et al. 2000; Baldoni et al. 2002),** AFLPs **(Angiolillo et al. 1999; Baldoni et al. 2000),** and rDNA and mtDNA polymorphisms **(Besnard and Bervillé 2002; Besnard *et al* . 2002 (a), 2002 (b**)).

The wild relatives of cultivated olive (oleasters) have been widely analysed using RFLP markers derived from mitochondrial, chloroplast and nuclear DNA,which, in addition to allozyme markers, provide evidence for the survival of indigenous oleaster populations, particularly in the western Mediterranean **(Lumaret and Ouazzani 2001; Lumaret *et al* . 2004).** Within wild populations, a clear distinction between the eastern and western Mediterranean has been noted **(Besnard and Bervillé., 2000; Besnard *et al* . 2002 (b); Bronzini de Caraffa *et al* . 2002).**

Internal transcribed spacer 1 (ITS-1) sequences, RAPD and inter-SSR (ISSR) markers have been deployed to evaluate the colonization history of ***O. europaea*** **(Hess *et al* . 2000).** Some ***Olea europaea*** retro elements have also been identified **(Hernandez *et al* . 2001)** and their copy number has been estimated**(Stergiou *et al* . 2002).**The development of SCAR markers has beenattempted from RAPDs **(Hernandez *et al* . 2001),** and one such has been reported by **Mekuria *et al* . (2001)** to be linked to tolerance to leaf peacockspot.

DNA fingerprinting is a powerful aid for the identification of olive oil provenance, since it can beused to generate a profile specific for any given plant genotype. Over the last decade, molecular markers have been widely applied also to characterize and identify olive cultivars. These analyses have utilized RAPDs **(Fabbri *et al* . 1995; Belaj *et al* . 1999; Mekuria *et al* . 1999; Barranco *et al.* 2000; Gemas *et al* . 2000; Belaj *et al.* 2001; Besnard *et al* . 2001c; Belaj *et al* . 2002; Guerin *et al* . 2002),** AFLPs **(Angiolillo *et al* . 1999; Rotondi *et al* . 2003; Owen *et al.* 2005; Montemurro *et al.* 2005), ISSRs (Hess *et al.* 2000; Pasqualone *et al* . 2001; Vargas and Kadereit 2001)** and SSRs**(Rallo *et al* . 2000; Sefc *et al.* 2000; Carriero *et al* . 2002; Cipriani *et al.* 2002; Bandelj *et al.* 2004).**

Thesame methods have also been applied to trace the geographic origin of batches of olive oil **(Muzzalupo and Perri 2002; Busconi *et al.* 2003; Breton *et al* . 2004; Pasqualone *et al.* 2004; Testolin and Lain., 2005).**

Single Nucleotide Polymorphisms (SNPs) are currently under development **(Reale *et al* . 2006)** in order to clearly distinguish inter-cultivar variability and characterize the clonal variants.

III- Microsatellite Markers

III-1-.Definition of the Microsatellites (also known as SSR)

Microsatellites are short segments of DNA that have a repeated sequence such as CACACACA, and they tend to occur in non-coding DNA. In some microsatellites, the repeated unit (e.g. CA) may occur four times, in others it may be seven, or two, or thirty.

The most common way to detect microsatellites is to design PCR primers that are unique to one microsatellite region (locus) in the genome and that base pair on either sides of the repeated portion (See the Figure below).

Therefore, a single pair of PCR primers will work for every individual in the species and produce different sized products for each of the different length microsatellites ***(Figure 7)*** .

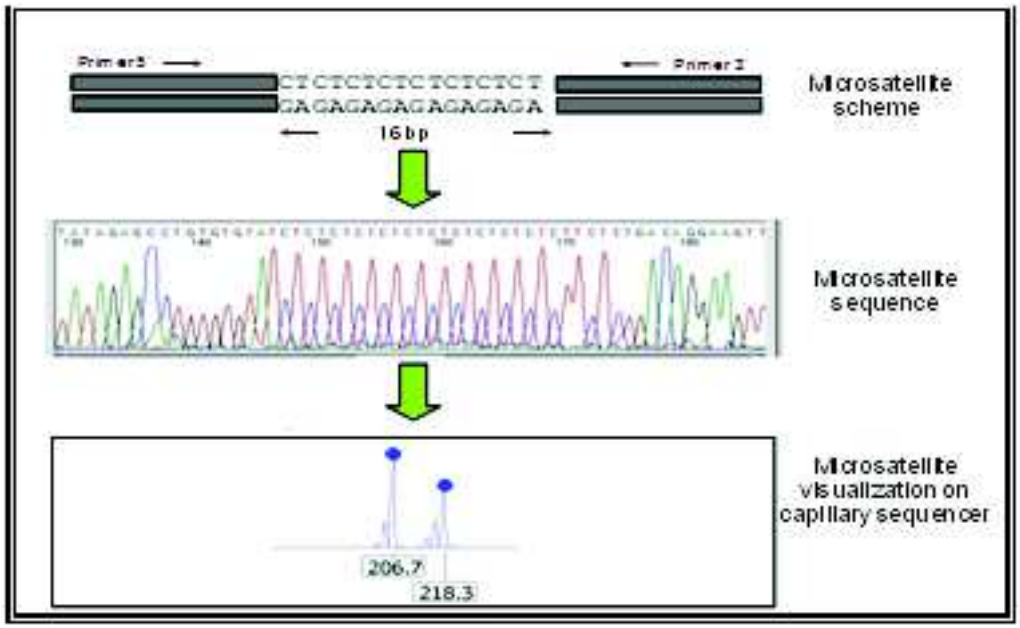

Figure 7 : *Microsatellite schema, sequence and visualization on capillary sequencer.*

Reference source : (Sarri *et al.,* 2006).

III-2- Microsatellite Types

III-2-1-.Dinucleotide

- Animals - CA
- Plants - TA, GA

III-2-2-.Trinucleotid

- GTG, CAG, and AAT
- Related to disease and cancers

III-2-3- Tetranucleotide

- GATA/GACA
- Highly polymorphic

III-3- Microsatellite Uses

III-3-1- Population Genetics

- Gene flow
- Stock Structure

III-3-2-.Genetic Probe

- Larvae
- Gut contents

- Scat
- Source populations

III-3-3-.Pedigree Maps

III-3-4-.Understanding Diseases

III-4- Microsatellite Advantages

- Highly Polymorphic
- Codominant
- In every organism examined to date
- Very abundant
- Random spacing in the genome
- Can find same loci in closely related speciesEasy and reliable scoring
- Highly sensitive
- Neutral markers

III-5- Microsatellite Disadvantages

- Expensive
- Time consuming
- Several loci are needed to obtain sufficient statistical power
- Current analyses methods do not distinguish between changes in flanking regions vs. changes within the microsatellite regions
- Different rates of evolution at different loci **(Lacape *et al* .2007).**

III-6- Utility of SSR loci in olive cultivar identification

SSR markers can be valuable for distinguishing and identifying olive varieties, since all cultivars are uniquely characterized. The cluster distribution emphasizes the existence of recognizable genetic similarities within varieties and the genetic heterogeneity between them. Discrimination of homonym cultivars by just a few primers demonstrates the presence of genetic differences between them.

The homonyms have usually been troublesome in olive cultivar identification as, historically, naming of cultivars have been based on common morphological traits (particularly of the fruit), toponyms or practical utility of the cultivars. Previous studies carried out with morphological**(Lombardo *et al* ., 2004)**and molecular markers**(Belaj *et al* ., 2001; Lopez *et al.,* 2004)** have demonstrated that generic names of olive cultivars include different genotypes.

Considering the small size of the samples analyzed, the levels of intracultivar variance may not be definitive. Since this variance was obtained analyzing accessions belonging to a limited geographical area and selected for the same purpose (oil production), it suggests that the distinct genotypes under the same denomination could have originated from the same genetic pool **(Besnard *et al* ., 2001(a)).**

Genomic variability, assessed by DNA molecular markers, can be a discriminating tool for genotypes selection to fulfill most important breeding objectives such as tolerance to

freezing and salinity stress, resistance to pathogens, and rooting ability. It would be important to improve the ex-situ plant germplasm collection and utilize it to adequately characterize all accessions and develop future breeding programs. In this respect, several Mediterranean cities have promoted olive germplasm collections, including Cordoba (Spain), Porquerolles (France), Marrakech (Morocco) and Cosenza (Italy), which hosts the majority of the Mediterranean varieties.

III-7-.Application of a common protocol for SSR fingerprinting in olive

Lack of agreement on the molecular markers to be used for olive fingerprinting has made impossible, up to now, to figure out the level and distribution of variability. Besides the important results obtained by several groups working independently at the characterization and identification of olive cultivars, the comparison of data among laboratories still represents a very difficult task.

The use of a common set of microsatellite markers and the application of stringent protocols

can represent a better world-wide applicable tool for olive DNA typing, either for population

genetic studies **(Breton *et al* . 2008)** and cultivars discrimination **(Baldoni *et al.* 2006; Belaj *et al* . 2004(b)).** The use of the selected SSRs, characterized by a 506 very high discrimination power, and theapplication of a common strategy for data comparison will finally allow data convergence, apreliminary condition for genotype identification and certification, as well as for dataexchange and managing, useful for the creation of an universal molecular database of olivegenetic resources.

III-8-.Microsatellite (SSR) protocol

Microsatellite (SSR) protocol used by the majority of European laboratories is written in the **(Table 1). (running fragments on ABI PRISM 3130, APPLIED BIOSYSTEMS).**

REACTION	1 sample (µl)	100 samples (µl)
Buffer 10X	2.5	250
Primer FW 5 pmol/ul	1.5	150
Primer RW 5 pmol/ul	1.5	150
MgCl2 50 mM	0.75	75
dNTP 2.5 mM TOTAL (*)	8	800
TAQ 5U/µl (Invitrogen)	0.5	50
H2O	9.25	925
Master Mix	**24**	**2400**
DNA 25 ng/µl	1	
TOTAL	25	

Table 1 : *Microsatellite (SSR) protocol(* ***Amplification reaction).***

Reference source : (Baldoni et al. 2009).

(*) (BE CAREFUL!!!):

2.5 mM TOTAL= 25 ul A (100mM)+25ul T (100mM)+ 25ul C (100mM)+ 25ul G (100mM) +3900 ul H2O

PCR CYCLES:

- 95°C 5 min
- 95°C 30 sec
- Ta°C(*) 30 sec per 35 cicli
- 72°C 30 sec
- 72°C 30-60 min
- 4°C ∞

(*) Use specific Ta for each SSR locus

III-9- Future Scope of Works

Projects are currently under development in order to address gaps in genetic mapping and molecular

breeding in olive. Three main areas of interest can be resumed:

1) completing the research on the evaluation, characterization and utilization of the available genetic resources, both on cultivated varieties and wild relatives;

2) continuing the project on genomic, functional and physical mapping;

3) establishing new breeding programs and completing those in progress by the extended application of marker-assisted selection.

4) examining the SSR markers retrieved from the literature and currently used in olive study, in order to select those most effective in characterizing the olive accessions and to make possible the comparison of data obtained by different laboratories.

Objectives

SSR markers are informative descriptors of the genetic variability of Algerian cultivated varieties of olives studied for the purpose of cultivar identification. These biotechnological tools can provide significant insights for research in crop breeding and germplasm conservation. The high genetic variability of olive trees will hopefully be exploited in breeding programs.

The use of microsatellite markers was confirmed to be a powerful tool not only for studying variation between varieties of the **(** ***Olea europaea*** **L.)** but also for characterizing intra-specific variations among cultivated olive accessions

The objectives of this work are

- The study of molecular characterization and identification of thirty four (**34)** Algerian olive varieties using five **(05)** SSR markers.

- Five SSR markers were genotyped in thirty four (**34)** Algerian varieties using PCR where the amplification was done in the thermal cycler performed in AB 9600 (Applied Biosystems).
- The bands amplified by PCR or amplified products are analysed by **automatic sequencer MegaBACE 3000 (Amersham Biosciences)**
- With this work we intended to construct a molecular data-base for Algerian olive cultivars using five **(05)** SSR markersalready selected and they are effective in characterizing seventy seven **(77)** cultivars of olive **(*Olea europea* L.)** from eleven **(11)**countries ***Croatia, Egypt, France, Greece, Israel, Italy, Morocco, Spain, Syria, Tunisia, Turkey, (Annex 2).***
- These molecular markers used for the identification of all the thirty four Algerian olive tree cultivars to study their genetic variability, and to search for synonymy and homonymy existing between them.

Materials and Methods

1- Experimental site

The experiments were performed at the ***Consiglio Nazionale delle Ricerche (CNR),*** National Research Council – Institute of Plant Genetics, Via Madonna Alta, 130, 06128, Perugia, Italy. In the laboratory of Olive Biotechnology, under the supervision of ***Dr. Luciana Baldoni*** and ***Dr Nicolò Cultrera*** .

2- Plant material

Thirty four olive tree accessions classified morphologically **(Table 2)** as belonging to the important cultivars grown in the same geographical area and corresponding to part of the regional autochthon Algerian olive germplasm were used for the study of the molecular characterization with a set of five markers (microsatellites SSR). Samples of olive leaves were harvested from growing plants from the olive germplasm collection ***(Figure 8)*** of the ITAF-Institut Technique de l'Arboriculture Fruitiére, Bejaia, Algeria ***(Figure 9)*** .

Figure 8 : *Collection field of the different olive varieties"ITAF," (Bejai),2009.*

Figure 9 : *Experimental station of "ITAF" at Takerietz (Bejaia).*

The agro- morphological characteristics of the different cultivars used in our experiment are given in **Table 2**. This agro- morphological studyhas been carried out in the ITAF-Institut technique de l'Arboriculture Fruitiére, Bejaia, Algeria by Dr. ***Mendil Mahmoud*** and Dr. ***Sebai Ahmed*** .

CULTIVARS	*SYNONYMS*	*ORIGIN*	*MORPHOLOGICAL CARACTERISTICS*			
			TREEE VIGOR	*FORM OF LEAVES*	*NUMBER OF FLOWERS*	*FORM OF FRUIT*
ABANI	LAABANI	VALEE OUED EL ARAB (KHENCHELA)	Medium	lanceolate	Medium	elongated
ABERKANE	AVERKANE	AKBOU (BEJAIA)	Medium	lanceolate	Low	elongated
AALEH	AALEH	CHERCHAR (KHENCHELA)	Medium	elliptic lanceolate	Medium	elongated
AGHCHREN D'EL OUSSER	-	BOUGAA (SETIF)	Low	elliptic lanceolate	Low	elongated
AGHCHREN DE TITEST	-	HAMMAM GUERGOUR(SETIF)	Low	lanceolate	Medium	spherical
AGHENFAS	AGHENFOUS	BOUGAA (SETIF)	Medium	lanceolate	Low	elongated
AGRAREZ	-	TAZMALT (BEJAIA)	Law	elliptic lanceolate	Low	spherical
AGUENAOU	AGNAW	BOUSSELAH (SETIF)	Medium	lanceolate	Low	ovoid
AKERMA	-	HAMMAM GUERGOUR (SETIF)	Medium	elliptic lanceolate	Low	elongated
AZERADJ	ARADJ ADJERAZ	KABYLIE SEDOUK (BEJAIA)	Law	elliptic lanceolate	Low	elongated
BLANQUETTE DE GUELMA	-	GUELMA	Medium	elliptic lanceolate	Medium	ovoid
BOUCHOUK GUERGOUR	-	GUERGOUR (SETIF)	Medium	elliptic lanceolate	Low	elongated
BOUCHOUK SOUMMAM	BOUCHOUK SIDI AICH AVOUCHOUK	SIDI AICH BEJAIA	Medium	lanceolate	Low	ovoid
BOUGHENFOUS	-	BOUANDAS	Medium	elliptic lanceolate	High	elongated
BOUICHRET	BOUTICHRAT AVOUICHERT	TAZMALT BEJAIA	Law	lanceolate	Low	elongated
BOUKAILA	-	CONSTANTINE	high	elliptic lanceolate	Medium	ovoid
BOURICHA	BOURICHA OLIVE D EL ARROUCH	EL HARROUCH (SKIKDA)	High	elliptic lanceolate	Medium	elongated
CHEMLAL	ACHAMLAL ACHAMLI ACHEMLAL	KABYLIE	High	elliptic lanceolate	Medium	elongated
FERKANI	FERKANE	FERKANE (TEBESSA)	Medium	elliptic lanceolate	Low	elongated
GROSSE DU HAMMA	QELB ETHOUR CŒUR DE BŒUF	HAMMA (CONSTANTINE)	Medium	lanceolate	Low	elongated
HAMRA	ROUGETTE OU ROUSSETTE	JIJEL	High	elliptic lanceolate	Medium	ovoid
LIMLI	IMELI LIMELI	SIDI AICH (BEJAIA)	Medium	elliptic lanceolate	Medium	elongated
LONGUE DE MILIANA	-	MILIANA	Medium	elliptic lanceolate	Medium	spherical
MEKKI	-	KHENCHELA	Medium	elliptic lanceolate	Low	ovoid
NEB DJEMEL	-	VALLEE D'OUE EL ARAB CHERCHAR-(KHENCHLA)	High	elliptic lanceolate	Medium	elongated
RONDE DE MILIANA	-	VALEE DE MILIANA (AIN DEFLA)	Law	elliptic lanceolate	Medium	elongated
ROUGETTE DE MITIDJA	-	PLAINE DE MITIDJA	Medium	elliptic lanceolate	Low	elongated
SIGOISE	OLIVE DE TLEMCEN OLIVE DU TELL	PLAINE DE SIG (MASCARA)	Medium	elliptic lanceolate	Medium	ovoid

Table 2 : *Morphological description of the olive tree cultivars included in our genetic diversity assessment.*

SOUIDI	-	CHERCHAR (KHENCHLA)	Medium	lanceolate
TABELOUT	TABELOUT ABELOUT	ZONE MONTAGNEUSE DU GOLF DE BEJAIA	High	elliptic lanceolate
TEFAH	ATEFAH TEFAHI	SEDOUK BEJAIA	High	elliptic lanceolate
TAKESRIT	-	EL KSEUR BEJAIA	Medium	elliptic lanceolate
ZELETNI	ZLITNI	CHECHAR KHENCHLA	Medium	elliptic lanceolate
SOUIDI	-	CHERCHAR (KHENCHLA)	Medium	lanceolate

The rest of Table 2

CULTIVARS	AGRONOMIC AND COMMERCIAL CARACTERISTICS			
	END USE	OIL YIELD (%)	FLOWERING	PRODUCTIVITY
ABANI	Oil	16 to 20	early with high intensity	High and alternating,
ABERKANE	Oil and table olive	16 to 20	early with law intensity	Law and alternating,
AALEH	Oil	18 to 22	early with high intensity	Medium and alternating,
AGHCHREN D'EL OUSSER	Oil and table olive	16 to 20	early with medium intensity	Medium and alternating,
AGHCHREN DE TITEST	Oil and table olive	14 to 18	early with law intensity	High and alternating,
AGHENFAS	Oil and table olive	16 to 20	early with law intensity	Medium and alternating
AGRAREZ	Oil and table olive	16 to 20	early with law intensity	Law and alternating
AGUENAOU	Oil and table olive	16 to 20	early with medium intensity	Medium and alternating
AKERMA	Oil and table olive	18 to 22	early with high intensity	Law and alternating,
AZERADJ	Oil and table olive	24 to 28	early with law intensity	Medium and alternating
BLANQUETTE DE GUELMA	Oil	18 to 22	early with law intensity	Medium and alternating
BOUCHOUK GUERGOUR	Oil and table olive	22 to 26	early with medium intensity	Law and alternating,
BOUCHOUK SOUMMAM	Oil and table olive	22 to 26	early with law intensity	Medium and few alternating
BOUGHENFOUS	Oil	22 to 26	late	Medium and alternating
BOUICHRET	Oil	20 to 24	early with law intensity	Medium and alternating
BOUKAILA	Oil	16 to 20	early with law intensity	High and alternating,
BOURICHA	Oil	18 to 22	early with high intensity	High and few alternating
CHEMLAL	Oil	18 to 22	late	High and few alternating
FERKANI	Oil	28 to 32	early with medium intensity	High and few alternating
GROSSE DU HAMMA	Oil and table olive	16 to 20	early with high intensity	Medium and alternating
HAMRA	Oil	18 to 22	early with medium intensity	Law and alternating,
LIMLI	Oil	20 to 24	early with high intensity	Medium and alternating
LONGUE DE MILIANA	Oil and table olive	16 to 20	early with medium intensity	Medium and alternating
MEKKI	Oil	12 to 16	early with law intensity	Medium and constante
	Oil	NEB DJEMEL	early with law intensity	Medium and alternating

Table 3 : *Agronomic and commercial description of the olive tree cultivars included in our genetic diversity assessment.*

RONDE DE MILIANA	Oil and table olive	16 to 20	early with medium intensity	Medium and alternating
ROUGETTE DE MITIDJA	Oil	18 to 20	early with law intensity	Law and alternating,
SIGOISE	Oil and table olive	18 to 22	early with medium intensity	Medium and alternating
SOUIDI	Oil	-	early with high intensity	Medium and alternating
TABELOUT	Oil	20 to 24	early with high intensity	Medium and alternating
TEFAH	Oil and table olive	18 to 22	Late with high intensity	Medium and alternating
TAKESRIT	Oil	16 to 20	early with medium intensity	Medium and alternating
ZELETNI	Oil	14 to 18	early with medium intensity	Medium and alternating

The rest of Table 3

The **Table 4** showed the list of the accessions analyzed in this study and collect from the ITAF olive germplasm collection of Bejaia region (Algeria).

Number	Name of the cultivars
1	ABANI
2	ABERKANE
3	AGHCHREN OUSSER
4	AGHENFAS
5	AGHTITEST
6	AGRAREZ
7	AGUENAOU
8	AIMEL
9	AKERMA
10	ALETH
11	AZZERADJ
12	BLANQUETTE GUELMA
13	BOUCHOUK GUERGOURE
14	BOUCHOUK LA FAYETTE
15	BOUCHOUK SIDI AICH
16	BOUICHERT
17	BOUKALIA
18	BOURICHA
19	CHEMLAL DE KABYLIE
20	FERKANI
21	GROSSE DU HAMMA
22	HAMRA
23	LIMLI
24	LONGUE DE MILIANA
25	MEKKI
26	NEB DJEMEL
27	RONDE DE MILIANA
28	ROUGETTE DE MITIDJA
29	SIGOISE
30	SOUIDI
31	TABELOUT
32	TASKRIT
33	TEFFAH
34	ZELETNI

Table 4 : *Olive accessions characterized in this study.*

Five published SSRs were pre-selected for their high level of polymorphism and easily scorable patterns. They were: DCA-01, DCA-09, DCA-16, DCA-18 ***(Sefc et al. 2000)*** and GAPU-89 ***(Carriero et al. 2002.*** **Table 5** contains the list of the five **(5)**microsatellites (SSR) used in our analysis.

SSR	Peak signal	Peak stuttering	No loci	Frequency of null alleles	Main drawbacks
DCA01	LOW	STRONG	1	0	-
DCA09	LOW	STRONG	1	0	-
DCA16	LOW	STRONG	1	0	-
DCA18	LOW	STRONG	1	0	-
GAPU89	LOW	STRONG	2	0	1

Table 5 : *Main characteristics of the 5 SSRs tested on 34 olive cultivars.*

Reference source : (Baldoni et al, 2009).

3- Experimental setup and data analysis

3-1- DNA extraction

Total genomic DNA from thirty four **(34)** Algerian cultivars was extracted from fresh young olive leaves harvested from olive trees growing in the olive germplasm collection of the Technical Algerian Institute of Arboriculture,(ITAF) Bejaia (Algeria), according to the protocol described in ***GenElute*** ^{TM} ***Plant Genomic DNA Miniprep Kit.***

About 100 mg of leaf tissue for each sample were chopped in a mortar with liquid nitrogen (Figure . The resulting powder was extracted in lysis buffer and were followed all the steps of the protocol. From each sample, were obtained about 800 ng of DNA (20 ng / ul). **(Figure 10)**

The DNA was extracted according to the following protocol written in User Guide (catalogue Numbers G2N10,G2N70, and G2N350).

Figure 10 : *The crushing of olive leaves with liquid nitrogen.*

3-1-1-.Procedure

1- Disrupt cells. Grind plant tissue into a fine powder in liquid nitrogen using a mortar and pestle. Transfer up to 100 mg of the powder to a Microcentrifuge tube. Keep the sample on ice for immediate use or freeze as –70 °C.

2- Lyse cells. Add 350 µl of Lysis Solution [Part A] and 50 µl of Lysis Solution [Part B] to the tube; thoroughly mix by vortexing and inverting. A white precipitate will form upon the addition of Lysis Solution (Part B). Incubate the mixture at 65 °C for 10 minutes with occasional inversion to dissolve the precipitate.

Optional digest with RNase.

3- This kit is designed to selectively isolate large DNA. If preparations are found to be contaminated with RNA, RNase A (not supplied) can be used to digest the RNA. Add 50 units of RNase A to the lysis mixture just prior to incubation at 65 °C.

Precipitate debris. Add 130 µl of Precipitation Solution to the mixture; mix completely by

inversion and place the sample on ice for 5 minutes. Ie at maximum speed (12,000-16,000 X g) for 5 minutes to pellet the cellular debris, proteins, and polysaccharides.

3-Filter debris. Carefully pipette the supernatant from step 3 onto a GenElute filtration column (blue insert with a 2 ml collection tube). Centrifuge at maximum speed for 1 minute.

This removes any cellular debris not removed in step 3. Discard the filtration column, but retain the collection tube.

4- Prepare for binding. Add 700 µl of Binding Solution directly to the flow-through liquid from step 4. Mix thoroughly by inversion.

5- Prepare binding column. Insert a GenElute Miniprep Binding Column (with a red o-ring) into a provided microcentrifuge tube, if not already assembled. Add 500 µl of the Column Preparation Solution to each miniprep column and centrifuge at 12,000 X g for 30 seconds to 1 minute. Discard the flow-through liquid.

Note **:** The Column Preparation Solution maximizes binding of DNA to the membrane resulting in more consistent yields.

6- Load lysate. Carefully pipette 700 µl of the mixture from step 5 onto the column prepared in step 6 and centrifuge at maximum speed for 1 minute. Discard the flow-through liquid; retain the collection tube. Return the column to the collection tube. Apply the remaining lysate from step 5 onto the column. Repeat the centrifugation as above and discard the flow-through liquid and collection tube.

7- First Column Wash. Prior to first time use, be sure to add ethanol to the Wash Solution Concentrate. Place the binding column into a fresh 2 ml collection tube and apply 500 µl of the diluted Wash Solution to the column. Centrifuge at maximum speed for 1 minute. Discard the flow through liquid, but retain the collection tube.

8- Second Column Wash. Apply another 500 µl of diluted Wash Solution to the column and

centrifuge at maximum speed for 3 minutes to dry the column. Do not allow the flow-through liquid to contact the column; wipe off any fluid that adheres to the outside of the column. **(Sambrook, J., *et al* .1989).**

Elute DNA

Transfer the binding column to a fresh 2 ml collection tube. Apply 100 µl of pre-warmed (65 °C) Elution Solution to the column and centrifuge at maximum speed for 1 minute. Repeat the elution. Do not allow the flow-through liquid to contact the column. Eluates may be collected in the same collection tube.

Alternatively, a second collection tube may be used for the second elution to prevent dilution of the first eluate.

The eluate contains pure genomic DNA. For shortterm storage of DNA, 2-8 °C is recommended. For long-term storage of DNA, -20 °C is recommended. Avoid freezing and thawing, which causes breaks in the DNA strand. Elution Solution will help stabilize the DNA at these temperatures.

DNA Precipitation (Optional)

The GenElute Plant Genomic DNA Kit is designed so that the DNA remains in solution, thus avoiding resuspension problems. However, if it is necessary to concentrate the DNA, ethanol precipitation in the presence of sodium acetate is recommended.

3 - 1 -2-.Alternative Disruption Procedures

The extraction of nucleic acid from plant tissue is complicated by the tough cell wall that surrounds most plant cells as well as the fibrous nature of many species. Several methods exist for the disruption of plant material. One of the most effective and commonly used methods is to grind the tissue in liquid nitrogen with a mortar and pestle. The GenElute Plant

Genomic DNA Miniprep Kit was developed based on this efficient method of disruption. However, other disruption techniques can be substituted for step 1 of the Procedure.

Good yields of high molecular weight DNA can also be obtained from freeze-dried tissue. Dried tissue should be ground into a fine powder with a mortar and pestle; up to 20 mg of this powder can be used in a single DNA preparation. Liquid nitrogen is not necessary during the grinding of freeze-dried tissue. After grinding the tissue into a powder, follow the Procedure

beginning with step 2.

3-1-3-.Results

Determine the concentration and purity of the plant DNA by spectrophotometric analysis and agarose gel electrophoresis. The ratio of absorbance at 260 nm to 280 nm (A260/A280) should be 1.7 to 1.9. The size and quality of the DNA can be determined by agarose gel electrophoresis or pulsed field electrophoresis**. (Birren B *et al* .1993).**

Plant	DNA Yield
Corn	7.5 µ g
Dianthus tissue culture	3.3 µg
Pepper	3.1 µg
Rice	5.9 µg
Soybean	5.7 µg
Tobacco	5.2 µg
Tomato	6.2 µg
Tomato (20 mg of freeze-dried leaf tissue)	5.7 µg
Wheat	11.5 µg

Table 6. : *Typical yields of DNA from various plant species per 100 mg of tissue.*

Reference source : (**Birren** et al,1993).

3-2- Multiplex polymerase chain reaction (PCR)amplification and SSR marker analysis

Multiplex polymerase chain reaction (PCR) reaction were performed in 25 µl containing 25 ng of olive genomic DNA. The DNA of each variety was analyzed with microsatellites DCA-01, DCA-09, DCA-16, DCA-18 **(Sefc *et al* . 2000)** and GAPU-89 **(Carriero *et al.* 2002**) using the following protocol: 20 ng of DNA sample were amplified using PCR **(Figure 11).**

Figure 11 : *The PCR instrument used in our experiment.*

3-2-1-.Reaction of amplification

Multiplex polymerase chain reaction (PCR) is a variant of PCR in which two or more loci are simultaneously amplified in the same reaction. Since its first description in 1988 this method has been successfully applied in many areas of DNA testing, including analyses of deletions mutations and polymorphisms ,or quantitative assays and reverse transcription PCR (**Henegariu *et al* . 1997).**

In our analysis, PCR was performed with three primer pairs at the same time using the QIAGEN Multiplex PCR Kit (QIAGEN) and according to the protocol of **standard Multiplex PCR.** Each reaction mixture (25 ul) consisted of 12.5 ul containing DNA polymerase HotStartTaq QIAGEN, QIAGEN Multiplex PCR buffer, and dNTP mix), 0.2 □ M of each primer and 25 ng of DNA.

For the amplification the thermal cycler was performed in AB 9600 (Applied Biosystems) according to the following cycles: an initial denaturation of DNA and activation of QIAGEN HotStartTaq DNA polymerase at 95 ° C for 15 min followed by 35 cycles of amplification (94 ° C for 30 s, 57 ° C for 90 s, and 72 ° C for 90 s), with final extension at 72 ° C for 10 min.

3-2-1-1-.Procedure

1- Thaw **2x QIAGEN Multiplex PCR Master Mix (if stored at –20°C), template DNA, RNase free water, and primer mix. Mix the solutions completely before use.**

It is important to mix the solutions completely before use to avoid localized concentrations of salts. Preparing a mixture of all primers avoids pipetting of individual primers for each experiment, reducing pipetting time and increasing reproducibility of results (for preparation of primer mix see Table 7,

2- Prepare a reaction mix according to Table 8 .

The reaction mix typically contains all the components required for multiplex PCR except the template DNA. Prepare a volume of reaction mix 10% greater than that required for the total number of reactions to be performed. For reaction volumes less than 50 µl, the 1:1 ratio of QIAGEN Multiplex PCR Master Mix to primer mix and template should be maintained as shown in **Table 8.**

Component	Volume/reaction	Final concentration
Reaction mix		
2x QIAGEN Multiplex PCR Master Mix*	25 µl	1x
10x primer mix, 2 µM each primer (see **Table 7**)	5 µl	0.2 µM†
RNase-free water	Variable	–
Template DNA Added at step 4	Variable	≤1 µg DNA/50 µl
Total volume	**50 µl‡**	

Table 7 : *Preparation of 10x Primer Mix (Containing Each Primer at 2 µM)*.*

· Provides a final concentration of 3 mM MgCl2.

† A final primer concentration of 0.2 µM is optimal for most primer–template systems. However, in some cases using other primer concentrations (0.1–0.3 µM) may further improve amplification performance.

‡ For volumes less than 50 µl, the 1:1 ratio of QIAGEN Multiplex PCR Master Mix to primer mix and template should be maintained.

Concentration of normalized primer stock	50 µM (50 pmol/µl)	100 µM (100 pmol/µl)
Each primer	20 µl	10 µl
TE buffer	Variable	Variable
Total volume	500 µl	500 µl

Table 8 : *Preparation of 10x Primer Mix (Containing Each Primer at 2 µM)* ****.***

· Allows preparation of a 10x primer mix containing up to 12 primer pairs (50 µM stock) or containing up to 25 primer pairs (100 µM stock).

3- Mix the reaction mix thoroughly and dispense appropriate volumes into PCR tubes or plates.

Mix gently, for example by pipetting the reaction mix up and down a few times. Due to the hot start, it is not necessary to keep samples on ice during reaction setup.

4- Add template DNA (≤1 µ g/50 µ l reaction) to the individual PCR tubes containing the reaction mix.

For multiplex RT-PCR, the volume of cDNA added (from the RT reaction) as template should not exceed 10% of the final PCR volume.

5- When using a thermal cycler with a heated lid, do not use mineral oil. Proceed directly to step 6. Otherwise, overlay with approximately 50 µl mineral oil.

6- Program the thermal cycler according to the manufacturer's instructions.

Optional: If a thermal cycler with a temperature gradient function can be used, determine the optimal annealing temperature by performing a gradient PCR.

7- Place the PCR tubes in the thermal cycler and start the cycling program as outlined

in Table 9.

Each PCR program must start with an initial heat-activation step at 95°C for 15 min to activate HotStarTaq DNA Polymerase.

After amplification, samples can be stored overnight at 2–8°C or at –20°C for long-term storage.

8- Analyze samples using an appropriate detection system, for example agarose gel electrophoresis (see Table 3, page 10 for choosing the optimal percentage of agarose), polyacrylamide gel electrophoresis*, or capillary electrophoresis.

The optimal amount of PCR product required to give a satisfactory signal with your detection method should be determined individually.

			Additional comments
Initial activation step:	**15 min**	**95°C**	HotStarTaq DNA Polymerase is activated by this heating step.
3-step cycling:			
Denaturation	30 s	94°C	
Annealing	90 s	57–63°C	If a gradient PCR cannt be performed, use 60°C As the starting annealing temperature. If the The lowest T_m* of your primer mixture is Below 60°C, use 57°C as starting annealing Temperature.
Extension approximately 1.5 kb in length.†	90 s	72°C	Optimal for targets up to
Number of cycles	30–45		The number of cycles is dependent on the amount of template DNA and the required sensitivity of your detection method. See Appendix C, page 36 for guidelines.
Final extension:	10 min	72°C	

Table 9 : *Universal Multiplex Cycling Protocol.*

· Tm determined according to the formula: Tm = 2°C x (number of [A+T]) + 4°C x (number of [G+C]).

† For targets longer than 1.5 kb, an extension time of 2 min may improve performance.

3-2-2- Resolving PCR product

The bands amplified by PCR or amplified products are analysed by **automatic sequencer MegaBACE 3000 (Amersham Biosciences) (Figure 12) .**

Figure 12 : *The MegaBACE 3000 instrument used in our experiment.*

3-2-2-1-.Principe of work of the sequencer MegaBACE 3000 (Amersham Biosciences)

MegaBACE 3000 DNA Analysis System is a high-throughput, fluorescence-based DNA system utilizing capillary electrophoresis with up to 96 capillaries operating in parallel. The system performs sample injection, gel matrix replacement, DNA separation, detection, and data analysis

3 - 2 - 2 -2-.Components

- Capillary electrophoresis instrument;
- 96 Capillary arrays;
- Instrument control and data analysis software;
- Computer workstation and monitor for instrument operation and data analysis.

3-2-3- Data analysis

The data obtained were analyzed using the software NTSYSpc version 2.02. To facilitate the discrimination between varieties, a similarity matrix was obtained with the program SIMQUAL.

While for the construction of the dendrogram above the matrix was analyzed by the method of UPGMA (Unweighted Pair Group Method using an Arithmetic average) of SAHN program that performs the sequential, agglomerative, hierarchical, and nested clustering methods as defined by **Sneath and Sokal (1973).**

- In *Annex* ***3*** *,* ***4*** and ***5,*** we have some examples of the manner that are representated the data in the sequencer :*The MegaBACE 3000* instrument used in our experiment.

After analyzing the data of Algerian varieties, they were subjected to further analysis along with a set of 100 varieties from some countries of the Mediterranean basin in particular, Italy**, Spain, Greece, Turkey, France, Syria, Egypt, Tunisia** and **Morocco** .

The country and the number of the varieties analyzed from each country are presented in the **Table 10.**

Country	Number of Varieties
Italy	50
Spain	22
Greece	10
Turkey	06
France	05
Syria	02
Egypt	01
Tunisia	03
Morocco	01

Table 10 : *The country and the number of the varieties analyzed.*

RESULTS AND DISCUSSION

I- Microsatellite polymorphism

Genomic DNA from thirty four **(34)** Algerian cultivars of ***Olea Europea*** **L** and five **(05)** microsatellites markers (SSR) were used in order to study the genetic diversity within Algerian and Mediterranean olive tree cultivars.

The five (05) tested microsatellites (SSRs) primers produced polymorphic amplification products in the cultivars studied. The total number of alleles amplified was **41** alleles with an average of **8.2** allele per locus, ranging from a minimum of **5** for **GAPU-89** to a maximum of **14** for **DCA-09.** The proportion of heterozygous loci was **32%,** with a maximum value of **70.5%** in DCA-01 and minimum of **0%** for DCA-09. **(Table 11 and 12).**

The number of alleles detected ranged between seven **(07)** for locus DCA-18, nine **(09)** for locus DCA-16, six **(06)** for locus DCA-01,five **(05)** for locus GAPU-89 and DCA-09 with an average of about fourteen **(14).**

	DCA-18	DCA-16	DCA-01	GAPU-89	DCA-09
	170	124	208	160	163
	172	148	216	162	173
	176	150	218	172	175
	178	156	230	178	177
	180	172	262	196	183
	184	174	270		185
	186	176			187
		178			191
		180			195
					196
					199
					201
					205
					207
Total	7	9	6	5	14

Table 11 : Allelic variability per SSR locus.

CULTIVAR	DCA-18		DCA-16		DCA-01		GAPU-89		DCA-09	
ABANI	184	184	150	150	208	216	160	172	163	187
ABERKANE	172	184	150	150	208	218	162	162	163	173
AGHCHREN OUSSER	176	184	176	176	208	216	172	178	163	199
AGHENFAS	172	180	156	174	208	216	162	162	195	207
AGHTIIEST	172	176	150	174	208	216	172	178	195	199
AGRAREZ	176	184	174	174	208	216	172	178	163	199
AGUENAOU	170	176	0	0	216	218	162	172	195	207
AIMEL	178	178	150	150	216	218	160	162	173	207
AKERMA	172	184	150	176	208	216	162	162	163	173
ALETH	184	184	150	150	208	216	160	172	163	187
AZZERADJ	176	184	174	174	208	216	172	178	163	199
BLANQUETTE GUELMA	178	178	180	180	208	208	162	178	191	195
BOUCHOUK GUERGOURE	172	184	150	150	208	216	162	178	195	199
BOUCHOUK LA FAYETTE	172	184	150	150	208	216	162	178	195	199
BOUCHOUK SIDI AICH	172	184	150	150	208	216	162	162	163	173
BOUICHERT	176	176	124	148	216	216	162	178	173	199
BOUKALIA	178	178	124	176	216	218	160	196	177	207
BOURICHA	176	176	124	124	216	218	160	162	173	185
CHEMLAL DE KABYLIE	172	172	150	150	216	218	162	162	173	177
FERKANI	176	178	150	150	216	230	162	162	173	196
GROSSE DU HAMMA	176	186	178	178	208	208	178	178	163	205
HAMRA	178	178	124	124	218	218	160	196	173	185
LIMLI	176	176	124	124	216	218	160	162	173	185
LONGUE DE MILIANA	178	184	150	174	208	208	178	196	173	195
MEKKI	170	170	150	150	208	218	162	172	173	207
NEB DJEMEL	178	178	150	150	208	208	162	162	173	195

Table 12 : *List of Algerian olive varieties analyzed with 5 SSR loci and length of the alleles obtained (expressed in base pairs).*

CULTIVAR	DCA-18		DCA-16		DCA-01		GAPU-89		DCA-09	
RONDE DE MILIANA	178	184	124	124	208	208	178	196	177	207
ROUGETTE DE MITIDJA	172	176	174	174	208	270	160	160	173	175
SIGOISE	172	180	156	174	208	216	162	162	195	207
SOUIDI	170	170	0	0	216	218	160	172	187	201
TABELOUT	172	178	124	172	216	218	160	172	173	183
TASKRIT	176	176	124	124	216	218	160	162	173	185
TEFFAH	172	184	174	174	208	208	178	196	173	195
ZELETNI	170	172	150	150	208	218	160	172	201	207

The rest of Table 12

II - Results

II - 1 - .Variety genetic relationship

II-1-1- Variety genetic relationship between the different 34 Algerian cultivars

In order to have an overview of the genetic similarities between the thirty four olive cultivars under study, **UPGMA** analysis was performed. The genetic similarities ranged from 00 (**Takesrit, Bouricha)**to 17,62 **(Tabelout).**

- UPGMA clustering resulted in a dendogram of genetic relationships that separated the 34 Algerian cultivars. **Figure 12** showed that some varieties were identical between them.
- Among these varieties, we have:
- **Takesrit,Limli and Bouricha,**
- **Aleth and Abani,**
- **Sigoise and Aghenfas,**
- **Bouchouk-Guergour and Bouchouk-Lafayette.**
- Another important case concerns the similarity between **Azzeradj**and **Agrarez** cultivars, which were equal to each other and very similar to **Aghchren Ousser**.
- Were obtain also and other case of similarity between the cultivars**Aberkane**, **Akerma**and **Bouchouk** -**Sidi Aiche**.
- All other nineteen **(19)** cultivars were clearly differentiated from each other. These cultivars are:AGHTITEST, AGUENAOU, AIMEL, BLANQUETTE GUELMA, BOUICHERT, BOUKALIA, CHEMLAL DE KABYLIE, FERKANI, GROSSE DU HAMMA, HAMRA, LONGUE DE MILIANA, MEKKINEB DJEMEL,RONDE DE MILIANA, ROUGETTE DE MITIDJA, SOUIDI, TABELOUT,TEFFAH and ZELETNI.

II-1-2- Variety genetic relationship between the different 34 Algerian cultivars and the other cultivars from the Mediterranean countries

- The comparison with the varieties of otherMediterranean countries (**Italy, Spain, Greece, Turkey, France**, **Syria**, **Egypt**, **Tunisia** and **Morocco)**has not revealed any cases of huge identities and similarities between them and Algerian cultivars but only a very few cases of similarities not significant were observed between these cultivars and some others from Algeria, in particular:
- **Souidi and Empeltre,**
- **Abani and Kalamata,**
- **Sigoise , Aghenfas and Hojiblanca.**
- The result of the analysis of the group **Aberkane,Akerma, Bouchouk and Sidi-Aiche** showed that these varieties are very close and similar to the Tunisian variety **Zaituna**.

III - Discussion

- These results show well that the use of markers **SSR** is very effective for the study of the molecular inter-varietal polymorphism of the olive tree contrary to the morphological and biochemical marker pens **(Ruby 1917., Grati *et al* 2000).**
- For that reason these marker are very used for the characterization and the identification of varieties.
- In general the results obtained with the molecular characterization of Algerian varieties correspond to those previously observed with the use of some morphological parameters **(Mendil and Seba, 2006)** but, unlike the latter, characters that are influenced by the agronomic and phenological stages of plants, the molecular data are stable and repeatable, thus allowing a reliable identification of different genotypes.
- The only discrepancy between molecular and morphological data and those that found for cultivars **Sigoise** and **Aghenfas**, which are revealed the same in genetic examination and are considered well differentiated from the morphological point of view.
- The identity of the cultivar **Sigoise** (whose name was given by the French during the colonization, from **1830** to **1962**), in fact, is of particular importance for the olive in Algeria, given that it represents **90%** of the West Olive area in Algeria, where the olive-growing tradition is new and therefore cannot be regarded as a cultivar indigenous to this region,
- While the cultivar **Aghenfas** is still very limited in Kabylia, in northeastern of Algeria, where the olive ultrasecolari origins.

It is therefore likely that **Aghenfas** represents the original variety from which the **Sigoise** is directly derived. The cultivar **Aghenfas** may have spread as the West where **Sigoise** would fit well to be widely used.

- The cultivar **Aghenfas** is not widely used in Kabylia like all other varieties of table olives (**Tefah**, **Azeradj**, etc..). This is due to the lack of interest of growers in this region for oil cultivar.
- In any case, in order to confirm the results and to characterize definitively the two varieties (**Aghenfas and Sigoise)** and decreeing her real identity, it will be needed

to increase the number of SSR markers examined and extend the investigation to different samples of these two varieties.

- As for other cases of identity between Algerian varieties, it was observed that the same varieties found from the same area of cultivation or areal neighbors.
- In fact, the other three cases of sinonomia can be explained by the geographical proximity of the growing areas of **Tizi-Ouzo**, **Bejaia**, etc..
- The cases of similarities observed with other cultivars of different countries may prove useful for studying the origin of the cultivars to study the behavior of similar cultivars in different growing areas.
- The potential genetic and agronomic germplasm native is still little known in detail and is not considered by the Algerian nursery sector despite its adaptability and hardiness.
- The preservation and dissemination of these indigenous varieties necessarily requires their transmission facilities with the creation of regional nurseries that use an identified and certified material.
- The results obtained, confirm the extraordinary potential of SSR markers. Both features, SSR transferability and polymorphism, are specially interesting for biodiversity studies. The discriminatory capacity of SSR has been reported by other authors **(Lopez *et al* .2004, Rallo *et al* .2000),** whose major application has been the identification and the discrimination of cultivars. The separation of microsatellite markers by sequencing apparatus revealed to be very suitable, since the detection of alleles was performed automatically using a computer software package and there was no need for manual scoring of the data.
- Microsatellites markers are useful, either alone or in combination with other molecular markers for fingerprinting of olive cultivars and can be used to generate a database for future identification.
- The detection method used confirmed that automated fluorescence detection is very reliable and fast technique.

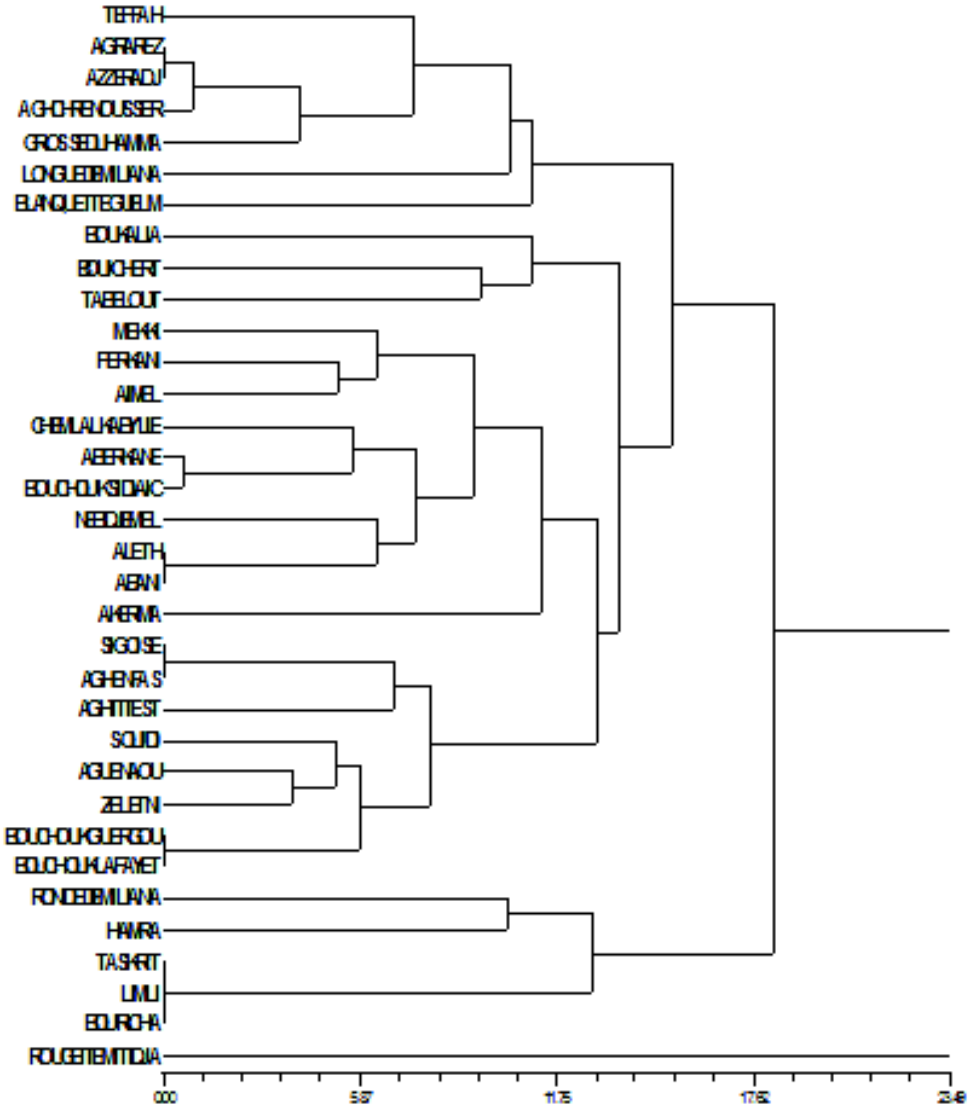

Figure 12 : *UPGMA Dendrogram showing genetic similarity among* ***34*** *Algerian varieties of olive.*

Genetic Similarity

Conclusion

- Olive (***Olea europea*** **L.)** is cultivated all around the Mediterranean basin. It is a highly variable specie with more than **2600** different cultivars, although many of these might be synonyms or ecotypes. Today the olive industry requires well-characterized cultivars with elite agronomic characteristics or cultivars adapted to modern intensive mechanized orchards. Molecular marker techniques have been applied to assist olive cultivar identification and origin. Since molecular markers are not environmental affected they are superior to biochemical markers.
- In order to establish genetic relationships and to individually identify 34 olive cultivars from Algeria, SSR molecular markers were used. Five **(05)** SSR markers were used in the thirty four **(34)** olive samples.
- Dendrograms were constructed and genetic similarities were calculated. Several unique bands and group of primers were able to individually discriminate all the thirty four **(34)**olives cultivars.

<u>This study has allowed the show that</u>

- DNA analysis of the main olive cultivars has allowed to highlight the richness and variability of the Algerian olive-groves. Have emerged from this study othe cases of synonymy and similarity, including to report the case of synonymy between **(Sigoise** and **Aghenfas)** which was found in contradiction with their morphological description. Clear, therefore, the importance of making new samples of these two cultivars to verify the result.
- The comparison with a varieties of other countries countries (**Italy, Spain, Greece, Turkey, France**, **Syria**, **Egypt**, **Tunisia** and **Morocco)** has not revealed any cases of identities and similarities but only a few cases of similarities but not significant were observed between these cultivars and some others from Algeria, in particular:
  - **Souidi and Empeltre,**
  - **Abani and Kalamata,**
  - **Sigoise , Aghenfas and Hojiblanca.**
- The goal for the future is to expand the exploration of Algerian olive territory to collect all the smaller local varieties for the study of agronomic and genetic characterization.
- Only the recovery and preservation of the large number of cultivars and local ecotypes will contribute to preserving the Algerian olive germplasm, for each genotype is a repository of valuable characters and, as such, is potentially useful for the development of olive production for local breeding programs of the species.
- The morphological and genetic characterization of olive germplasm is an important step forward for the development of Algerian olive sector but now need to create a National Center of Conservation of the Olive to the conservation, evaluation, selection and development of many cultivars and local ecotypes.
- Have the molecular profiles of olive cultivars is very important to carry out inspections of compliance genetics of plants propagated by nurseries and to prevent trade disputes.

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ANNEX

Annex 1 . Number of olive cultivars and their synonyms in the world (FAO, 1989).

Country	Autochthonous		Imported		Total		Total Names
	Cultivars	Synonyms	Cultivars	Synonyms	Cultivars	Synonyms	
Afghanistan	—	—	8	0	8	0	8
Albania	22	34	21	4	43	38	81
Algeria	41	50	19	7	60	57	117
Angola	—	—	—	—	—	—	—
Argentina	14	4	94	73	108	77	185
Australia	2	—	40	40	42	40	82
Azerbaijan	7	9	31	33	38	42	80
Brazil	—	—	12	5	12	5	17
Bhutan	—	—	4	—	4	—	4
Burma	—	—	6	—	6	—	6
Chile	2	2	11	5	13	7	20
China	13	9	67	50	80	59	139
Columbia	1	—	6	1	7	1	8
Croatia	33	69	31	38	64	107	171
Cyprus	1	—	15	7	16	7	23
Egypt	6	4	17	7	230	11	34
France	88	300	24	21	112	321	433
Georgia	2	1	6	10	8	11	19
Greece	52	84	—	—	52	84	136
India	—	—	19	2	19	2	21
Iraq	10	7	10	4	20	11	31
Iran	9	4	6	3	15	7	22
Israel	31	48	29	15	60	63	123
Italy	538	1302	6	4	544	1306	1850
Japan	3	1	7	5	10	6	16
Jordan	5	6	2	1	7	7	14
Lebanon	5	11	11	5	16	16	32
Libya	10	11	23	18	33	29	62
Malta	—	—	—	—	—	—	—
Mexico	—	—	13	1	13	1	14
Morocco	6	10	20	7	26	17	43
Nepal	—	—	5	—	—	—	5
New Zealand	—	—	8	1	8	1	9
Pakistan	1	—	16	1	17	1	18
Peru	4	—	5	1	9	1	10
Portugal	24	95	18	28	42	123	165
Russia	—	—	2	3	2	3	5
San Marino	—	—	6	—	6	—	6
Saudi Arabia	—	—	4	—	4	—	4
Slovenia	7	27	16	25	23	52	75
South Africa	—	—	9	3	9	3	12
Spain	183	456	8	4	191	460	651
Switzerland	—	—	2	—	2	—	2
Syria	23	23	7	4	30	27	57
Tunisia	44	80	22	16	66	96	162
Turkey	45	66	11	3	56	69	125
Turkmenistan	—	—	5	—	5	—	5
Uruguay	—	—	—	—	—	—	—
U.S.A.	6	2	42	39	48	41	89
Ukraine	16	10	24	30	40	40	80
Yugoslavia	19	2	30	9	49	11	60
Totals	**1275**	**2631**					**5331**

Annex 2. List of the 77 olive cultivars used for the evaluation of the SSR.

725 Table 1. List of the 77 olive cultivars used for the evaluation of the SSR marke
726 cultivars used for the SSR selection are reported in bold.

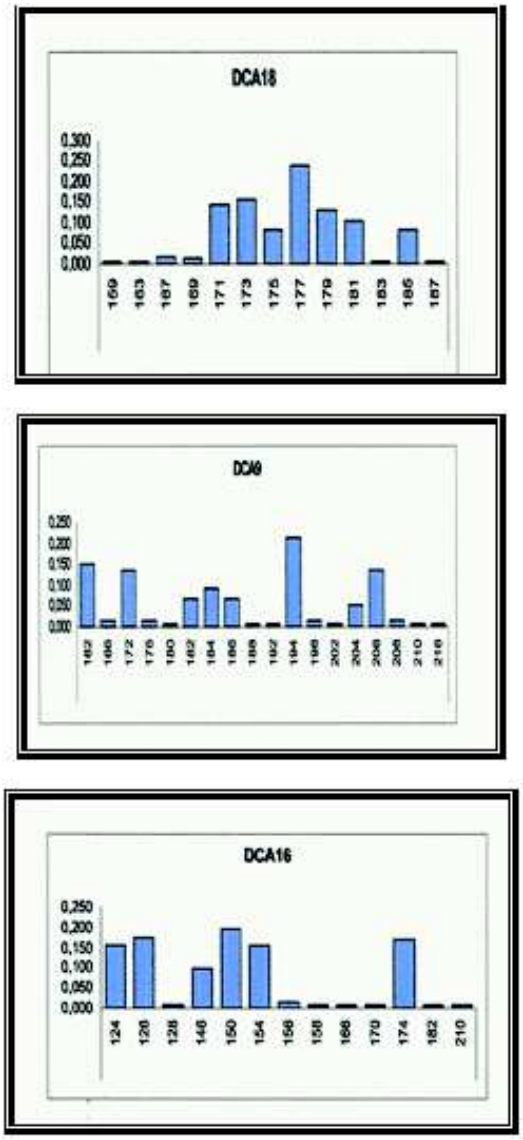
Cultivar	Number of clones	Country of main diffusion	Repository of samples/ Register number
Istarska Belica		Croatia	WOGB[1]
Oblica		Croatia	WOGB
Toffahi		Egypt	WOGB
Bouteillan		France	WOGB
Lucques		France	WOGB
Oliviere		France	WOGB
Picholine		France	WOGB
Verdale		France	WOGB
Amigdalolia		Greece	WOGB
Kalamon		**Greece**	**WOGB/105**
Kerkiras		Greece	WOGB
Konservolia		**Greece**	**WOGB/219**
Koroneiki		**Greece**	**WOGB/218**
Mastoidis		**Greece**	**WOGB/345**
Maureya		Greece	WOGB
Mirtolia		Greece	WOGB
Merhavia		Israel	WOGB
Ascolana Tenera		Italy	WOGB
Biancolilla		**Italy**	**CRA-OLI[2]/347**
Borgiona		Italy	CRA-OLI
Bosana		Italy	CIFCO[3]
Caiazzana		Italy	CRA-OLI
Canino	**3**	**Italy**	**CRA-OLI/32**
Capolga		Italy	CRA-OLI
Carolea		**Italy**	**WOGB/736 - CRA-OLI/19**
Cassanese		Italy	CRA-OLI
Cellina di Nardo'		Italy	CRA-OLI
Dolce Agogia	3	Italy	IGV[4]
Frantoio	**4**	**Italy**	**WOGB/80 - CRA-OLI/92**
Gargna'		Italy	IGV
Gentile di Chieti		Italy	IGV
Leccino	**3**	**Italy**	**WOGB/82 - CRA-OLI/93**
Mignola di Cartoceto		Italy	CRA-OLI
Moraiolo	**10**	**Italy**	**WOGB/78 - CRA-OLI/94**
Nostrale di Rigali	1	Italy	CRA-OLI
Nostrana di Brisighella		Italy	IGV
Ogliarola Salentina		**Italy**	**CRA-OLI/64**
Olialonga		Italy	CIFCO
Orbetana		Italy	CRA-OLI
Ottobratica		Italy	CRA-OLI
Passalunara		Italy	IGV
Piantone di Mogliano		Italy	CRA-OLI
Raia	4	Italy	CRA-OLI
Raio	3	Italy	CRA-OLI
Semidana		Italy	CIFCO
Zaituna		Italy	IGV
Picholine Marocaine		**Morocco**	**WOGB/101**
Arbequina		**Spain**	**WOGB/231**
Blanqueta		Spain	WOGB
Changlot Real		Spain	WOGB
Cornezuelo de Jaen		Spain	WOGB
Cornicabra		**Spain**	**WOGB/10**
Empeltre		Spain	WOGB
Farga		Spain	WOGB
Gordal Sevillana		Spain	WOGB
Hojiblanca		**Spain**	**WOGB/2**

The rest of Annex 2

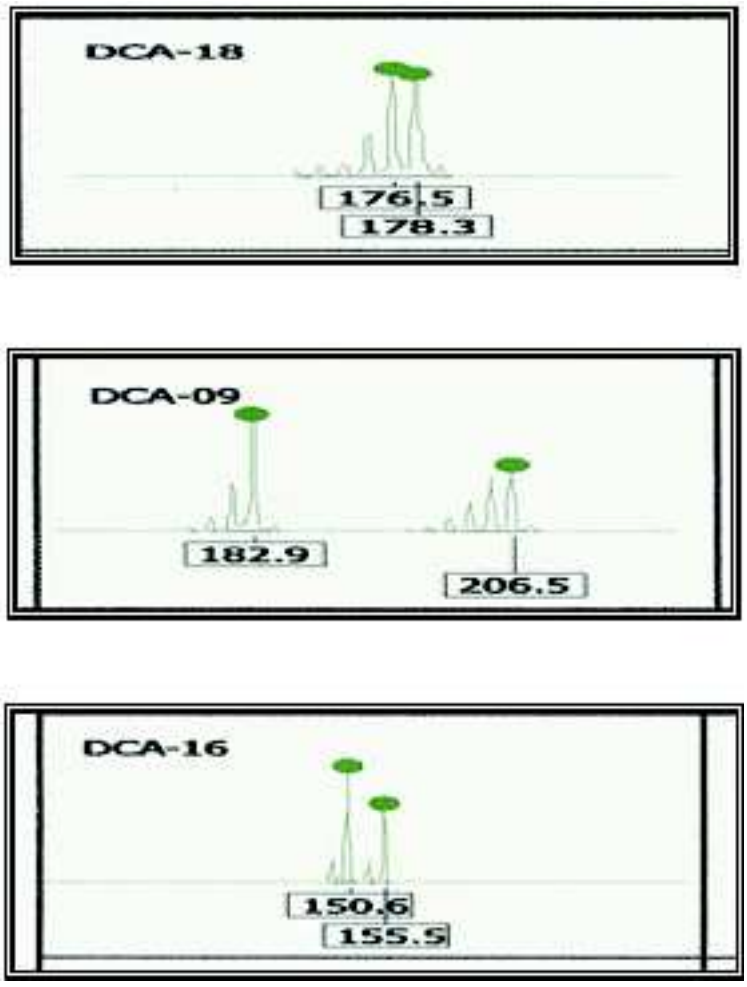
echin de Granada	Spain	WOGB
echin de Sevilla	**Spain**	**WOGB/5**
lanzanilla Cacereña	**Spain**	**WOGB/430**
lanzanilla de Jaen	Spain	WOGB
lanzanilla de Sevilla	Spain	WOGB
'cual	**Spain**	**WOGB/9**
icudo	Spain	WOGB
oyal de Cazorla	Spain	WOGB
evillenca	Spain	WOGB
'erdial de Huevar	Spain	WOGB
'illalonga	Spain	WOGB
.aissy	Syria	WOGB
aity	Syria	WOGB
hemlali	**Tunisia**	**WOGB/744**
uslati	Tunisia	WOGB
almati	Tunisia	WOGB
yyalik	**Turkey**	**WOGB/97**
emir Sofralik	Turkey	WOGB
lemecik	**Turkey**	**WOGB/93**
'slu	Turkey	WOGB
'un Celebi	Turkey	WOGB

WOGB: World Olive Germplasm Bank, Córdoba, Spain
CRA-OLI: CRA – Centro di Ricerca per l'Olivicoltura e l'Industria Olearia, Rende (CS), Italy
CIFCO, Consorzio Interprovinciale Frutticoltura, Cagliari e Oristano, Italy
CNR-IGV: CNR - Istituto di Genetica Vegetale, Perugia, Italy
Samples of four cultivars were obtained from two different collections: no discrepancies were observed among them.

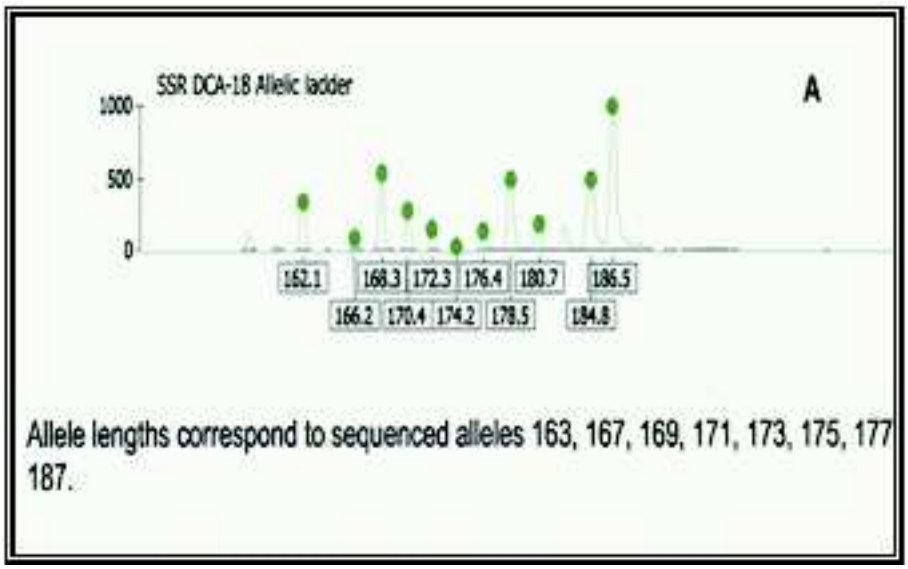
Annex 3. Allele frequency at the 03 selected SSR loci analysed on the enlarged set of 34 Algerian genotypes.



Annex 4. Pherograms of 03 microsatellites used in the study coming out from an automatic sequencer.



Annex 5. **Allele lengths correspond to sequenced alleles 163, 167, 169, 171, 173, 175, 177, 179, 181, 185,187of the SSR DCA-18 .**


SSR DCA-18 Allelic ladder
A
1000
500
0
162.1
166.2
168.3
170.4
172.3
174.2
176.4
178.5
180.7
184.8
186.5
Allele lengths correspond to sequenced alleles 163, 167, 169, 171, 173, 175, 177
187.