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AFFIRMATION OF DNA BARCODING AS A POWERFUL TOOL IN CATALOGUING MEDICINAL AND AROMATIC PLANTS IN MEDITERRANEAN FORESTS

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~ Στους γονείς οφείλομεν το ζην, στους δε διδασκάλους το ευ ζην~

Μέγας Αλέξανδρος, 356-323 π.Χ.

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I. Introduction

1. History of Medicinal and Aromatic plants

1.1 In ancient times

In 1960, at the cave site of Shanidar in what is now north-eastern Iraq, the skeleton of an adult male was discovered, lying on his left side in a partial foetal position. He had been buried some 60,000 years ago. Routine soil samples were gathered for pollen analysis in an attempt to reconstruct the site's palaeoclimate and vegetational history. In some of the samples concentrated clumps of pollen were found suggesting that entire flowering plants had been buried close to the man. Though the source of the pollen is hotly debated, a study of the particular flower types suggested that the flowers may have been specifically chosen for their medicinal properties. Yarrow (Achillea spp.), St. Barnaby's thistle (Centaurea spp.), groundsel (Senecio spp.) and rose mallow (Hibiscus spp.) amongst others were represented in the pollen samples, all of which have long known curative powers as stimulants, astringents and anti-inflammatories (Solecki 1976).

Imag. 1 *The entrance to Shanidar Cave in northern Iraq- Partial skeleton in situ in Shanidar Cave.*

The history of medicinal plants is essentially the history of mankind itself, every culture and civilization has used plants extensively since prehistory. A cultural fact useful to explain what may have happened in the past is the study of several contemporary rural sites in South America, where each ethnic group has an extensive knowledge of local flora, cultivates its own medicine garden and provides information on the medicinal powers of diverse native plants.

Cultural tradition and personal dedication to experiment with diverse plants can explain the accumulation of knowledge by native people aware of the potential contribution of nature to human's life. That ability also found in wild animals and even in some domesticated animals (cats, dogs), is a faculty used by these ethnic groups habituated to live closed to nature, compared to urbanized people who have lost this capacity, becoming unable to recognize which plants to choose for a natural remedy.

There is no doubt that the earliest drug used as an analgesic was the product of sugar fermentation in solution by yeast (ethanol). Alcohol was produced “when crushed grapes of berries in water were left standing in a warm place”, as far back as 6400BC. The technique of distillation introduced to Europe by Arabic cultures during the middle ages, enabled alcohol to be concentrated at high levels. Since the very beginning alcoholic beverages became the ‘wonder drink’ capable of producing relaxation, elevation of mood, increased appetite and release of inhibition. Later alcohol has been used as an anxiolytic, soporific, hypnotic, analgesic, astringent, bactericidal and also as a solvent (Ortega 2004).

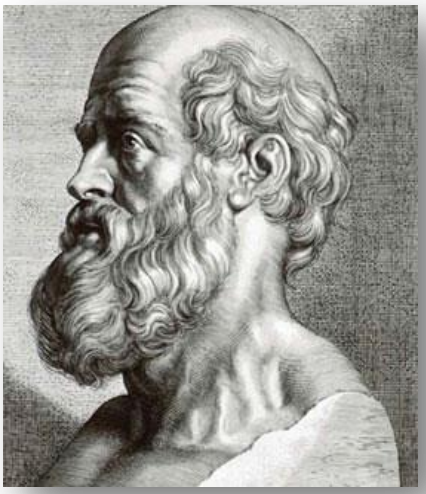
Undisputedly, the history of herbology is inextricably intertwined with that of modern medicine. Many drugs listed as conventional medications were originally derived from plants. Salicylic acid, a precursor of aspirin, was originally derived from white willow bark and the meadowsweet plant. Cinchona bark is the source of malaria-fighting quinine. Vincristine, used to treat certain types of cancer, comes from periwinkle. The opium poppy yields morphine, codeine, and paregoric, a treatment for diarrhea Laudanum, a tincture of the opium poppy, was the favored tranquilizer in Victorian times. Even today, morphine-the most important alkaloid of the opium poppy-remains the standard against which new synthetic pain relieves is measured (Ortega 2004).

In 2735 B.C., the Chinese emperor Shen Nong wrote an authoritative treatise on herbs that is still in use today, the use of Ma Huang (known as ephedra in the Western world), mostly used against respiratory distress. Ephedrine, extracted from ephedra, is widely used as a decongestant. In 1800 B.C records of King Hammurabi of Babylon include instructions for using medicinal plants. He prescribed the use of mint for digestive disorders. Modern research has confirmed that peppermint does indeed relieve nausea and vomiting by mildly anesthetizing the lining of the stomach (Ortega 2004).

The entire Middle East is characterized by a rich history of herbal healing. There are texts still survived from the ancient cultures of Egypt, Mesopotamia and India that describe and illustrate the use of many medicinal plant products, including castor oil, linseed oil, and white poppies. In the scriptural book of Ezekiel, which dates from the sixth century B.C., it can be find this admonition regarding plant life: "..and the fruit thereof shall be for meat, and leaf thereof for medicine." Egyptian hieroglyphs show physicians of the first and second centuries A.D. treating constipation with senna pods, and using caraway and peppermint to relieve digestive upsets (Ortega 2004).

1.2 Arabic and Greek legacies

Approximately 1000 years ago, Baghdad became a recognized focal point of medicinal learning. Muslim, Jewish and Christian scribes and scholars cooperated in making available a wealth of texts, by Persians-Mesopotamian, Byzantine-Greek and Indian traditions. The theory and practice of medicine in the first millennium was preserved for us in a series of books such as The ROYAL Book of All Medicine by Ali ibn Abbas al-Majusi (Haly Abbas, d 994), the Canon of Medicine by ibn Sina (Avicenna, d. 1037) etc.. The essence of these medicinal writings flowed west to Damascus, Cairo, Palermo and Cordoba having been translated exerting a strong influence on the first university curricula.



Imag. 2 *Hippocrates- also considered as the 'Father of Medicine'*

The emerging universities of the 12th century European western culture, received the strong influence of the writings of Hippocrates of Cos (4th B.C.); the founder of allopathic medicine, is considered to be the first Unani *(Ἰωνία or Ἰωνίη)* physician and Galen of Pergamum (2nd C.E.) translated into Latin. (Baytop, 1999). The Hippocratic foundations were complemented, possibly by Byzantine teachers before the 8th century, with Galen's "Art of Medicine" or "Articella".

1.3 In the Middle Ages

Remarkable is during the Middle Ages too, home-grown botanicals were the only medicines readily available, and for centuries, no self-respecting household would be without a carefully tended and extensively used herb garden. For the most part, herbal healing lore was passed from generation to generation by word of mouth. Mother taught daughter; the village herbalist taught a promising apprentice.

By the seventeenth century, the knowledge of herbal medicine was widely disseminated throughout Europe. In 1649, Nicholas Culpeper wrote A Physical Directory, and a few years later produced The English Physician. This respected herbal pharmacopeia was one of the first manuals that the layperson could use for health care, and it is still widely referred to and quoted today. Culpeper had studied at Cambridge University and was meant to become a great doctor, in the academic sense of the word. Instead, he chose to apprentice to an apothecary and eventually set up

his own shop. He served the poor people of London and became known as their neighborhood doctor. The herbal he created was meant for the layperson.

The first U.S. Pharmacopeia was published in 1820. This volume included an authoritative listing of herbal drugs, with descriptions of their properties, uses, dosages, and tests of purity. It was periodically revised and became the legal standard for medical compounds in 1906. But as Western medicine evolved from an art to a science in the nineteenth century, information that had at one time been widely available became the domain of comparatively few. Once scientific methods were developed to extract and synthesize the active ingredients in plants, pharmaceutical laboratories took over from providers of medicinal herbs as the producers of drugs. The use of herbs, which for most of history had been mainstream medical practice, began to be considered unscientific, or at least unconventional, and to fall into relative obscurity.

1.4 Herbal Medicine Today

During the past decade, traditional systems of medicine have become a topic of global importance. Current estimates suggest that, in many developing countries, a large proportion of the population relies heavily on traditional practitioners and medicinal plants to meet primary health care needs. Although modern medicine may be available in these countries, herbal medicines (phytomedicines) have often maintained popularity for historical and cultural reasons. Concurrently, many people in developed countries have begun to turn to alternative or complementary therapies, including medicinal herbs.

Imag. 3 *Flag of the World Health Organization*

The World Health Organization (W.H.O.) estimates that 4 billion people, 80% of the world population, presently use herbal medicine for some aspect of primary health care. Herbal medicine is a major component in all indigenous peoples' traditional medicine and a common element in Ayurvedic, homeopathic, naturopathic, traditional oriental, and Native American Indian medicine. W.H.O. notes that of 119 plant-derived pharmaceutical medicines, about 74% are used in modern medicine in ways that correlated directly with their traditional uses as plant medicines by native cultures. Major pharmaceutical companies are currently conducting extensive research on plant materials gathered from the rain forests and other places for their potential medicinal value.

Rather than using a W.H.O.le plant, pharmacologists identify, isolate, extract, and synthesize individual components, thus capturing the active properties. This can create problems, however. In addition to active ingredients, plants contain minerals, vitamins, volatile oils, glycosides, alkaloids, bioflavanoids, and other substances that are important in supporting a particular herb's medicinal properties. These elements also provide an important natural safeguard. Isolated or synthesized active compounds can become toxic in relatively small doses; it usually takes a much greater amount of a W.H.O.le herb, with all of its components, to reach a toxic level. Herbs are medicines, however, and they can have powerful effects.

2. Efficacy of Medicinal and Aromatic plants

2.1 MAPs utilization

Medicinal and aromatic plants (MAPs) as mentioned above have been used by mankind for millennia; their use is as old as humanity itself. From the pre-historic era, men have been using plants as medicine and in recent years, interest in the exploitation of plants as pharmaceuticals, herbal remedies, flavourings, dietary supplements, homeopathics, medicinal and herbal teas, liqueurs, spirits, sweets, aromas and essences, perfumes, cosmetics, coloring agents, varnishes, fireworks, and detergents and other natural products has greatly increased (Iqbal 1993; Walter 2001; Rao & Arora 2004).

Whereas in some goods the herbal ingredients are evident, e.g. in teas or in herbal remedies where they are declared on the packaging, in other products the botanically source is more secret: the bitter taste of Campari is based on the Common Centaury (Centaurium erythraea), and the fenugreek (Trigonella foenum-graecum) contains steroid-saponins which are extracted for use in oral contraceptives. The use of botanical raw material is in many cases much cheaper than to use chemical alternative substances (Lange 2004).

Through evolution, plants have developed large numbers of chemical substances to defend themselves against insect pests, and fungal and other pathogenic diseases. Some of these chemicals can also fight against micro-organism and other diseases in the human body. They represent an

important source of natural drugs. Their highly complex molecular structures often surpass the imagination of the chemist and cannot easily be reproduced in the laboratory (Seters 2003).

2.2 Species in Use & Economic value

The range of species used and their scope for healing is vast. Cures as yet undiscovered may exist in plants as yet undescribed. Currently, it is estimated that the number of higher plant species used worldwide for medicinal and aromatic purposes is more than 50,000 (Schippmann et al., 2002). This equates to approximately 20% of the world's vascular flora and constitutes the biggest spectrum of biodiversity used by people for a specific purpose (Hamilton et al., 2006).

Medicinal and aromatic plants harvested from the wild remain of immense importance for the well-being of millions of people around the world. MAPs are clearly an important global resource in terms of healthcare but they are also an important economic resource, traded extensively on scales ranging from the local to the international. Internationally, the trade in medicinal and aromatic plants is estimated to be worth $60 billion per year (World Bank, 2004) increasing at a rate of 7% a year (Koul and Wahab, 2004). Loss of habitat combined with over-harvesting threatens the survival of many of these plant species.

Country of import	Quantity [tonnes]	Value [US$]	Country of export	Quantity [tonnes]	Value [US$]
Hong Kong	59,950	263,484,200	China	150,600	266,038,500
USA	51,200	139,379,500	Hong Kong	55,000	201,021,200
Japan	46,450	131,031,500	India	40,400	61,665,500
Germany	44,750	104,457,200	Mexico	37,600	14,257,500
Rep. Korea	33,500	49,889,200	Germany	15,100	68,243,200
France	21,800	51,975,000	USA	13,050	104,572,000
China	15,550	41,602,800	Egypt	11,800	13,476,000
Italy	11,950	43,006,600	Bulgaria	10,300	14,355,500
Pakistan	10,650	9,813,800	Chile	9,850	26,352,000
Spain	9,850	27,648,300	Morocco	8,500	13,685,400
UK	7,950	29,551,000	Albania	8,050	11,693,300
Malaysia	7,050	38,685,400	Singapore	7,950	52,620,700
Total	320,550	930,524,400	Total	368,100	847,980,800

Tab. 1 *The 12 leading countries of import or export of MAP material classified as pharmaceutical plants (SITC.3: 292,4= commodity groups HS 1211). The countries are listed according to descending order of average trade volumes, 1991-2003 (COMTRADE database, United Nation Statistics Division, New York).*

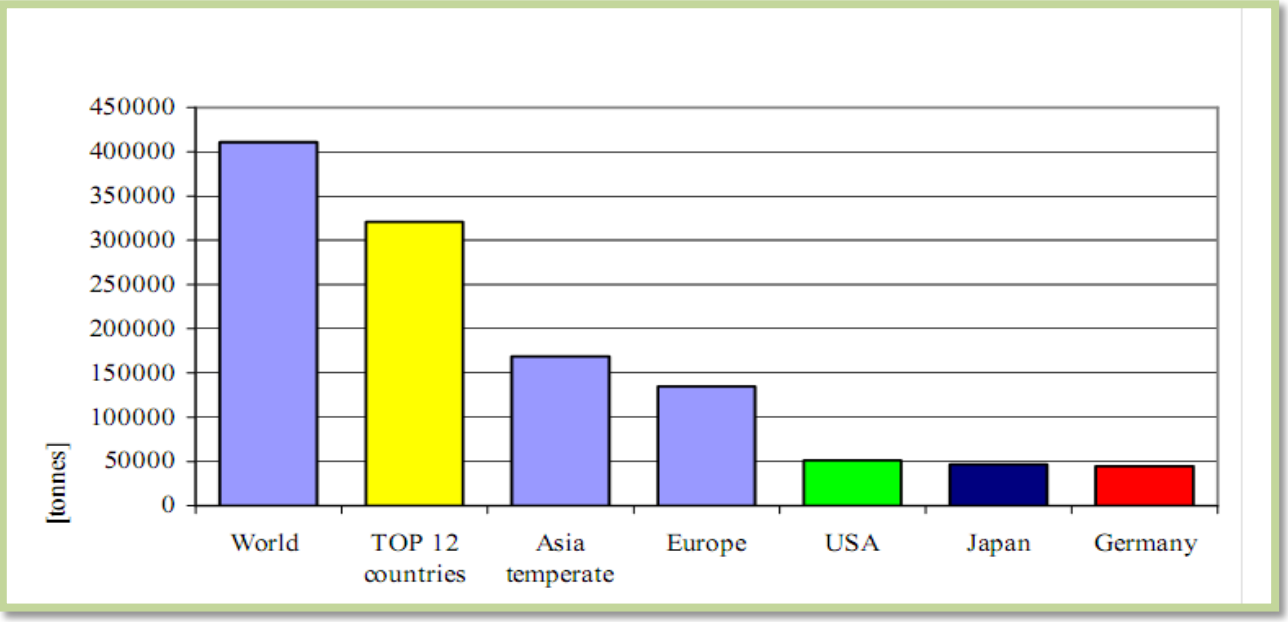


Fig. 1 *The dominating actors, countries and regions, in the international trade in pharmaceutical plants (SITC.3: commodity group 292.4) and their average import quantities in tones for the period 1991-2003(COMTRADE database, United Nation Statistics Division, New York.).*

About 2,000 medicinal and aromatic plant species are used on a commercial basis in Europe, of which two-thirds are native to Europe. In the EU, medicinal and aromatic plants are cultivated on an estimated 70,000 ha. Leading species are: lavender (Lavandula spp.), Opium Poppy (Papaver somniferum), Caraway (Carum carvi) and Fennel (Foeniculum vulgare). France and Spain are EU countries with many hectares under cultivation. However, in Spain wild-harvesting and cultivation of medicinal and aromatic plants has declined. There is some cultivation in Germany, where leading producers of herbal medicines have their own plantations for popular products. Eastern European countries such as Bulgaria, Hungary and Albania are major EU suppliers of material from medicinal and aromatic plants.

In 2000, Germany was, by far, the leading EU importer of medicinal & aromatic plants. Between 1998 and 2000, however, Germany saw its share in EU imports decrease from 38 percent to 29 percent, while the United Kingdom experienced an increase from 7 to 12 percent. The Netherlands was small importer of medicinal & aromatic plants, being only the 12th leading EU importer.

The major importing markets are the EU and USA. In these countries, increased demand for medicinal plants is being fuelled primarily by consumer interest in natural products and remedies, as well as by increasing concerns about the possible side effects of allopathic medicines. Major

developing countries such as China and India are exporting medicinal plants, herbal tonics, cosmetics, perfumes etc. There are therefore good prospects for export growth from LDCs in this market. However, markets in developed countries for herbal medicine -especially in Europe and the USA - are highly regulated and are very difficult to penetrate, particularly for developing countries and LDCs W.H.O.se products have not undergone the stringent tests applied by developed country pharmaceutical manufacturers before mass production (India Trade Promotion Organization-ITPO) (http://www.tradeportalofindia.com/contentmgmt/Desktops2.html?compid=itpo&itemcode=I087).

3. Ethnobotany of Mediterranean region

The Mediterranean Basin biodiversity hotspot is the second largest hotspot in the world and the largest of the world's five Mediterranean-Climate regions. The hotspot covers more than 2 million square kilometers and stretches west to east from Portugal to Jordan and north to south from northern Italy to Cape Verde (Mittermeier et al. 2004). It is one of the areas with the greatest diversity on the planet, and thus it is considered that it should be maintained as a conservation sanctuary (Myers et al., 2000). Approximately, 30,000 plant species occur and more than 13,000 are found nowhere else, or endemic, to the hotspot, yet many more are being discovered every year (Radford et al., 2011). The Mediterranean Basin is the third richest hotspot in the world in terms of its plant diversity (Mittermeier et al. 2004). Therefore, the radical trapping properties of the Mediterranean flora, comprising more than 10000 plant species with various properties (anti-inflammatory, diuretic and so on), deserve further investigation.

Arguments such as species richness and uses, migration, cultural shift or the disappearance of the communities are put forward to prioritize ethnobotanical studies in places such as Amazon. These same reasons continue to be valid in industrialized countries or emerging countries, such as those of Mediterranean Basin, where the alternation of the physical and biological environment, rural depopulation, the new means of communication, etc. are causing an accelerated loss of traditional knowledge, making these types of studies perhaps even more urgent.

3.1 Toxicity complication of MAPs & identification problems

However, several spontaneous plants are potentially toxic for human beings (Ngogang J. et al. 2008; Tinggi U. 2003; Vetter J. 2000; Xia Q. et al. 2008). The accidental ingestion of toxic plant portions (for instance seed, fruit, root, etc.) can cause severe poisoning or even death (McIntire MS. et al. 1990; Vetter J. 2000). Hence, plant medicinal or aromatic materials may be substituted accidentally by taxa from closely related species or adulterated intentionally by materials from unrelated plants. In the last years, the plant exposures are among the most frequent poisoning cases

reported by poisoning control centers (Mrvos R. 2001). More precisely, in 2002, 63 people were reported with syptoms of general malaise, nausea and vomiting after consumption of herbal tea which was inadvertently mixed with neurotoxic Japanese star anise (*Illicium anisatum*) (Johanns E.S. et al. 2002). Adulteration resulting in an epidemic of severe kidney damages caused by aristolochic acid was first reported in Belgium in 1993 (Vanherweghem J.L. et al. 1993), followed by Hong Kong and Korea (Lo S.H. et al. 2004; Lee S. et al. 2004) in 2004.

Nevertheless, the identification of herbal medical materials using traditional, organoleptic and chemical methods can be difficult, particularly for those materials derived from the processed parts of a plant or in powder form. Therefore, an accurate, universal, stable and specific marker allowing non-specialists to identify the source species from a tiny amount of tissue is undoubtedly beneficial. Molecular technology is believed to be a reliable alternative tool for the identification of herbs (Shaw et al. 1997; Kaplan et al. 2004) and DNA barcoding is the latest move towards the generation of universal standards (Chen et al. 2010; Heubl, 2010). DNA barcoding has become a major focus in the biodiversity and conservation fields in recent years.

3.2 Selected plant species for study

Few plant species that provide medicinal herbs have been scientifically evaluated for their possible medical application. Safety and efficacy data are available for even fewer plants, their extracts and active ingredients, and the preparations containing them. Furthermore, in most countries the herbal medicines market is poorly regulated, and herbal products are often neither registered nor controlled. Assurance of the safety, quality, and efficacy of medicinal plants and herbal products has now become a key issue in industrialized and in developing countries. Both the general consumer and health-care professionals need up-to-date, authoritative information on the safety and efficacy of medicinal plants.

During the fourth International Conference of Drug Regulatory Authorities (ICDRA) held in Tokyo in 1986, W.H.O. was requested to compile a list of medicinal plants and to establish international specifications for the most widely used medicinal plants and simple preparations. Guidelines for the assessment of herbal medicines were subsequently prepared by W.H.O. and adopted by the sixth ICDRA in Ottawa, Canada, in 1991.

As a result of ICDRA's recommendations and in response to requests from W.H.O.'s Member States for assistance in providing safe and effective herbal medicines for use in national healthcare systems, W.H.O. has published 4 volumes on selected medicinal plants so far; volume 1 includes 28 monographs; volume 2 contains an additional 30 monographs; volume 3 provides 31 monographs

and volume 4 contains 28 new monographs published, a total of 118 monographs in four volumes (W.H.O. 2009).

Consequently, in this doctorate thesis, it has been decided to be included for analysis some of these plant species that have been proposed by the published lists of W.H.O., naturally occurred in Mediterranean Basin (mainly in Italy and Greece, most of them) divided into different categories according to their importance in medicine.

3.3 Plant species monographs

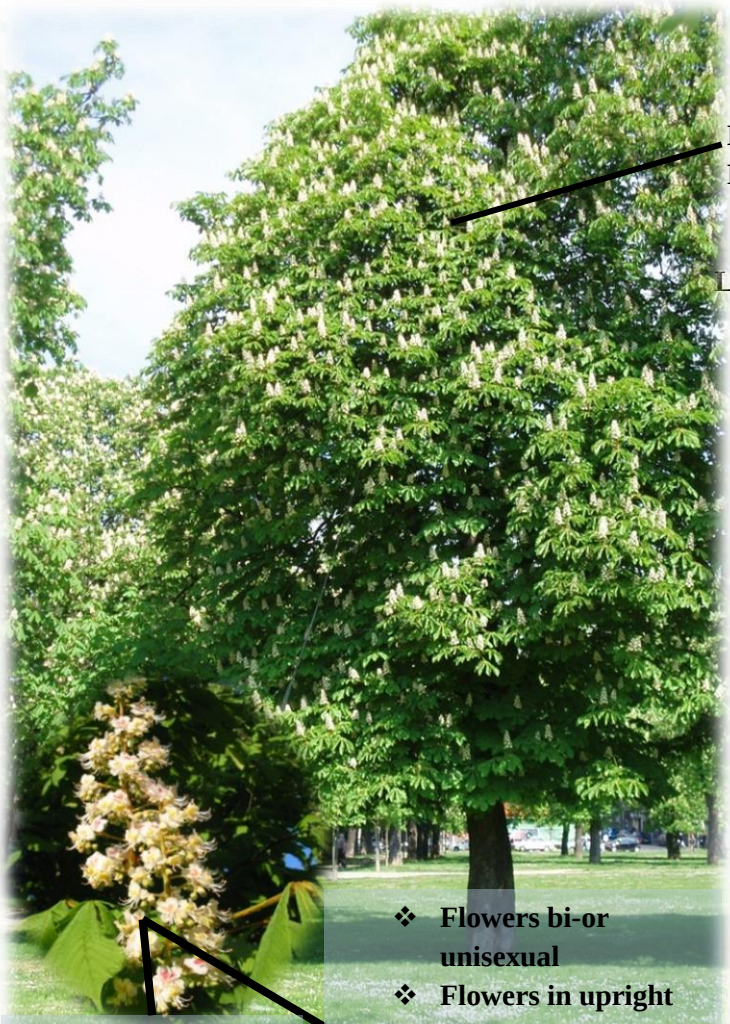
3.3.1 AESCULUS HIPPOCASTANEUM L.

English name: *Horse Chestnut*, Family: *Sapindaceae*

Seed

Fruit shell with short spikes

Habitat: *Native to south-eastern Europe (Balkans), widely planted as an ornamental tree in parks and along roads, occasionally naturalized*



H. up to 20m, F.S. May-June

Leaves palmate

- **Flowers bi-or unisexual**
- **Flowers in upright racemes**
- **Leaflets up to 20cm long**

Petals white with a distinct pink mark 10-15mm long

Health tip:

Horse Chenstnut preparations are available for most of the plant's main indications. This is a traditional household remedy: use a tincture of Horse Chenstnut leaves, flowers and bark soaked in brandy as a rub to ease rheumatic pain.

While conventional medicine uses mainly the saponin (aescin) containedin the seeds, folk medicine also utilizes the leaves, flowers and bark of the Horse Chenstnut tree. Aescin, also known as horse chestnut extract, is used in preparations for venous insufficiency, varicose veins, oedema and haemorrhoids. In addition Horse Chestnut rubs and bath oils are recommended for bruices and to improve circulation. Folk medicine uses the leaves for rheumatic complaints, haemorrhoids, thrombosis and other venous diseases.

3.3.2 ALTHAEA OFFICINALIS L.

English name: Marsh Mallow, Family: Malvaceae

Marsh Mallow has been used as a medicinal plant since antiquity and is still an accepted remedy in herbal medicine today. A tea made from its leaves, flowers or roots helps alleviate oral and throat infections as well as gastro-intestinal complaints. The plant may even have immune-boosting properties. Marsh Mallow tea with honey is a popular cough remedy and Marsh Mallow poultices are applied in folk medicine for a variety of skin conditions.

Habitat: Moist ground, salt marshes, on loamy soils. Asia, Eastern Europe, coastal areas

- ***Escapes from gardens often found in the wild***
- ***Entire plant is covered in a soft down***
- ***Outer sepals joined at base, petals lobed at the top***

Anthers purple

Flower 3-5cm across

Flowers pale pink, in dense clusters

H 60-1, 20 cm FS. July-Sept.

Root up to 50cm

Health tip:

Soak 1-2 tsp of chopped Marsh Mallow root in a cup of cold water for about 1h. then heat slowly to the desired temperature and strain. Alternatively, place 1-2 tsp. of Marsh Mallow leaves in a cup and add hot (but not boiling) water. Strain after 10'.

3.3.3 BERBERIS VULGARIS L.

English name: Common Barberry, Family: Berberidaceae

H. 2-3m
FS. May-

The refreshingly tart fruits of this shrub are rich in vitamin C and can be used in jams and compotes. Folk medicine uses them as a laxative and for liver and spleen problems. The poisonous bark of the root, too, was used in the past as a remedy for gall bladder complaints, jaundice, digestive disorders, diarrhea, kidney stones, rheumatism and numerous other ailments.

Long, very sharp spines, often in threes

Habitat: Forests margins, hedgerows, open pine forests. Europe to western Asia.

- ***Deciduous***
- ***Leaves in bunches***
- ***Flowers have an intense, slightly unpleasant fragrance***

Tough leaf, 2-6cm long

Margin with prickly teeth

Clusters with many yellow flowers

6 petals and 6 sepals

Flowers in pendent racemes

Oblong berries, up to 1cm long

3.3.4 BETULA PENDULA ROTH.

English name: Silver Birch, Family: Betulaceae

H. 8-25m
FS. April-

Dried Silver Birch leaves are mildly diuretic, without over stimulating the kidneys and are recommended for urinary tract infections. In folk medicine, an infusion from the leaves is used to ease rheumatic pain and gout and a decoction from the leaves and bark is applied as a skin tonic. The fresh sap, harvested in early spring, was recommended as a remedy for thinning hair

Leaves almost diamond-shaped

Habitat: Mixed and deciduous forests, rough grassland, heaths and moors, wasteland. Europe, western Asia

- ***deciduous***
- ***male catkins pendant***
- ***tiny seeds with 2 small, transparent wings on either side***

Crown, elongated, sparse

Male flowers yellowish-brown

Twigs thin, usually hanging

Branches mostly projecting from trunk at a sharp

Female flowers initially upright, later drooping

Male catkins 10cm long

3.3.5 CASTANEA SATIVA

English name: Sweet Chesnut, Family: Fagaceae

H. up to 30m
FS. June

Chestnuts are not just a popular stuffing ingrendient for the Christmas turkey, in the tree's native regions they are also known as a gentle household remedy for diarrhea. However, the real medicinal power is in the leaves, which are rich in tannins and are known in folk medicine as a remedy for coughs and sore throats (as a gargle), circulatory problems, aching legs and diarrhea.

Habitat: Native to south-western Asia and southern Europe

- ***Male flowers in long upright catkins***
- ***Female flowers below male ones***
- ***Very spiky fruit shell***

Health tip:

Chestnut leaf tea for respiratory complaints: use 2 tsp of finely chopped leaves per cup, add cold water and bring to the boil. Strain immediately and leave to cool. Drink 2-3 cups per day at intervals.

3.3.6 CERATONIA SILIQUA L.

English name: Carub, Locust Tree, Family: Fagaceae

The seeds of this tree have a constant weight of 0.18gr/the equivalent of 1 carat/and have in the past been used to assess the value of jewels. A flour extracted from the seeds is largely indigestible and is used as a bulking and gelling agent in baby and diet foods and pharmaceutical products. In some parts of the world, the seed shells are used as household remedy for diarrhea.

H. 2-10m
FS. July-September

Habitat: Maquis, rocky slopes. On nutrient-poor soils. Mediterranean region, western Asia, northern Africa

- ***evergreen***
- ***leaves even-pinnate***
- ***flowers grow directly from the branches***
- ***very distinctive due to late flowering season and prolific flowers***
- ***poisonous***

Smooth margin

Oval leaflet

Flowers without petals yellowish-white, up to 2cm in size

Flowers grow directly from the branches

Fruits

Seed pods up to 20cm long

3.3.7 CERCIS SILIQUASTRUM L.

English name: Judas Tree, Family: Fagaceae

H. 3-10m
FS. May

Generally with multiple stems

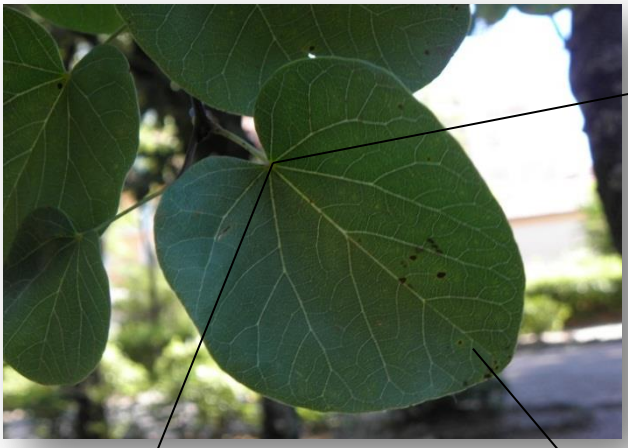
Crown broadly spreading, often irregular

Back in Biblical times, this tree ornamented the gardens of Judea and according to legend, Judas hanged himself from one. The flowers were supposedly white originally, taking on their pink hue after the crucifixion of Christ. The flowers can be used to garnish salads, but the fruits are inedible.

Pea like flowers up to 2cm long, pink

Habitat: Originally in the eastern Mediterranean. As an ornamental tree in southern and central Europe. Flowers in clusters on twigs and trunk

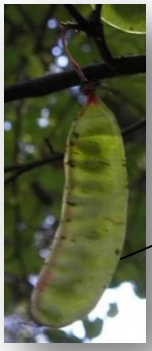
- ***Hardly but prefers mild locations***
- ***One of the most popular ornamental trees in southern Europe***
- ***Fruits on the tree in winter***



Leaf up to 11cm long, round to

Base heart-shaped

Rough, leathery, hairless

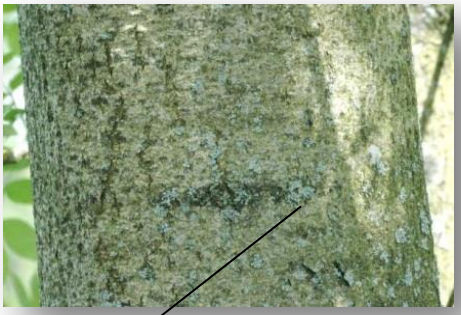


6-15cm long, flat, hard

3.3.8 FRAXINUS EXCELSIOR L.

English name: Common Ash, Family: Oleaceae

The leaves and bark of the Common Ash are used in folk medicine and homeopathy, though not in conventional medicine. The leaves contain tannins, mucilage and plant acids. They are taken in teas as a diuretic and gentle laxative, to relieve rheumatic complaints, gout and stones and are used to treat slow-healing wounds.



H. up to 40m
FS. April-May

Flowers in dense clusters

Habitat: Mixed and lowland forests, along rivers and streams, Europe, Asia Minor to Caucasus.

- ***deciduous***
- ***colonizing tree, also on dry ground***
- ***flowers appear before leaves***

Bunches of Ash keys

Ash fruits, known as 'keys'

Leaflet

Leaves odd-pinnate

3.3.9 FRAXINUS ORNUS L.

English name: Manna Ash, Family: Oleaceae

The Manna produced by this tree has nothing but its name in common with the Biblical manna. Nevertheless, this dried exudate from the bark was a popular delicacy in mediaeval times-imports of manna from Sicily is recorded in Venice as early as the 9th century. Its main ingredient is mannitol, a sugar alcohol, which is used as a gentle laxative and a sugar substitute for diabetics.

Habitat: Forest margins, hedgerows, lowland forests, along the banks of streams. On nutrient-rich soils. Europe, Asia Minor.

- *Green brunches angular with narrow cork wings*
- *Leaves opposite*

H. 5-15m
FS. May-June

Leaves odd-pinnate

Scented flowers

Fruits up to 4cm long, hang in bundles

Curiosity:

In early history the Greeks used to make lances and spears from the wood of this tree. Nemesis, the Greek Goddess of justice, carried a branch of the tree in her hand as a symbol of her fair decisions.

Manna from dried bark exudate

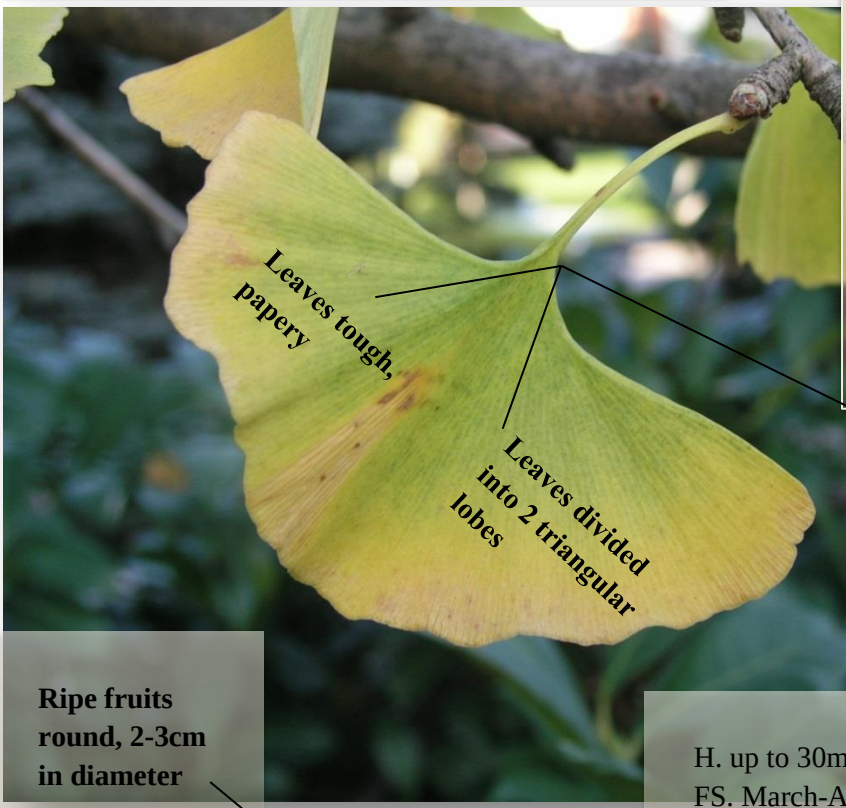
3.3.10 GINKGO BILOBA L.

English name: Ginkgo, Family: Ginkgoaceae

Ginkgo leaves are prescribed in Traditional Chinese Medicine for asthmatic complaints. In western herbal medicine, preparations from Ginkgo leaves are sold as a herbal supplement, said to stimulate brain activity. Studies have shown that Ginkgo improves blood flow to the brain but claims that this may assist in the treatment of Alzheimer's disease have yet to be scientifically proven.

Habitat: Native to China, grown worldwide as an ornamental tree.

- ***Deciduous, golden-yellow autumn color***
- ***Botanically related to conifers, despite being a deciduous, broad leaved tree***
- ***Male and female flowers on separate plants***



Distinctive, fan-shaped leaves

Ripe fruits round, 2-3cm in diameter

H. up to 30m
FS. March-April

Curiosity:

Ancestors of the Ginkgo, which were very similar to today's species, populated the Earth back in the Jurassic period. The Ginkgo is the sole survivor of this group, making it a living fossil. One Ginkgo tree even survived the atom bomb attack in Hiroshima and the tree has been a symbol of hope for the Japanese ever since.

3.3.11 Glycyrrhiza glabra

H. up to 30m
FS. March-April

English name: Liquorice, Family: Fagaceae

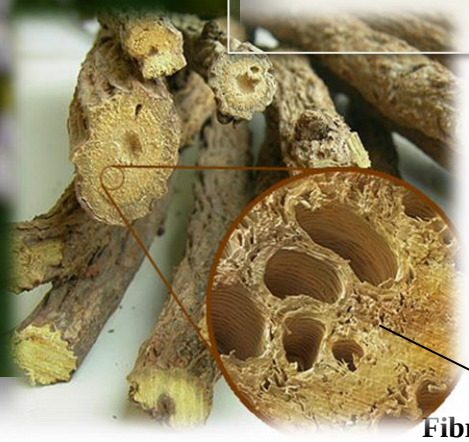
In addition to its use in confectionery, Liquorice root is applied medicinally for its expectorant and demulcent properties, e.g. in cough syrups and gastritis remedies. The root contains glycyrrhizin, a substance that is 50 times sweeter than sucrose and has long been used as a flavouring in other medicines to mask their unpleasant taste. It should be used in moderation, however, as prolonged use can raise blood pressure and affect hormonal balance.

Leaflets long, oval

Habitat: Eastern Mediterranean, South-Eastern Asia.

- ***Root yellow inside, sweet-tasting***
- ***Leaves pinnate***
- ***Leaflets long, oval, underside feels sticky***

Racemes 8-15cm tall



Flowers in tall, upright racemes

Fibrous root

3.3.12 Humulus lupulus

English name: Hops, Family: Cannabaceae

H. 200-400cm
FS. July-Aug

Only the unfertilized female plants are cultivated for beer-making. The bitter agents accumulated in their flower heads (known as 'cones' or 'strobiles') are responsible for the drink's taste. In herbal medicine, the plant is used as a mild sedative and sleep aid. Folk medicine recommends a tea made from Hops for loss of appetite and as a digestive. In mediaeval times it was used to curb sexual desire. Skin contact with the plant can cause dermatitis in some people.

Habitat: Lowland forests, forest margins, wasteland sites, on fences, mainly in cultivation. Throughout Europe, south-western Asia, North America

- ***Male and female flowers on separate plants***
- ***Climbing and trailing plant***

Upper leaves simple

Lower leaves with 3-5 lobes

Hop cones (female flowers) pendant

3.3.13 Ilex aquifolium C.

English name: Holly, English Holly Family: Aquifoliaceae

H. 1-6m
FS. May-June

The leaves of the Holly tree, not its poisonous berries, are the parts of most medicinal interest. They are used in homeopathic remedies for flue, conjunctivitis and other eye infections; folk medicine recommends them for fevers, rheumatic complaints and bronchitis; and in Bach flower remedies, Holly helps to overcome irritability, anger, envy and suspicion.

Male

Leaves dark green, shiny, leathery

Habitat: forests. In sandy or stony ground. Western and Central Europe., including Britain, from Norway to Germany south to the Mediterranean and North-Western Africa

- ***Male and female flowers on separate plants***
- ***Native, evergreen woodland plant***

Leaves evergreen with sharp, spiny margins

Bright red berries

Curiosity:

In some areas, believers still carry holly twigs to church on Palm Sunday to have them sanctified. At Christmas, springs of holly make popular evergreen decorations, particularly in Britain. They symbolize the continuation of life during winter.

3.3.14 Laurus nobilis L.

English name: Bay Laurel, Bay Tree Family: Lauraceae

H 2-10m
FS March-April

In Greek mythology, Bay-or Laurel-is sacred to Apollo and his son Asclepius, the gods of healing and medicine. It was held in high esteem as a medicinal plant throughout the Middle Ages and beyond. Nowadays, Bay leaves are mostly known as an aromatic flavoring in soups and stews. In folk medicine, Bay was used to treat stomach complaints and to ease rheumatic pain.

Habitat: Forests in the Mediterranean, in northern Europe as an ornamental garden plant.

- *Evergreen*
- *Male and female flowers on separate plants*

Leaf margins

Fruits fleshy, oval

Leaves stiff, leathery, lanceolate

Flowers yellow to white

Flowers in clusters

Curiosity:

Laurel wreaths crowned the heads of the victorious Julius Caesar and other Roman commanders. In Greece, successful athletes were also awarded this decoration. Later, students were honored with the 'baccalaureus', a laurel wreath with berries. The academic title of Bachelor was derived from this.

3.3.15 Lavandula angustifolia Mill.

English name: Common Lavender, Family: Lamiaceae

H 50-100cm
FS July-Sept

Anyone who has ever walked among the rows and rows of lavender in the French Provenance-and has seen the abundance of lavender-related products in the local souvenir shops-is unlikely to ever forget this plant. A tea from lavender flowers is said to stimulate the appetite, settle the stomach, calm the nerves and help promote sleep. Lavender,

Young leaves downy

Habitat: rocky ground, garigue. Native to southern Europe. Commonly grown as a garden ornamental shrub.

- ***Compact, aromatically scented shrub***
- ***Flowers in whorled spikes on long stalks, rising tal above the shrub itself***

Small labiate flowers, about 10-12mm long

Flowers in whorled terminal spikes

Health Tip:

For a soothing lavender bath, add 50-60gr of lavender flowers to 1l. of water. Bring to the boil, then leave to settle for 10min. Strain and add to your bathwater. Relax in the bath and go to bed afterwards.

3.3.16 Lavandula latifolia Medik.

English name: Spike Lavender, Family: Lamiaceae

H 50-100cm
FS July-Sept

The medicinal properties of Spike Lavender are similar to those of Common Lavender. Although the flowers yield more essential oil, it is considered of inferior quality and less fragrant than that of Common Lavender. It is used externally to treat wounds, burns and rheumatic pain; added to a steam inhalation bath it soothes catarrhs of the respiratory system; and a drop of Lavender oil on a spoonful of sugar is said to aid digestion.

Habitat: Evergreen shrubland, maquis. Southern Europe to Balkans

- ***Compact, low-growing shrub, strong, aromatic fragrance.***
- ***Bracts lanceolate***

Flowers in spikes

Small labiate flower, 8-10mm long

Underside of leaves downy

3.3.17 Olea europaea spp. europaea L.

English name: Olive Tree, Family: Oleaceae

H 6-15m
FS May-June

The Olive Tree has been known since biblical times. In Mediterranean folk medicine, the leaves are sometimes used in herbal teas. However, mostly the oil is used: as a lubricant, to relieve constipation or, mixed with garlic, to treat joint and muscle aches. In conventional medicine it is used as carrier oil for fat-soluble medications and in the treatment of skin conditions. Yet, above all, olive oil is valuable and cholesterol-lowering cooking oil. In Bach flower remedies, Olive is recommended for exhaustion and mental fatigue.

Habitat: Evergreen maquis, primarily in cultivation. Mediterranean region

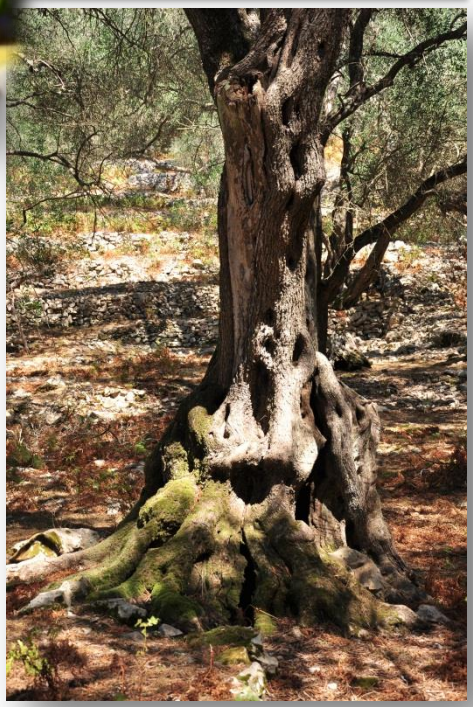
- ***Cultivated since antiquity***
- ***Evergreen***
- ***One of the healthiest oils for use in cooking and salads***

Flower with 4 lobes

Underside of leaves downy

Leaves evergreen

Olives first green then deep brown to black



Health Tip:

Olive oil vinaigrette: mix two finely chopped shallots with some mustard and garlic to taste. Add vinegar and season with salt and black pepper. Leave for a few minutes to allow the flavors to mingle and then add 50ml of good quality cold-pressed, extra-virgin olive oil.

3.3.18 Passiflora incarnatal L.

English name: Purple Passionflower, Family: Passifloraceae

H 8-10m
FS May-July

The name 'Passionflower' was coined by Christian missionaries who saw in its flowers the symbols of the passions of Christ: the five stamens and three styles symbolize the wounds and the nails, the double raw of colored filaments represents the crown of thorns, the tendrils are the whips and the lobed leaves resemble the clutching hands of the soldiers. The leaves and flowers are sold in herbal preparations and tea mixes to relieve nervous disorders.

Habitat: Central America, southern USA, Mexico, in Europe as a garden plant

- ***Ornamental flower with Passion-of-Christ symbolism***
- ***Climbing plant***

Flower pink or white, 3-5cm across

5 stamens

Corona of fine filaments

Leaves with 3 deep lobes

3.3.19 Pistacia lentiscus L.

English name: Mastic Tree, Family: Anacardiaceae

H 1-3m
FS March-June

The Greeks, on the island of Chios in particular cultivate tree-like specimens, to extract the Mastic resin. Mastic, the resin from this tree, is obtained from incisions made into the bark. It was once used as glue for plasters and false beards. Mastic is still used as a folk remedy for coughs, bronchitis, stomach complaints and diarrhea. The leaves are said to help lower blood pressure.

Habitat: Dry forests, maquis, garigue. Mediterranean regions

- ***Evergreen***
- ***Male and female flowers on separate plants***
- ***Black berries, 4mm in size***

Leaves leathery

Leaf rachis winged

Leaves even-pinnate

Small stone fruits, initially red, black when ripe

Male flowers reddish

Although Pistacia lentiscus L. is a common species in Mediterranean evergreen with sclerophyllous formations normally producing resin, mastic is secreted only from trees on the southern part of Chios, where large-scale mastic productions takes place. Many ancient writers, among them Dioscorides, referred to mastic gum, which was considered a panacea for many maladies. (Article of askitis!!!)

Imag. 4 *Hardened resin, flowed from the incisions, after 10-20 days*

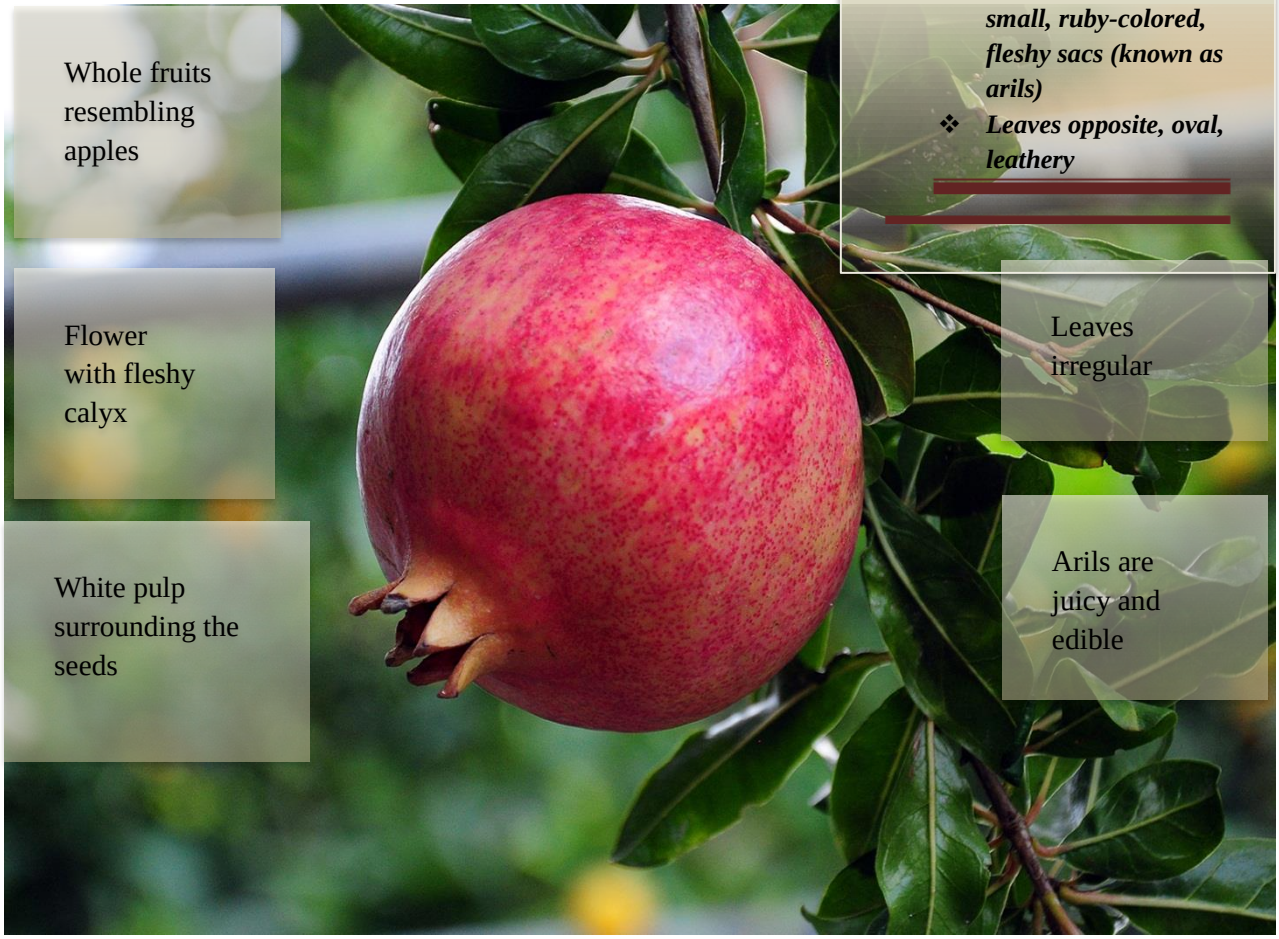
3.3.20 Punica granatum L.

English name: Pomegranate, Family: Punicaceae

The Pomegranate fruit has been a symbol of fertility since antiquity. The bark of the stem and roots contain up to 20% tannins. The plant also contains several alkaloids and one of them, isopelletierine, has a paralyzing effect on tapeworm. Pomegranate was therefore administered to that effect, followed by a laxative to flush out the parasites. However this method is no longer used in modern medicine, due to its dangerous side effects.

Habitat: Native to South-Western Asia; introduced in the Mediterranean.

- ***Seeds embedded in small, ruby-colored, fleshy sacs (known as arils)***
- ***Leaves opposite, oval, leathery***



3.3.21 Rosmarinus officinalis L.

English name: Rosemary, Family: Lamiaceae

Rosemary's essential oil is said to improve circulation, alleviate rheumatic and neuralgic pain, aid digestion, relieve flatulence and have antispasmodic properties. It is used as a tonic and mood-enhancer when feeling depressed, mentally tired or nervous. However, it should not be used as a herbal remedy when pregnant.

Habitat: Evergreen shrubland. Mediterranean. Commonly grown in gardens as ornamental shrub and medicinal or culinary herb.

- ***Evergreen, aromatically scented shrub***
- ***Leaves curled down along the sides***

Flower 10-12mm long

2 protruding stamens

Leaves needle - shaped

H 50-200cm
FS Jan-Dec

3.3.22 Sambucus nigra L.

English name: Elder, Family: Caprifoliaceae

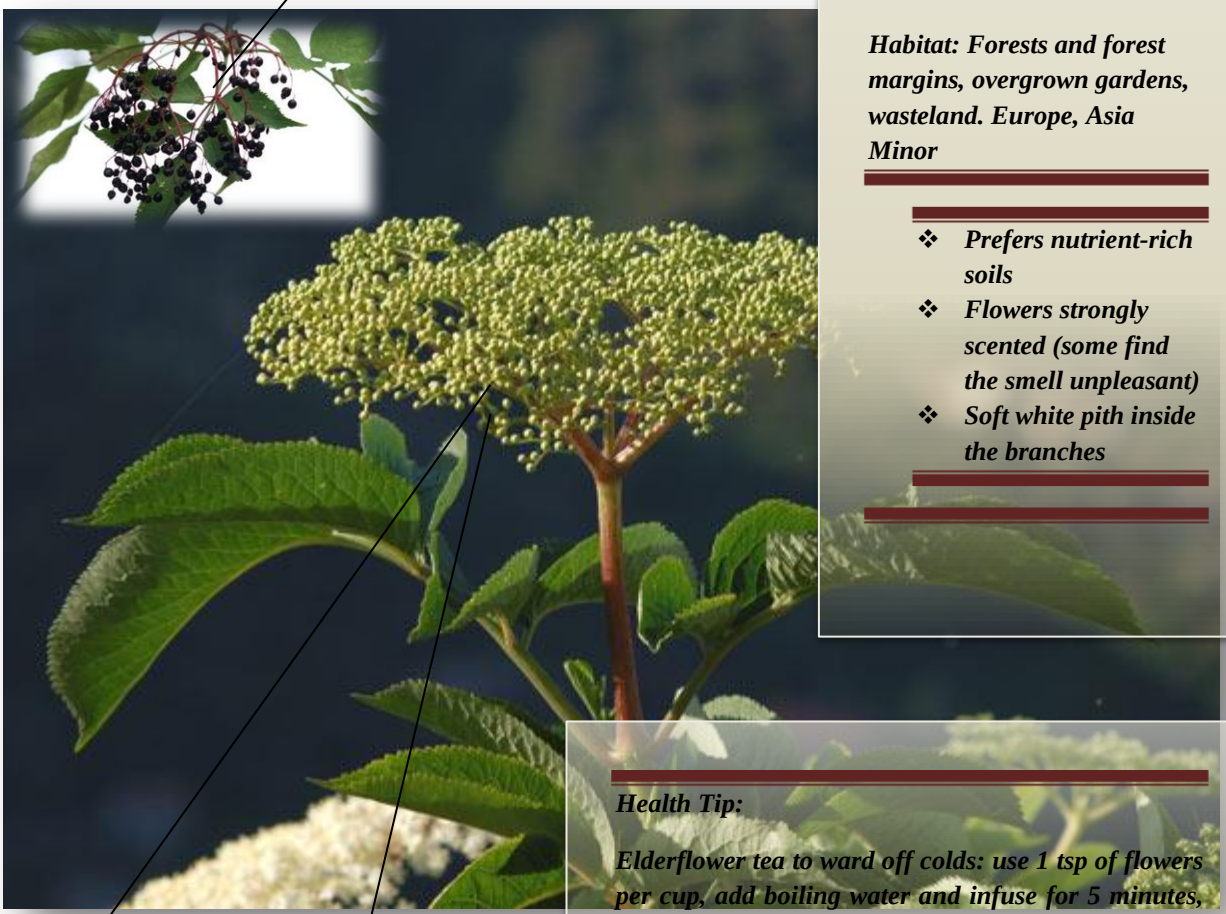
H 3-7m
FS June-July

An Elder bush used to be part of every country garden and was thought to ward off evil spirits and protect against witchcraft. Virtually all parts of the plant can be used medicinally. Elderflower tea has a diaphoretic action and is taken as a remedy for colds, as are the leaves. Elderberry juice is recommended for headaches, constipation and as a diuretic and diaphoretic. The root and bark are said to relieve rheumatic pain. According to the great mediaeval scholar Albertus Magnus, the bark, scraped off from the top down, acts as a laxative.

Habitat: Forests and forest margins, overgrown gardens, wasteland. Europe, Asia Minor

- ***Prefers nutrient-rich soils***
- ***Flowers strongly scented (some find the smell unpleasant)***
- ***Soft white pith inside the branches***

Elderberries
4-6mm in size



Flowers in a large, flat umbel

Umbels 10-25cm across

Health Tip:

Elderflower tea to ward off colds: use 1 tsp of flowers per cup, add boiling water and infuse for 5 minutes, then strain. Drink several cups throughout the day. To induce sweating, double the amount of flowers per cup and drink as hot as possible.

3.3.23 Vitex agnus castus L.

English name: Agnus Castus, Family: Lamiaceae

The plant is also known as Chaste Tree or Monk's Pepper, as its fruits used to be chewed by mediaeval monks in a bid to curb sexual desire. An extract from the fruits is used in modern herbal medicine for premenstrual tension, painful or irregular periods and to relieve the symptoms of the menopause. The leaves were used as a folk remedy against fever.

Leaves palmate

Habitat: Along riverbanks, on damp ground. Mediterranean to northern India

- ***deciduous***
- ***Flowers blue, violet or pink***
- ***Berries purplish-black, 5mm in diameter***

Petals 6-9mm long

Flowers in a branching spike

Fruits

H 1-6m
FS June-Nov

3.3.24 Ziziphus jujuba Mill.

English name: Jujube, Red date, Chinese date Family: Rhamnaceae

H 5–10 m
FS May-July

The plant's name is derived from Greek 'ζίζυφον', zizyfon and is well known for both its culinary and medicinal properties. The freshly harvested as well as the candied dried fruits are often eaten as a snack, or as a tea remedy. The fruits are used in Chinese and Korean traditional medicine, where they are believed to alleviate stress and traditionally for antifungal, antibacterial, antiulcer, anti-inflammatory, sedative, antispastic, antifertility, contraception, hypotensive and antinephritic, cardiotonic, antioxidant, immunostimulant, and wound healing properties. In Persian traditional medicine it is used in combination with other herbal medicines to treat colds, flu and coughing.

Habitat: Originated in southern Asia, introduced in southeastern Europe, an invasive species in Madagascar

- ***Flowers blue, violet or pink***
- ***Berries purplish-black, 5mm in diameter***

Leaves with 3 conspicuous veins at the base, and a finely toothed margin.

Fruit edible oval drupe 1.5–3cm

Leaves shiny-green, ovate-acute 1–3 cm long

Curiosity:

The jujube's sweet smell is believed to make teenagers fall in love, and as a result, in the Himalaya and Karakoram regions, boys take a stem of sweet-smelling jujube flowers with them or put it on their hats to attract girls. Moreover, in the traditional Chinese wedding ceremony, the jujube was often placed in the newlyweds' bedroom as a good luck charm for fertility, along with peanuts, longan, and chestnuts, punning on an invocation to "have an honored child soon".

Wolfgang 2008; Spohn M. & Spohn R. 2008

Medicinal Species	Pharmaceutical Name	Utlilization Part(s)	Uses	Medicinal application, use instructions and dosage	Family	Habitat	Cit
Aesculus hippocastaneum	Hippocastani cortex, flos, folium, siccum normatum, semen	Cork, leaves, seeds, flowers	Medicinal, Culinary Supplements, Herbal, Cosmetics	Venous insufficiency, varicose veins, oedema, antihaemorrhoidal	Sapindaceae	Native In Greece	WHO, Cat 1
Althaea officinalis	Althaea Folium, Althaea Radix	Leaves, roots	Medicinal, Culinary Supplements, Herbal, Cosmetics	Antitussive, gastro-intestinal complaints, immune boosting properties	Malvaceae	Native	WHO, Cat 1
Berberis vulgaris	Berberides cortex Berberides radix	Cork, roots, fruit	Medicinal, Herbal, Aromatics	Laxative, jaundice, digestive disorders, antidiarrhoeal, kidney stones, antirheumatism	Berberidaceae	Native	WHO, Cat 2
Betula pendula	Betulae folium	Leaves	Medicinal	Diuretic, urinary tract infections, antirheumatism, antiarthritics, skin tonic, remedy for hair thinning	Betulaceae	Native	REFIT, Cat.1
Castanea sativa	Castanea folium Castanea turiones recentes	Leaves, fresh gems	Medicinal, Herbal, Culinary Supplements	Antidiarrhoeal, Anti-inflammatory, Astringent, Expectorant	Fagaceae	Native	REFIT, Cat.2
Ceratonia siliqua	Algarrobo, Caroube, Carouge	Fruit, leaves, bark	Medicinal, Industrial	Astringent, Antitussive, Antidiarrhoeal	Fabaceae	Native	Kivcak et al. 2001
Cercis siliquastrum	Cercis siliquas-trum	Fresh gemes, bark	Medicinal	Antidiarrhoeal, Gastric Anti-inflammatory	Fabaceae	Native	Roberts 2000
Fraxinus excelsior	Fraxini folium Fraxini cortex Fraxini semen Fraxini turiones recentes	Leaves, bark	Medicinal	Diuretic, gentle laxative, antirheumatism, astringent	Oleaceae	Native	REFIT, Cat.2
Fraxinus ornus	Manna	Bark	Medicinal	Gentle laxative, sugar substitute for diabetics	Oleaceae	Native	REFIT, Cat.2
Ginkgo biloba	Ginkgo extractum siccum, Gingko Folium	Leaves	Medicinal, Herbal, Cosmetics	Antiasthmatic, impoves blood flow in the brain	Ginkgoaceae	Cultivated	WHO, Cat 1
Glycyrrhiza glabra	Liquiritiae radix	Roots, rhizomes, juices	Medicinal, Herbal	Expectorant, Demulcent	Fabaceae	Native	WHO, Cat 2
Humulus lupulus	Lupuli strobulus Lupuli flos, Lupuli herba	Cones (female inflorescences), flower, leaves	Medicinal, Culinary Supplements, Herbal, Cosmetics	Mild sedative,sleep aid, digestive, widely cultivated for beer-making	Cannabaceae	Native	WHO, Cat 2
Ilex aquifolium	Acebo, Agrifolio, Ailes de Chauve-Souris	Leaves, flowers	Medicinal, Herbal	Conjunctivitis, analgesic, antirheumatism, Anti-inflammatory, sedative	Aquifoliaceae	Native	Wolfgang 2008
Laurus nobilis	Lauri folium Lauri turiones Lauri fructus	Leaves,fruit	Medicinal	Gastric Anti-inflammatory, External Anti-inflammatory, Analgesic and skeletal muscle relaxant, Respiratory complaints	Lauraceae	Native	Camejo-Rodrigues et al., 2003
Lavandula officinalis	Aetheroleum lavandulae, flos lavandulae	Inflorescences	Medicinal, Industrial, Cosmetics, Herbal	Mild sedative, subtle sleep aid, aromatically scented widely used in cosmetics	Lamiaceae	Native	WHO, Cat 1
Olea europea	Oleae folium Oleae fructus Olivae oleum	Leaves,fruit,tender buds, fruit juice	Cosmetics Medicinal, Culinary Supplements	Antihypertensive, Antidiarrhoeal, Anthelminthic, For burns, Laxative, Antihaemorrhoidal	Oleaceae	Native	Montilla et al. 2003
Passiflora incarnata	Passiflorae Herba	Leaves, Flowers	Medicinal, Culinary	Sedative, Skeletal muscle relaxant	Passifloraceae	Cultivated	WHO, Cat 2

			Supplements, Herbal, Cosmetics				
Pistacia lentiscus	Mastic tree, Lentisc	Bark (resin, Mastic), Leaves	Medicinal, Culinary Supplements, Industrial, Herbal	Anti-inflammatory, Analgesic, Gastric Anti-inflammatory, Antidiarrhoeal, Antihypertensive	Anacardiaceae	Native	Lev & Ama, 2002, 2008; Al-Habal et al., I984
Punica granatum	Cortex granati, Pericarpium granati	Bark, Stem, Roots	Medicinal, Culinary Supplements, Herbal, Cosmetics	Sedative, Laxative, Anthelminthic	Punicaceae	Cultivated	WHO, Cat 2
Rosmarinus officinalis	Aetheroleum rosmarini, flos rosmarini	Epigeal parts	Medicinal, Industrial, Cosmetics	Vasotonic, Digestive, Anticatarrhal, Antihypertensive, Gastric Anti-inflammatory, Anti-inflammatory	Lamiaceae	Native	WHO, Cat 2
Sambucus nigra	Sambucus flos	Leaves, flowers, cork, branches	Medicinal, Herbal, Aromatics, Cosmetics	Gastric Anti-inflammatory, External Anti-inflammatory, Skeleto-muscular disorders, Headache, Toothache, Analgesic	Caprifoliaceae	Native	WHO, Cat 2
Vitex agnus castus	Fructus Agni Casti	Leaves, gems, fruit, roots	Medicinal, Herbal	Premenstrual tention, Analgesic, Relieve symptoms of the menopause, Antipyretic	Lamiaceae	Native	WHO, Cat 1
Ziziphus jujube	Fructus Ziziphy	Mature fruit	Medicinal, Culinary Supplements	Antipyretic, diuretic, emmenagogue, expectorant, sedative and tonic, Treatment of asthma, bronchitis, diabetes, eye diseases, inflammatory skin conditions, liver disorders, scabies, ulcers and wounds	Rhamnaceae	Native	WHO, Cat 2

Tab. 2 *Medicinal properties of the analyzed plant species*

4. Molecular technology as a reliable tool towards the generation of universal standars.

4.1 DNA barcoding, a powerful tool for species identification

Term: DNA barcoding is a technique for species identification, carried on by DNA sequences from a short segment of the genome, with the intention of advocating a broad range of environmental and conservation data in which conventional taxonomic classification is not relevant or applicable (Eby J. et al. 2011).

The primary goals of DNA barcoding are species identification of known specimens and discovery of overlooked species for enhancing taxonomy for the benefit of science and society (Kress and Erickson 2008). Using barcoding, a species can be identified from a tiny amount of tissue, from seeds or from sterile, juvenile or fragmentary materials when morphological identification is difficult or even impossible. (Valentini et al. 2008). DNA barcodes will aid the detection, monitoring and management of biodiversity, which is currently threatened by climate change and other human impacts.

4.2 Selection of barcode locus

However, the selection of a barcode locus is complicated by the trade-off that arises between the need for universal application and maximal rates of sequences divergence (Kress WJ et al. 2005).

The crucial criteria for barcode loci are universality, good sequence quality and coverage and species discrimination. (Erickson et al. 2008; Kress and Erickson 2008). Ideally, the barcode region would be routinely retrievable with a single primer pair, sufficiently variable to obtain maximum discrimination among species and universally applicable so that even a sample with no morphological characters can be identified. The usability of DNA barcoding was first demonstrated in animals. A portion of the mitochondrial coxI (cytochrome c oxidase subunit 1) gene was largely applied as DNA barcode sequence for animal identification (Hebert P.D.N. et al. 2003) but in land plants, this sequence is highly invariant and therefore unsuitable for use as DNA barcodes.

The difficulty of finding a single-locus barcode has suggested a multilocus approach, focusing on the plastid genome as currently the most effective strategy for barcoding plant species (see Hollingsworth et al. 2009, and citations therein), although there is still much debate concerning the most suitable regions to be used.

From the broad pool of loci recently considered (Kress et al. 2005; Shaw et al. 2007; Chase et al. 2007; Newmaster et al. 2008), the greatest interest was aroused by three candidate loci: rbcL (an easy-to-align coding region), a section of matK (a rapidly evolving coding region), and trnH-psbA, (one rapidly evolving intergenic spacer). Based on the relative ease of amplification, sequencing, multialignment, and on the amount of variation displayed (sufficient to discriminate among sister species without affecting their correct assignation through infra-specific variation), many research groups have proposed different combinations of these loci (Kress & Erickson 2007; Lahaye et al. 2008; Fazekas et al. 2008; Hollingsworth et al. 2009; CBOL Plant Working Group 2009).

Various biological contexts (e.g., sampling strategies) have been used to compare the performance of plant barcoding loci. A sound assessment of the universality of regions is usually given by the 'species pairs' and 'floristic' approaches. The former involves analyzing pairs of related species from multiple phylogenetically divergent genera and may be defined as a 'methodological' protocol; the latter involves sampling multiple species within a given geographical area and represents an example of how barcoding might be applied in practice. However, only limited insights into species-level resolution are usually provided by both approaches as individual genera are not sampled in depth to provide the estimates of barcode efficacy to inter-specific discrimination. Conversely, a third method, the 'taxon-based' approach, involves sampling multiple species within a given taxonomic group. This provides limited insights into universality but offers more definitive information on discrimination power at species level.

To date, the species pairs (e.g. Kress et al. 2005; Kress& Erickson 2007), floristic (e.g. Fazekas et al. 2008; Lahaye et al. 2008; Gonzalez et al. 2009), and taxon-based (e.g. Newmaster et al. 2008; Newmaster & Ragupathy 2009) approaches have all provided useful insights into the potential performance of varying combinations of barcoding loci. The most frequently recommended combinations for broad future applications appear to be the following: rbcL + trnH-psbA (Kress & Erickson 2007), matK + trnH-psbA (Newmaster et al. 2008; Lahaye et al. 2008), rbcL + trnH-psbA + matK or rbcL + matK (Hollingsworth et al. 2009) until the CBOL Plant Working Group (2009) proposed rbcL + matK as the most convenient combination in terms of universality, sequence quality and discrimination power. Nevertheless, Fazekas et al. (2008) argued that 'regardless of the region(s) ultimately adopted for plant barcoding, there will always be some species that would be better resolved by some other region', as even the worst performing region in a data set provided informative characters for some species for which the best performing region did not.

In the present doctorate thesis, for the first time, it has been tested the universal application of barcoding in medicinal and aromatic plants in Mediterranean forests of Italy and Greece. The

objectives of the current study is (i) to provide a test for future in situ application of DNA Barcodes by evaluating the efficacy of species discrimination with the use of three plastid regions (trnH-psbA, rbcL, matK), under the criteria of methods and natural co-occurrence of the species in a real ecosystem and (ii) to examine the main potential drawbacks of barcoding certain taxonomic groups (medicinal & aromatic plants) which may prove exceptionally challenging because of intrinsic bio-ecological factors.

4.3 Characteristics of the selected barcode locus

The matK region, about 800bp in size, is one of the most rapidly evolving regions in chloroplasts and often provides high resolution, which means good species identification (Fazekas et al. 2008, Lahaye et al. 2008). Although matK region is useful to determine species identity and the geographical origin of medicinal and aromatic plants, appears several disadvantages such as unavailability of universal primers suitable for all taxa and technical difficulties due to low PCR amplification success (especially outside angiosperms) (CBOL Plant working group 2009).

Additionally the rbcl region is shorter, about 500bp in size and its use has several advantages. Firstly the availability of standard universal primers, secondly, high amplification success rate in PCR in a broad range of flowering plant, gymnosperm and cryptogam species and last but not least, excellent quality of sequences. However, its species discrimination power, in contrast to matK, is less. In general the rbcl barcode provides the path into a family, genus and sometimes also into species (Kress et al. 2005).

Nevertheless, the plastid region trnH-psbA, (Lahaye et al. 2008, Hollingsworth et al. 2009), its size ranges between 340-660bp, shows the highest amplification success and discrimination rate among the other two loci (Kress et al. 2005, Kress&Erickson, 2007). Therefore, this intergenic spacer appears to be a useful region for the differentiation of medicinal and aromatic plants from their adulterants. Although its high discrimination power, one serious disadvantage of trnH-psbA is that it does not generate bidirectional unambiguous sequences (CBOL Plant Working Group, 2009). The presence of a poly-A/T structure in a region of this intergenic spacer reduces the success rate of DNA sequencing (Zhu et al., 2010). In addition, nucleotide insertions and deletions are frequently found in this region, making sequence alignment difficult such that manual editing of sequence traces is preferred. (Zhu et al.., 2010).

II. Materials and Methods

1. Plant material

More than 100 specimens were sampled through Italy and Greece, in different biogeographic regions (three provenances from Italy and one from Greece) of each country (Tab.3). They were identified directly in the field and taxa were used to assemble a herbarium. Green tissues were lyophilized, and stored at the DNA Bank of Forest trees. The relative efficacy of the three most widely used barcoding markers trnH-psbA, rbcL and matK is being evaluated by comparing inter- and intra- specific sequence diversity. So far, more than 38 medicinal and aromatic plant species have been collected and being analyzed derived from 22 genera, most of them collected from at least 4 different regions through Italy and Greece (some representatives from Western Mediterranean were included for comparison) with a total number of 30 different provenances belonging to 17 families. Most of the specimens were collected in the wild. Due to the significant quantity as well as the rarity of the plant samples some major institutes (ETHIAGE/Greece), botanical gardens (Viterbo, Padova, Pisa, U.S.A., Canada, Finland) and national parks (Parco Nazionale Colli Euganei di Padova) respond to our call providing a crucially important assistance to our demand for samples.

Imag. 5 *Samples collection in botanical garden of Padova, Italy*

Imag. 6 *Samples collection in botanical garden of Ethiage, Thessaloniki, Greece*

Family	Genus	Species	Origin
Anacardiaceae	**Pistacia**	terebinthus L.	Greece
		terebinthus L.	Minorca, Spain
Aquifoliaceae	**Ilex**	aquifolium L.	Botanic. Garden PA
		aquifolium L.	Sardinia, Italy
		aquifolium L.	Latium (C. Italy)
		aquifolium Loud. var. Marginata	Botanic. Garden PA
		latifolia Thunb.	Botanic. Garden PA
Berberidaceae	**Berberis**	vulgaris L.	Botanic. Garden PA
		vulgaris L.	Botanic. Garden VT
		vulgaris L.	Trento
		aristata DC.	Botanic. Garden PA
Betulaceae	**Betula**	pendula Roth.	Greece
Cannabaceae	**Humulus**	lupulus L.	Umbria (C. Italy)
		lupulus L.	Botanic. Garden PA
Caprifoliaceae	**Sambucus**	nigra L.	Umbria (C. Italy)
		nigra L.	Umbria (C. Italy)
		nigra L.	Sardinia, Italy
		nigra L.	Veneto (NE Italy)
		nigra L.	Greece
		ebulus L.	Latium (C. Italy)
		ebulus L.	Veneto (NE Italy)
		racemosa L.	Botanic. Garden PA
Fabaceae	**Ceratonia**	siliqua L.	Greece
		siliqua L.	Malta
		siliqua L.	Puglia
	Cercis	siliquastrum L.	Abruzzo (SE Italy)
		siliquastrum L.	Botanic. Garden PA
		siliquastrum L.	Greece
Fagaceae	**Castanea**	sativa Mill.	Latium (C Italy)
		sativa Mill.	Greece
	Glycyrrhiza	glabra L.	Botanic. Garden PA
		echinata L.	Botanic. Garden PA
		echinata L.	Botanic. Garden VT
Ginkgoaceae	**Ginkgo**	biloba L.	Botanic. Garden PA
		biloba L.	Latium (C. Italy)
Lamiaceae	**Lavandula**	angustifolia Mill.	Botanic. Garden PA
		angustifolia Mill.	Greece
		angustifolia Mill.	Latium (C Italy)
		angustifolia Mill.	Sicily (S Italy)
		dentata L.	Greece
		dentata L.	Botanic. Garden PA
		latifolia Medik.	Latium (C Italy)
		latifolia Medik.	Botanic. garden PS
		stoechas L.	Greece
		stoechas L.	Botanic. Garden PA
	Vitex	agnus-castus L.	Greece
		agnus-castus L.	Botanic. Garden PA
		agnus-castus L.	Botanic. Garden VT
		glabrata R.Br.	Botanic. Garden PA
	Rosmarinus	officinalis L.	Greece
		officinalis L.	Sardinia, Italy
		officinalis L.	Botanic. Garden PA
Lauraceae	**Laurus**	nobilis L.	Latium (C. Italy)
		nobilis L.	Botanic. Garden PA

Malvaceae	**Althaea**	officinalis L.	Greece
		officinalis L.	Botanic. Garden PA
		officinalis L.	Botanic. Garden VT
		cannabina L.	Botanic. Garden PA
Oleaceae	**Fraxinus**	ornus L.	Greece
		ornus L.	Apulia (S. Italy)
		ornus L.	Marche (CE Italy)
		ornus L.	Botanic. Garden PA
		ornus L.	Latium (C. Italy)
		angustifolia Vahl	Latium (C. Italy)
		angustifolia Vahl	Sardinia, Italy
		angustifolia M.Bieb. ex Willd. subsp. Oxycarpa	Botanic. Garden PA
		excelsior L.	Botanic. Garden PS
	Olea	europaea L.	Greece
		europaea L.	Minorca, Spain
		europaea L.	Sardinia, Italy
		europaea L.	Sicily (S. Italy)
		europaea L.	Botanic. Garden PA
Passifloraceae	**Passiflora**	incarnata L.	Botanic. Garden PA
		incarnata L.	Latium (C. Italy)
		edulis Sims, 1818	Botanic. Garden PA
		edulis Sims, 1819	Botanic. Garden FIN
Punicaceae	**Punica**	granatum L.	Latium (C. Italy)
		granatum L.	Sardinia, Italy
		granatum L.	Latium (C. Italy)
		granatum L.	Apulia (S. Italy)
Rhamnaceae	**Ziziphus**	jujuba Mill.	Latium (C. Italy)
		jujuba Mill.	Botanic. Garden PA
		jujuba Mill.	Greece, Itea
Sapindaceae	**Aesculus**	hippocastaneum L.	Latium (WS Italy)
		hippocastaneum L.	Botanic Garden PA
		hippocastaneum L.	Greece, Lidoriki
		indica (Wall. ex Camb.) Hook.f.)	Botanic. Garden PA
		indica (Wall. ex Camb.) Hook.f.)	Botanic. Garden USA
		indica (Wall. ex Camb.) Hook.f.)	Botanic. Garden CA

Tab. 3 *List of analyzed specimens divided in different families*

2. DNA Extraction, Polymerase Chain Reaction (PCR), Amplification and Sequencing

DNA extraction was performed on all the collected samples. Unfortunately, in some particular plant groups (e.g. Pistacia lentiscus L. etc.) we did not manage to obtain DNA, using a variety of different protocols, probably, due to co-extraction of high quantities of tannins, polyphenols and polysaccharides (Shepherd et al., 2002). Nevertheless, we managed to claim more than 250 DNA sequences of different taxa so far.

DNA extractions were performed with the DNeasy Plant Minikit (QIAGEN), following the manufacturer's instructions. The universal applicability of the technical analyses was considered a prerequisite for exploring the barcoding potential in a practical floristic case study. Uniform PCR procedures were performed for all taxa and barcoding loci. DNAs (ca. 40 ng) were amplified with RTG PCR beads (GE Healthcare) in 25 µl final volume according to the manufacturer's protocol. . Thermocycling conditions were as follows: 94 C for 3 min, followed by 35 cycles of 94 C for 30 s, 53 C for 40 s and 72 C for 40 s, with a final extension step of 10 min at 72 C. Primers for the investigated barcoding region are shown in Tab. 4. PCR products were cleaned with Illustra DNA and Gel Band Purification Kit (GE Healthcare) and eluted in 30 µl type 6 elution buffer. A part of the standardized aliquots were then submitted to Eurofins MWG Operon (http://www.eurofinsdna.com) for sequencing and the rest to Macrogen Inc. (http://www.macrogen.com/eng/macrogen/macrogen_main.jsp). Electropherograms were edited with CHROMAS 2.01 (http:// www.technelysium.com.au) and checked visually.

Marker region	Primers	Reference
MatKim	CGTACAGTACTTTTGTGTTTACGAG	*(Kim unpublished)*
	ACCCAGTCCATCTAAATCTTGGTTC	
rbcl	ATGTCACCACAAACAGAAAC	
	TCGCATGTACCTGCAGTAGC	*(Kress et al. 2005)*
trnh-psbA	CGCGCATGGTGGATTCACAATCC	
	GTTATGCATGAACGTAATGCTC	*(Shaw et al. 2007)*

Tab. 4 *List of primers used for amplification of the three candidate DNA barcoding markers*

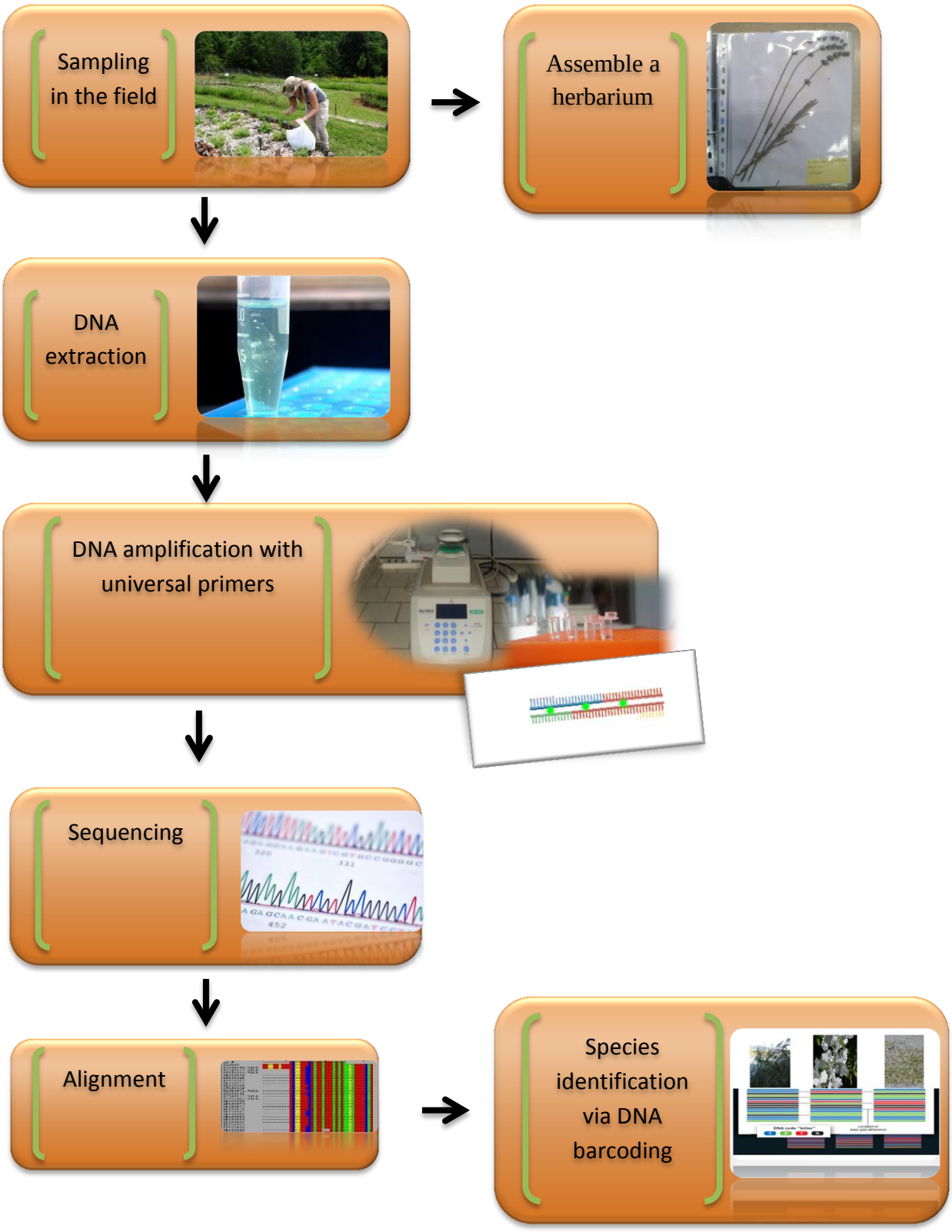


Fig. 2 *Methodology of medicinal plant species identification via DNA barcoding.*

3. Data analysis

The obtained sequences were analyzed with differing approaches to explore intra and interspecific divergence and discrimination failure or success in global dataset of each taxonomic group.

- *Haplotypes with BLASTClust:* This approach gives an estimate of observed variability within dataset without multiple sequence alignment using different levels of the stringency. In particular BLASTClust was performed (http://toolkit.tuebingen.mpg.de/blastclust) with sequence length covered 100% and percent identity threshold 100% to cluster unaligned identical sequences and to evaluate if, in my dataset, sequences from different species are identical, highlighting problematic limitation for discrimination ability (Tab.6).
- *Presence of barcoding gap:* According to the proposal of CBOL Plant Working Group (2009), species discrimination is successful when the minimum uncorrected interspecific p-distance in a species group is higher than the maximum intraspecific distance within each species. Species discrimination power of the investigated loci was thus calculated using the distance approach with the uncorrected p-distance; it was also calculated the Kimura2-parameter (K2P) genetic distances for comparison. The sequence divergences between and within species were calculated for each barcode locus with MEGA 5.0 (Tamura et al. 2007) (Fig.5-8).
- *Species monophyly with supermatrix approach*: This "tree-based" approach is based on support of species monophyly (Fazekas et al. 2008), however the primary purpose of this analysis being not to draw evolutionary relationships but merely to detect species identification by verifying that different accessions (or provenances) of the same species formed a well-supported single clade, representative of the specific taxon. The analysis was performed as described in Kress et al. 2009 that overcome the limitations when phylogenetic reconstruction includes many divergent taxa, and that combining a coding sequence, that may be globally aligned, with the non-coding sequences that are aligned in nested groups. Sequences were aligned with MAFFT method (*multiple sequence alignment based on fast Fourier transform*) (http://toolkit.tuebingen.mpg.de/mafft) under default parameters; alignments were visualized and checked with SeaView (Galtier et al. 1996). Sequences of the rbcL and matK were unambiguously aligned with each other in a global alignment. TrnH-psbA sequences were partitioned by genera and then manually built various separate taxonomically structured sequence files. Finally *supermatrix* algorithms in R-package Phylotools (Zhang et al. 2010), automatically distributes sequences into nested sub-blocks of homologous sequence alignment (Fig.9). The generated supermatrix was used as imput in

RaxML (Stamatakis et al. 2008) to reconstruct a tree based approach. The locus combination was partitioned for independent model assessment of each marker; 1,000 bootstrap replicates were performed to estimate clade robustness with a minimum collapse bootstrap of 70.

- *Assessment discrimination using GenBank:* It was finally used GenBank database to provide an additional test of discrimination ability and a practical application of Barcode. GenBank (http://www.ncbi.nlm.nih.gov), is a comprehensive global database, that contains publicly available nucleotide sequences for almost 260.000 formally described species, during December 2012. Before the discrimination assessment, the database was screened for the presence of the marker sequences at the species and/or genus level relatively to my dataset, using the NCBI Taxonomy database (http://www.ncbi.nlm.nih.gov/taxonomy). The ability to discriminate of every single marker was evaluated in GenBank using megaBLAST algorithm (http://blast.ncbi.nlm.nih.gov) with default parameters and adjusted to retrieve 5.000 sequences. A query sequence was considered as successfully discriminated if the top Bit-score obtained in GenBank matched the name of the species. When more than one species shared a top Bit-Score or the species scored lower, the result was considered an identification failure (Tab.7-10).

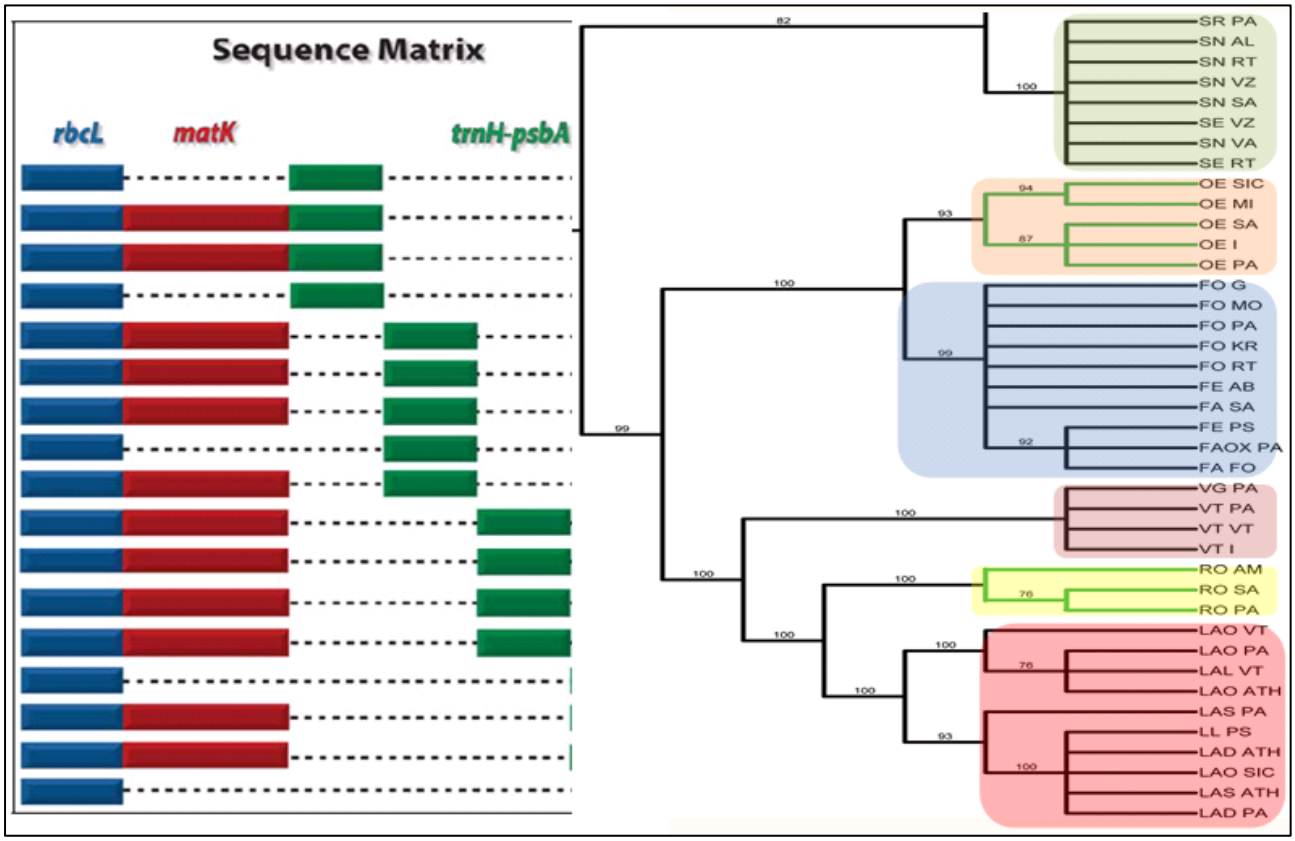


Fig. 3 *Schematic representation of species monophyly with supermatrix approach.*

	MatK	Rbcl	Trnh-psbA
Investigated Species (N)	**91**	**89**	**89**
% PCR Success	**91%**	**100%**	**98%**
% Sequencing Success	**87%**	**98%**	**94%**
Sequence length (range)	**797-817**	**688**	**277-648**
Number of Sequences obtained (S)	**72**	**87**	**82**
Number of Haplotypes (H)	**38**	**46**	**57**
Discrimination Success (H/S)	**53%**	**53%**	**70%**
Overall marker Efficiency (H/N)	**42%**	**52%**	**64%**

Tab. 5 *Markers' main features and discrimination ability within the investigated dataset.*

III. Results

1. Sequencing characterization

The investigated barcoding regions displayed different levels of data recovery (Tab. 5). Each of the three barcoding loci was successfully amplified using standard primer pairs and PCR protocols. Rbcl showed the highest amplification level (100%) among the three primers, followed by trnh-psbA (98%) and MatK (91%). Trnh-psbA failed only in the case of *Althaea officinalis* L. (Botanical Garden of Padova) and *Cercis siliquastrum* L. (Abruzzo, Italy) whereas MatK didn't amplify 8 plant samples including the whole group of Berberis species (3 *Berberis vulgaris* L. individuals and 1 *Berberis aristata* DC.), all the *Cercis siliquastrum* L. group (3 specimens) and *Punica granatum* L. from South Italy (Monopoli).

Concerning the DNA sequences success, a high number of sequences were managed to be obtained; 87 rbcl DNA sequences were achieved from a total number of 89, (98%), succeeded by 82 of trnh-psbA (94%) and 72 MatK sequences (87%). Regarding the results of rbcl primer, only two plant individuals didn't manage to be sequenced; *Aesculus hippocastaneum* L. from Central Greece and *Glycyrrhiza echinata* L. from Central Italy (Viterbo). On the other hand, trnh-psbA failed in 5 species; *Berberis vulgaris* L. (Botanical Garden of Viterbo), *Glycyrrhiza echinata* L. (Botanical Garden of Viterbo) as well, *Glycyrrhiza glabra* L. (Botanical Garden of Padova), *Passiflora edulis* Sims, 1818 (Botanical Garden of Helsinki, Finland)and *Ziziphus jujuba* Mill. from Greece. Finally, MatK, couldn't evaluate the sequences of 11 individuals; *Aesulus indica* (Wall. ex Camb.) Hook.f. (Botanical Garden of Vancouver, Canada), *Althaea cannabina* L. (Botanical Garden of Padova), 3 samples of Lavandula group, *Lavandula dentata* L. and 2 species of *Lavandula angustifolia* Mill. collected from Greece, Sicily and Botanical Garden of Padova respectively and *Rosmarinus officinalis* L. from Greece. In addition, it failed in the whole group of *Ginkgo biloba* L. (2 representatives) as well as in the one of Vitex genus; 2 *Vitex agnus-castus* L. from Botanical Gardens of Viterbo and Padova and *Vitex glabrata* R.Br. also collected from the latest. In total, 241 cpDNA sequences were obtained from the three tested loci.

Electropherograms revealed full overlap between complementary sequences among the three primer pairs across all taxa. Overall, sequence quality for the rbcl marker was high: 100% of sequence reads were full length followed by MatKim primer, with high enough quality of sequences recovery, except from the case of Lavandula genera, where in some of the tested species it failed, due to the dense, background noise making it impossible to be read. Finally, concerning the case of trnH-psbA primer, it demonstrated the poorest DNA sequence quality in respect to the other two loci. As many authors have mentioned so far, the presence of a poly-A/T structure in a region of this intergenic spacer is pretty common and as a result it reduces the success rate of DNA sequencing (Armenise et al. 2012, Zhu et al., 2010). Here, this phenomenon was observed intensely in the Sambucus species (Fig. 4), thus the entire sequence was completed from conjoining partial bidirectional reads (Kress&Erickson 2007).

The multialignment of the two coding sequences (rbcL and MatKim) was straightforward. All rbcl sequences were 688 bp long. In contrast, matK and trnH-psbA sequences were more various, due to the presence of numerous insertion/deletions, and multialignment was achieved only after manual adjustment. Length range of the matK sequences was from 797 bp, in the case of *Althaea officinalis* L. specimens, to 817 bpm in the case of *Pistacia terebinthus* L. individuals. Regarding to the sequences obtained with trnH-psbA, due to their high diversity, it was decided to be separated in different plant groups according to their genera. Thus, each group was multialigned separately and the sequences length ranged from 277 bp (*Ginkgo biloba* L.) to 648 bp (*Pistacia terebinthus* L.).

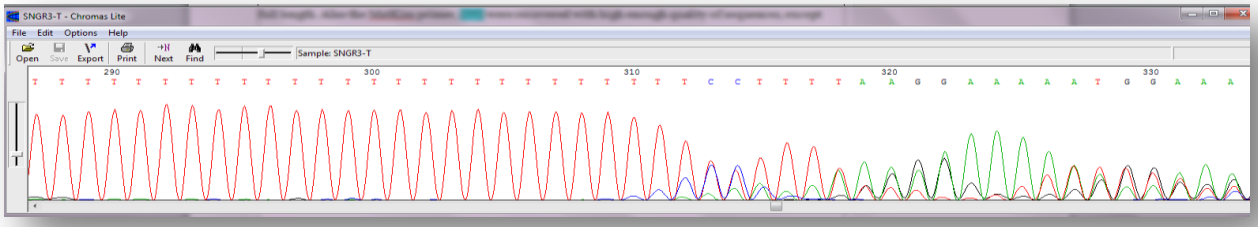


Fig. 4 *Presence of a poly T structure in trnH-psbA marker in a Sambucus species causing the degradation of the remaining sequence.*

2. Markers' discrimination ability

The alignment–free method implemented in BLUST Clust produced haplotypes from the sequences obtained with each marker. TrnH-psbA displayed the highest discrimination success 70% as it generated 57 haplotypes from 82 DNA sequences. It failed in discriminating the genera of; Ilex (*I. aqustifolium* L., *I. latifolia* Medik.), Lavandula (*L. angustifolia* Miller, *L. dentata* L., *L. stoechas* L., *L. latifolia* Medik.), Vitex (*Vitex agnus-castus* L., *Vitex glabrata* R.Br.) and two of the three tested species of Fraxinus genus (*F. angustifolia* Vahl, 1804 and *F. excelsior* L.). In addition, both rbcl and MatK demonstrated 53% of discrimination power, generating 46 haplotypes, deriving from 87 DNA sequences and 38 haplotype from 72 DNA sequences respectively. Rbcl locus failed in distinguishing; Fraxinus genus (*F. angustifolia* Vahl, 1804, *F. excelsior* L. and *F. ornus* L.), Lavandula genus (*L. angustifolia* Miller, *L. dentata* L., *L. stoechas* L. and *L. latifolia* Medik.), two of the three analyzed species of Sambucus genus (*S. nigra* L. and *S. ebulus* L.), Vitex genus (*Vitex agnus-castus* L., *Vitex glabrata* R.Br.) and finally Althaea genus (*A.officinalis* L. and *A. cannabina* L.). Moreover, MatK failed in resolving the genera of; *Sambucus* (*S. nigra* L. and *S. ebulus* L.) two of the three representative species tested in this study, Fraxinus (*F. angustifolia* Vahl, 1804 and *F. excelsior* L.), Ilex (*I. aqustifolium* L., *I. latifolia* Medik.) and finally in two of the four tested species of Lavandula genus (*L. latifolia* Medik. and *L. angustifolia* Miller).

On the other hand, multiregion combination of the three tested loci, rbcl + Matk + trnh-psbA, (Tab.6) produced 75 total haplotypes, considerable higher discrimination power regarding to the number of haplotypes that each marker generated. Focusing on a more detailed evaluation within the three analyzed

markers, it can be assumed that; all three loci separately and in combination failed to distinguish 2 species of Fraxinus genus (*F. angustifolia* Vahl, 1804 and *F. excelsior* L.) while in all the tested cases apart the one from rbcl, the third analyzed species of Oleaceae family, F. ornus was perfectly separated from its congeneric species. Moreover, Ilex, (*I. aqustifolium* L., *I. latifolia* Medik.), Lavandula (*L. angustifolia* Miller, *L. dentata* L.,) except MatK that just mismatched *L. angustifolia* Miller with *L. latifolia* Medik. and Vitex genera (*Vitex agnus-castus* L., *Vitex glabrata* R.Br.) weren't separated in any case. Regarding the case of Lavandula species, only MatK locus was able to discriminate *L. dentata* L. totally and contributed partially to the *L. stoechas* L. classification in the locus combination. On the other hand, Sambucus species were perfectly separated, producing 7 haplotypes; 2 species of Sambucus nigra, both from Italy, shared the same haplotype whereas the others; 3 more *S. nigra* L., 2 *S. ebulus* L. and 1 *S. racemosa* L. individuals produced 6 different haplotypes respectively. Furthermore, 3 *Althaea officinalis* L. individuals were distinguished by *Althaea cannabina* L. species, something that it wasn't achieved by rbcl locus. Also, *Berberis vulgaris* L. species were successfully resolved from *Berberis aristata* DC., generating 4 different haplotypes (as the number of the tested individuals) as well as *Passiflora incarnata* L. from *Passiflora edulis* Sims produced 4 different haplotypes (as the number of the tested samples) respectively. Finally, Aesculus spp. (*Aesculus hippocastaneum* L., from *Aesculus indica* (Camb.) Hook.) were efficiently discriminated producing 6 haplotypes equal with the number of the analyzed specimens.

3. Barcoding gap

Species discrimination was also calculated using the criteria reported by CBOL plant working group i.e. discrimination was considered successful, if the minimum interspecific K2P (Kimura 2 parameter) distance involving a species was larger than its maximum intraspecific K2P distance (barcoding gap). Thus, in this study, to evaluate the barcoding gap, the minimum inter- and maximum intraspecific divergences were tested for each locus. This approach was applied only in taxa for which interspecific comparisons could be done due to the presence of congeneric species in my database. Moreover, in the case of trnh-psbA locus, on account of the different length and the high variability of the mentioned primer sequences, all interspecific distances were calculated 'between species within genera'. The three potential DNA barcodes displayed different levels of divergence among the investigated genera (Fig.5-8).

Interspecific divergences resulted larger than intraspecific values in 31/61cases of congeneric comparison (51%). In the rest of the specimens, interspecific values were absent (in most of the cases) while in a few examples were even higher.

In particular, average minimum interspecific divergence for both rbcl and MatK in *Aesculus hippocastaneum* L., was 0,007 while maximum intraspecific arrived at 0,002, 0,03 and 0,005 respectively for *Aesculus indica* (Wall. ex Camb.) Hook.f., 0,008 and 0,006 for *Althaea officinalis* L., 0,1 and 0 for *Betula pendula* Roth., 0,06 and 0 for *Ceratonia siliqua* L. and 0,019 and 0,001 for *Cercis siliquastrum* L.. Moreover, presence of DNA barcoding gap was observed also in *Castanea sativa* L. resulted minimum interspecific 0,07 and 0

maximum intraspecific divergence, in *Fraxinus ornus* L. with 0,001 and 0 respectively, in *Ginkgo biloba* L. with 0,1 and 0, in Humulus lupulus L. with 0,09 and 0, in Laurus nobilis L. with 0,1 and 0, in Olea europaea L. with 0,007 and 0,0005, in Punica granatum L. with 0,1 and 0, in Pistacia terebinthus L. arriving at 0,07 and 0,02 correspondingly, in Rosmarinus officinalis L. 0,04 and 0,0005 and finally in Ziziphus jujuba Mill. minimum interspecific scored 0,09 maximum intraspecific divergence 0,05. (Fig.8). Regarding the results of trnh-psbA locus, its sequences were treated separately, due to the difficulty in multialignment, because of the high variability of the different genera, thus, it was determined just in the genera with multiple species representatives. As a result of the above limitations, DNA barcoding gap presence was achieved by trnh-psbA only in 2 genera (Aesculus and Sambucus) from a total number of 9 with minimum interspecific divergence 0,01 and 0,03 while maximum intraspecific arrived at 0,005 and 0,02 for each one respectively (Fig.7).

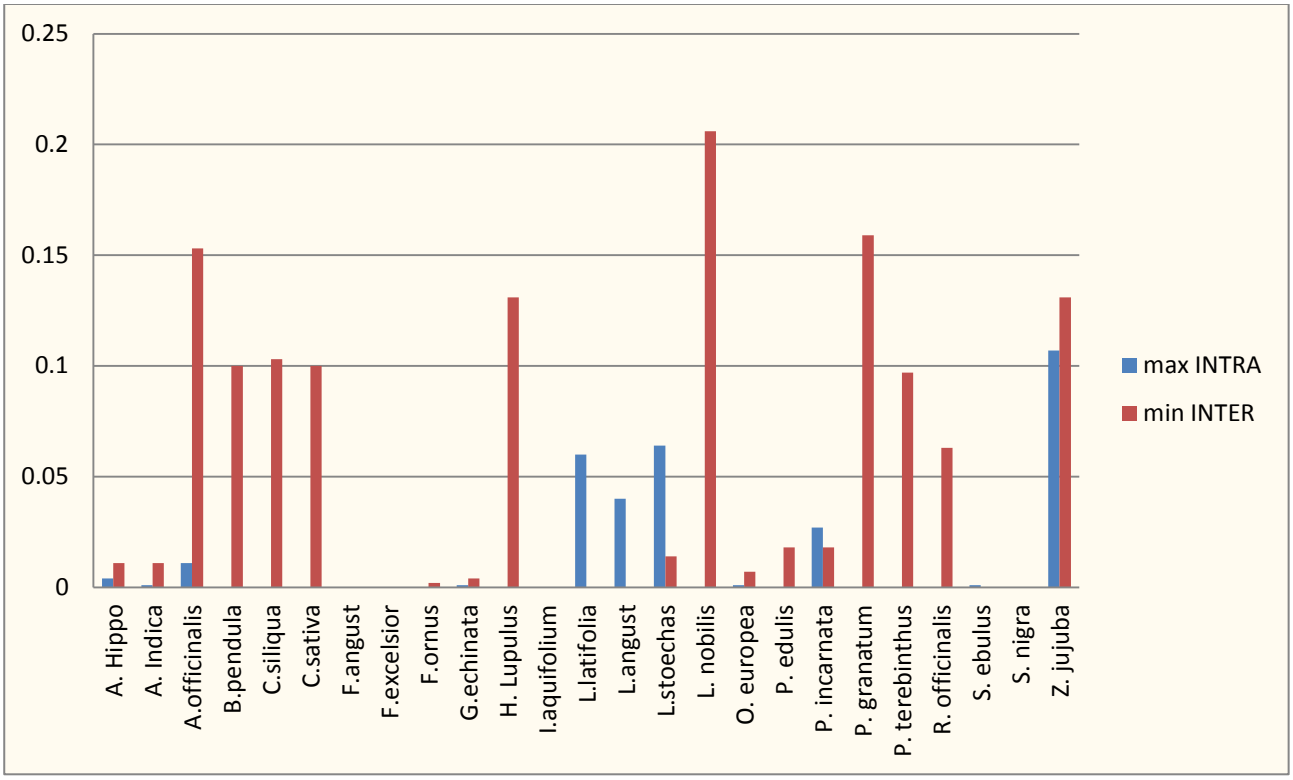


Fig. 5 *Relative distribution of interspecific divergence between congeneric species (red) and intraspecific distances (blue) using Kimura 2 parameters model for MatK locus. Ratios could not be determined in some species where there was either single accession or sequencing failure in some loci.*

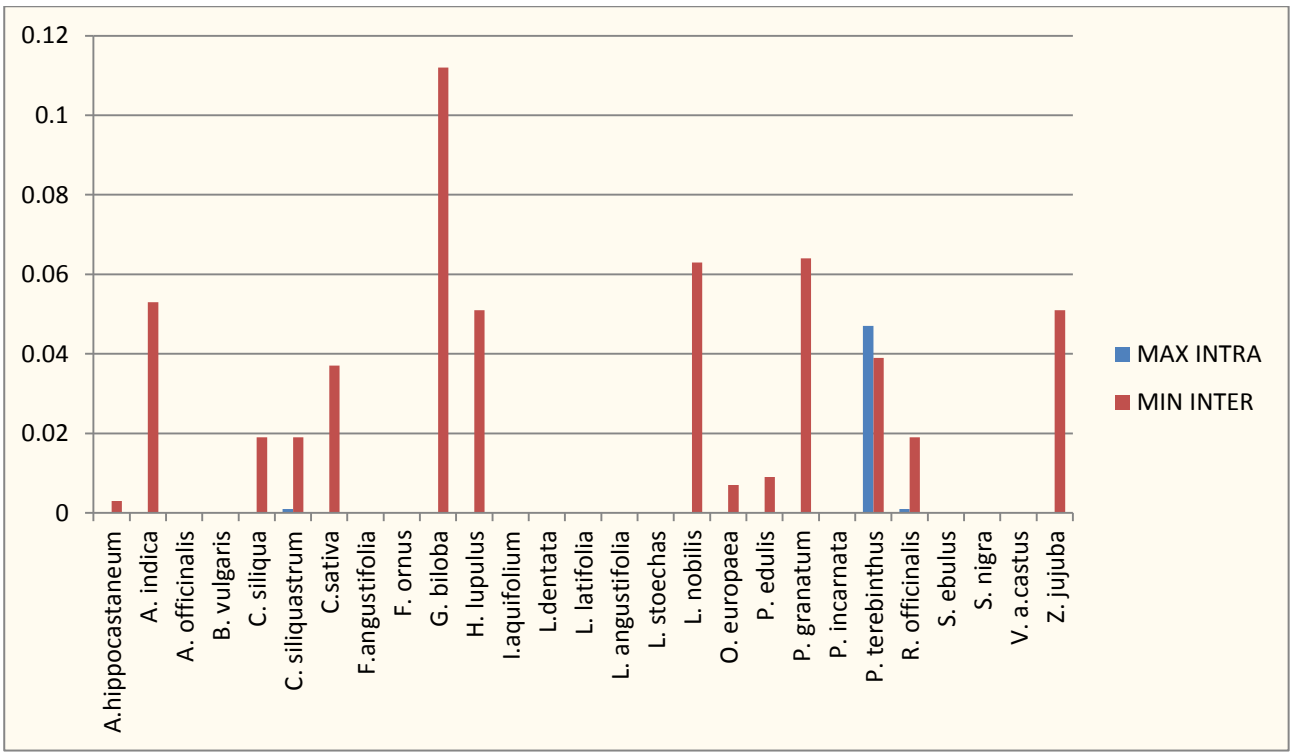


Fig. 6 *Relative distribution of interspecific divergence between congeneric species (red) and intraspecific distances (blue) using Kimura 2 parameters model for rbcl locus. Ratios could not be determined in some species where there was either single accession or sequencing failure in some loci.*

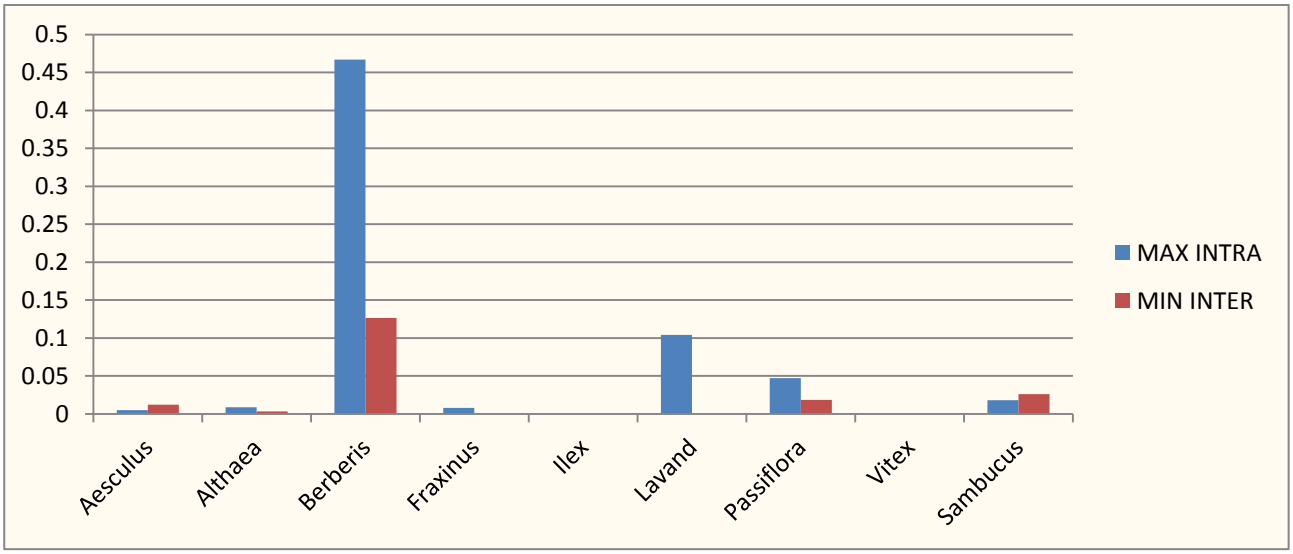


Fig. 7 *Relative distribution of interspecific divergence between congeneric species (red) and intraspecific distances (blue) using Kimura 2 parameters model for trnh-psbA locus. Ratios could not be determined in some species where there was either single accession or sequencing failure in some loci.*

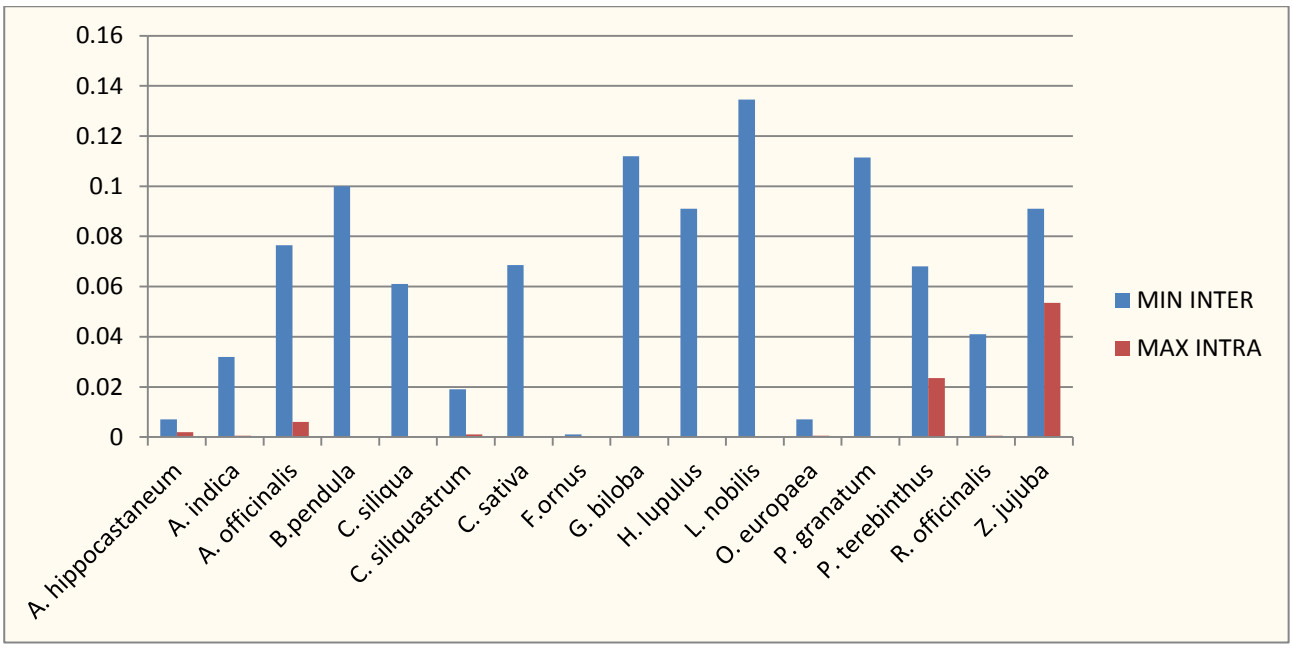


Fig. 8 *Mean value of interspecific divergence between congeneric species (red) and intraspecific distances (blue) using Kimura 2 parameters model for both rbcl and MatK loci only in the groups where barcoding gap is present.*

4. Tree-based analysis

With the phylogenetic approach (Fig. 9), all the investigated samples grouped in clusters according to their different genera with a minimum collapse bootstrap value of 70. Families and genera were easily recognized (93%-100% bootstrap support). Berberis sp. appeared polyphyletic, as evidenced in previous studies (Roy S. et al. 2010). Within *Aesculus hippocastaneum* L. species, the individual from Greece, collected in the wild, was surprisingly separated from all the other *Aesculus hippocastaneum* L. individuals. Nevertheless, 16 species in 15 multispecific genera appeared monophyletic with high bootstrap support (>93%) while nine genera (Aesculus, Althaea, Berberis, Ilex, Sambucus, Fraxinus, Vitex, Passiflora and Lavandula) in total, failed in resolving some of their species. In the case of Aesculus genus, Aesculus hippocastaneum L. individuals were clearly separated from 3 Aesulus indica (Wall. ex Camb.) Hook.f. samples except from the occasion of the already mentioned specimen from Greece. In addition, within Althaea genus, while the two species of *Althaea officinalis* L. from Padova and Viterbo were clearly separated from the species of *Althaea cannabina* L. with a high score of 95 bootstrap, the third representative of *Althaea officinalis* L. from Greece collected in the wild, seems to be discrete from the whole genus with a bootstrap score of 76. In the cases of; Ilex, Sambucus and Vitex genera, *Ilex latifolia* Thunb. species failed to be separated from the other *Ilex aquifolium* L. species, *Sambucus nigra* L. from *S. ebulus* L. and *S. racemosa* L. specimens as well as three *Vitex agnus-castus* L. samples from *Vitex glabrata* R.Br. Regarding Berberis

genus, two of the tested representatives Berberis aristata DC. and Berberis vulgaris L. both of them collected from the botanical garden of Padova, mismatched with Passiflora (P. incarnata L.) group while the other two analyzed individuals of B. vulgaris L. from Trento and botanical garden of Viterbo respectively, were placed each one separately, constructing 3 different groups in total. Finally, Fraxinus and Lavandula genera, failed as well in discriminating the correct plant species as each one of them constructed two different groups mismatching the tested species.

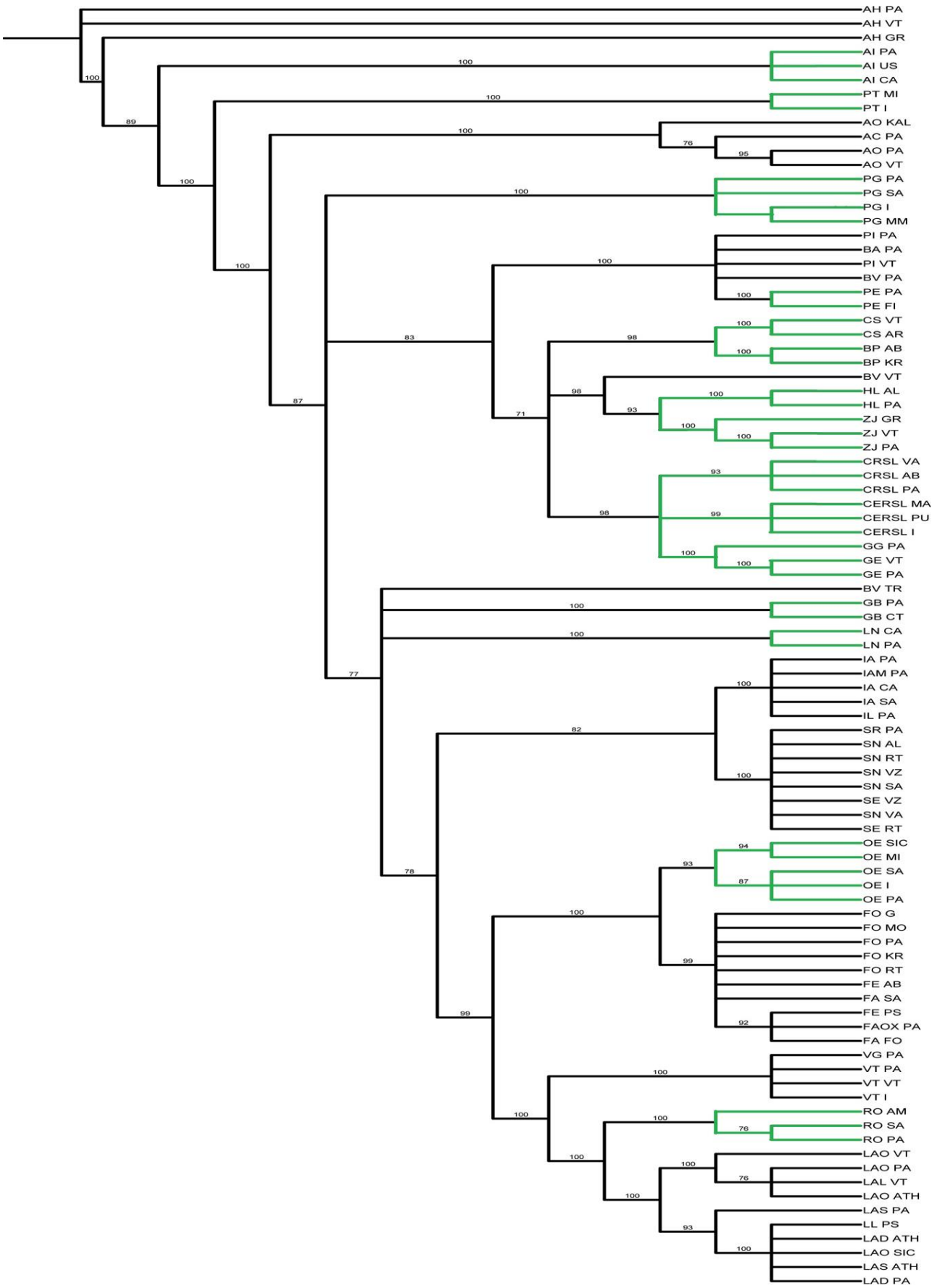


Fig. 9 *Species monophyly through inference of RAxML dendrogram. Green color indicates the successful species discrimination within genera.*

List of Haplotypes

Haplotypes	Species	Haplotypes	Species
Hap_1: 1	Aesculus hippocastaneum L. (It)	Hap_29: 1	Glycyrrhiza echinata L. (It)
Hap_2: 1	Aesculus hippocastaneum L. (It)	Hap_75: 1	Glycyrrhiza echinata L. (It)
Hap_72: 1	Aesculus hippocastaneum L. (Gr)	Hap_30: 1	Glycyrrhiza glabra L. (It)
Hap_3: 1	Aesculus indica (Wall. ex Camb.) Hook.f.) (It)	Hap_31: 2	Ginkgo biloba L.(It), Ginkgo biloba L.(It)
Hap_4: 1	Aesculus indica (Wall. ex Camb.) Hook.f.) (U.S.A.)	Hap_32: 1	Humulus lupulus L. (It)
Hap_5: 1	Aesculus indica (Wall. ex Camb.) Hook.f.) (CA)	Hap_33: 1	Humulus lupulus L. (It)
Hap_6: 1	Althaea officinalis L. (Gr)	Hap_34: 5	Ilex aquifolium L.(It.) Ilex aquifolium L.(It.), Ilex aquifolium L.(It.), Ilex aquifolium Loud. var. Marginata, Ilex latifolia Thunb.
Hap_7: 1	Althaea officinalis L. (It)	Hap_35: 1	Lavandula dentata L. (Gr)
Hap_8: 1	Althaea officinalis L. (It)	Hap_36: 2	Lavandula dentata L. (It), Lavandula angustifolia Mill. (It)
Hap_9: 1	Althaea cannabina L. (It)	Hap_37: 1	Lavandula latifolia Medik. (It)
Hap_10: 1	Betula pendula Roth. (Gr)	Hap_38: 1	Lavandula latifolia Medik. (It)
Hap_74: 1	Betula pendula Roth.	Hap_39: 1	Lavandula angustifolia Mill. (Gr)
Hap_11: 1	Berberis aristata DC. (It)	Hap_40: 1	Lavandula angustifolia Mill. (It)
Hap_12: 1	Berberis vulgaris L. (It)	Hap_41: 1	Lavandula angustifolia Mill. (It)
Hap_13: 1	Berberis vulgaris L. (It)	Hap_42: 1	Lavandula stoechas L. (Gr)
Hap_14: 1	Berberis vulgaris L. (It)	Hap_43: 1	Lavandula stoechas L. (It)
Hap_19: 1	Cercis siliquastrum L. (It)	Hap_44: 1	Laurus nobilis L. (It)
Hap_20: 1	Cercis siliquastrum L. (Gr)	Hap_45: 1	Laurus nobilis L. (It)
Hap_21: 1	Cercis siliquastrum L. (It)	Hap_46: 2	Olea europaea L. (Gr), Olea europaea L. (It)
Hap_15: 2	Castanea sativa Mill.(Gr), Castanea sativa Mill.(It)	Hap_47: 2	Olea europaea L. (Sp), Olea europaea L. (It)
Hap_16: 1	Ceratonia siliqua L. (Gr)	Hap_48: 1	Olea europaea L. (It)
Hap_17: 1	Ceratonia siliqua L. (Ma)	Hap_49: 3	Punica granatum L. (Gr), Punica granatum L. (It), Punica granatum L. (It)
Hap_18: 1	Ceratonia siliqua L. (It)	Hap_50: 1	Punica granatum L. (Sp)
Hap_22: 3	Fraxinus angustifolia Vahl (It) Fraxinus angustifolia M.Bieb. ex Willd. subsp. Oxycarpa (It), Fraxinus excelsior L. (It)	Hap_51: 1	Passiflora incarnata L. (It)
Hap_23: 1	Fraxinus angustifolia Vahl (It)	Hap_52: 1	Passiflora incarnata L. (It)
Hap_73: 1	Fraxinus excelsior L. (It)	Hap_53: 1	Passiflora edulis Sims, 1818 (It)
Hap_24: 1	Fraxinus ornus L. (Gr)	Hap_54: 1	Passiflora edulis Sims, 1818 (Fn)
Hap_25: 1	Fraxinus ornus L. (It)	Hap_55:1	Pistacia terebinthus L. (Gr)
Hap_26: 1	Fraxinus ornus L. (It)	Hap_56:1	Pistacia terebinthus L. (Sp)
Hap_27: 1	Fraxinus ornus L. (It)	Hap_57: 1	Rosmarinus officinalis L. (Gr)
Hap_28: 1	Fraxinus ornus L. (It)	Hap_58: 1	Rosmarinus officinalis L. (It)

Haplotypes	Species	Haplotypes	Species
Hap_59: 1	Rosmarinus officinalis L. (It)	Hap_66: 1	Sambucus nigra L. (It)
Hap_60: 1	Sambucus ebulus L. (It)	Hap_67: 1	Vitex agnus-castus L. (Gr)
Hap_61: 1	Sambucus ebulus L. (It)	Hap_68: 1	Vitex agnus-castus L. (It)
Hap_62: 1	Sambucus racemosa L. (It)	Hap_69: 2	Vitex glabrata R.Br. (It), Vitex agnus-castus L. (It)
Hap_63: 2	Sambucus nigra L. (It), Sambucus nigra L. (It)	Hap_70: 2	Ziziphus jujuba Mill.(It), Ziziphus jujuba Mill.(It)
Hap_64: 1	Sambucus nigra L. (It)	Hap_71: 1	Ziziphus jujuba Mill.(Gr)
Hap_65: 1	Sambucus nigra L. (Gr)		

Tab. 6 *Haplotypes list (gaps considered) of the three tested loci, generated using Blastclust. Green=success red=failure blue=singleton-sampled species*

It=Italy, Gr=Greece, Sp=Spain, Ma=Malta, Fn=Finland, Ca=Canada

5. GenBank assessment

The number and percentage of specimens present in GenBank, at the species and genus level, varies for each locus and is summarized in (Tab. 7) All the specimens in the dataset are present at the genus level (100%) for rbcl marker while for MatK 97% (70/72). These two markers showed also high and comparable coverage at the species level (85% and 83%, respectively). In contrast, GenBank provides trnH-psbA sequences for 76% of the studied species while 94% (77/82) at the genus level. Pistacia sp. genus wasn't present in the database of NCBI in both MatK and trnH-psbA loci.

When submitted to GenBank, all the markers showed a low ability to identify my samples at the species level (37% with matK, 43% with rbcl and 65% with trnH-psbA); as expectable, a much better identification was achieved at the genus level (80% with matK, 86% with rbcl and 93% with trnH-psbA).

Samples without species sequence representation in GenBank are shown in (Tab. 8). This fraction is low for all markers as well as in most of these cases the presence of the genus rank was ascertained in GenBank (100% rbcl, 83% for matK and 75% for trnH-psbA) and identification success was generally high (75% with matK, 70% with trnH-psbA, and 69% with rbcl).

Universal Primer pairs	Species Presence in NCBI	Genera Presence in NCBI	Species success in NCBI	Genera success in NCBI
rbcl	74/87 (85%)	87/87 (100%)	32/74 (43%)	75/87 (86%)
trnH-psbA	62/82 (76%)	77/82 (94%)	40/62 (65%)	74/80 (93%)
MatK	60/72 (83%)	70/72 (97%)	22/60 (37%)	56/70 (80%)

Tab. 7 *Specimens present in GenBank at the species and genus level for each locus and ability of the barcoding markers to identify the samples.*

Marker region	Species not present in GenBank	Genera present in GenBank (of the species not present)	BLAST success at genera level
MatK	12/72 (17%)	10/12 (83%)	9/12 (75%)
rbcl	13/87 (15%)	13/13 (100%)	9/13 (69%)
trnH-psbA	20/82 (24%)	15/20 (75%)	14/20 (70%)

Tab. 8 *Specimens not present in GenBank at species level and ability of the barcoding markers to identify such samples at the genus level.*

In the cases where the proper genus was missing from the GenBank, megaBLAST associated the sequence with a closely related genus from the same family. More specifically, in the case of *Pistacia terebinthus* L. (Anacardiaceae) for both MatK and trnh-psbA loci, two genera from the same family (Rhus and Toxicodendron respectively) were associated to the query sequences and in the case of the trnH-psbA sequences of *Ceratonia siliqua* L.(Fabaceae) megaBLAST found Cassia grandis, a species that belongs to the same family as the mentioned species too. Overall wrong genera identification from different families didn't occur in any case of the three tested markers.

To sum up all the obstacles encountered by the tested markers (amplification/sequencing difficulties or missing data) and provide a clearer picture of the "practical" results, actually achieved, I report the species identification power of the single and combined DNA regions on the GenBank database for the whole dataset (Tab. 9). TrnH-psbA had the highest score (45%), whereas rbcl and matK correctly assigned (36% and 24% of samples, respectively). Species identification with two-marker combinations ranged from 43% (matK+rbcl) to 55% for both rbcl+trnH-psbA and matK+ trnH-psbA primer pairs respectively. Finally the three-marker combination slightly improved the success rate scored by providing the highest species resolution (60%).

NCBI identification success at species level

Species	Rbcl	MatK	Trnh-psbA	Rbcl + MatK	Rbcl + Trnh-psbA	MatK + Trnh-psbA	Rbcl + MatK+ Trnh-psbA
Pistacia terebinthus L.	M. NBCI	M. NBCI	M. NBCI	-	-	-	-
Pistacia terebinthus L.	M. NBCI	M. NBCI	M. NBCI	-	-	-	-
Ilex aquifolium L.	-	-	-	-	-	-	-
Ilex aquifolium L.	-	-	-	-	-	-	-
Ilex aquifolium L.	-	-	-	-	-	-	-
Ilex aquifolium Loud. var. Marginata	M. NBCI	M. NBCI	M. NBCI	-	-	-	-
Ilex latifolia Thunb.	-	-	-	-	-	-	-
Berberis vulgaris L.	M. NBCI	SE. FAIL	M. NBCI	-	-	-	-
Berberis vulgaris L.	M. NBCI	SE. FAIL	M. NBCI	-	-	-	-
Berberis vulgaris L.	M. NBCI	SE. FAIL	M. NBCI	-	-	-	-
Berberis aristata DC.	-	SE. FAIL	-	-	-	-	-
Betula pendula Roth.	-	-	-	-	-	-	-
Betula pendula Roth.	SE. FAIL	-	SE. FAIL	-	-	-	-
Humulus lupulus L.	+	+	+	+	+	+	+
Humulus lupulus L.	+	+	+	+	+	+	+
Sambucus nigra L.	+	-	+	+	+	+	+
Sambucus nigra L.	+	-	+	+	+	+	+
Sambucus nigra L.	+	-	-	+	+	-	+
Sambucus nigra L.	+	-	+	+	+	+	+
Sambucus nigra L.	+	-	+	+	+	+	+
Sambucus ebulus L.	-	+	-	+	-	+	+
Sambucus ebulus L.	-	+	-	+	-	+	+
Sambucus racemosa L.	-	-	-	-	-	-	-
Ceratonia siliqua L.	+	+	M. NBCI	+	+	+	+
Ceratonia siliqua L.	+	+	M. NBCI	+	+	+	+
Ceratonia siliqua L.	+	+	M. NBCI	+	+	+	+
Cercis siliquastrum L.	-	SE. FAIL	SE. FAIL	-	-	-	-
Cercis siliquastrum L.	-	SE. FAIL	+	-	+	+	+
Cercis siliquastrum L.	-	SE. FAIL	+	-	+	+	+
Castanea sativa Mill.	-	-	+	-	+	+	+
Castanea sativa Mill.	-	-	+	-	+	+	+
Glycyrrhiza glabra L.	-	-	SE. FAIL	-	-	-	-
Glycyrrhiza echinata L.	-	M. NBCI	-	-	-	-	-
Glycyrrhiza echinata L.	SE. FAIL	M. NBCI	SEQ. FAIL	-	-	-	-

Ginkgo biloba L.	+	SE. FAIL	+	+	+	+	+
Ginkgo biloba L.	+	SE. FAIL	+	+	+	+	+
Lavandula angustifolia Mill.	-	-	-	-	-	-	-
Lavandula angustifolia Mill.	-	SE. FAIL	-	-	-	-	-
Lavandula angustifolia Mill.	-	-	-	-	-	-	-
Lavandula angustifolia Mill.	-	SE. FAIL	-	-	-	-	-
Lavandula dentata L.	M. NBCI	M. NBCI	M. NBCI	-	-	-	-
Lavandula dentata L.	M. NBCI	M. NBCI	M. NBCI	-	-	-	-
Lavandula latifolia Medik.	-	-	M. NBCI	-	-	-	-
Lavandula latifolia Medik.	+	+	M. NBCI	+	+	+	+
Lavandula stoechas L.	-	-	+	-	+	+	+
Lavandula stoechas L.	-	SE. FAIL	+	-	+	+	+
Vitex agnus-castus L.	+	SE. FAIL	M. NBCI	+	+	-	+
Vitex agnus-castus L.	+	SE. FAIL	M. NBCI	+	+	-	+
Vitex agnus-castus L.	+	SE. FAIL	M. NBCI	+	+	-	+
Vitex glabrata R.Br.	M. NBCI	+	-	+	-	+	+
Rosmarinus officinalis L.	-	+	+	+	+	+	+
Rosmarinus officinalis L.	-	-	+	-	+	+	+
Rosmarinus officinalis L.	-	-	+	-	+	+	+
Laurus nobilis L.	+	-	+	+	+	+	+
Laurus nobilis L.	+	-	+	+	+	+	+
Althaea officinalis L.	-	-	-	-	-	-	-
Althaea officinalis L.	-	-	SE. FAIL	-	-	-	-
Althaea officinalis L.	-	-	+	-	+	+	+
Althaea cannabina L.	M. NBCI	SE. FAIL	-	-	-	-	-
Fraxinus ornus L.	-	-	+	-	+	+	+
Fraxinus ornus L.	-	-	+	-	+	+	+
Fraxinus ornus L.	-	-	+	-	+	+	+
Fraxinus ornus L.	-	-	+	-	+	+	+
Fraxinus ornus L.	-	-	+	-	+	+	+
Fraxinus angustifolia Vahl	-	-	-	-	-	-	-
Fraxinus angustifolia Vahl	-	-	-	-	-	-	-
Fraxinus angustifolia M.Bieb. ex Willd.	-	-	-	-	-	-	-
Fraxinus excelsior L.	-	-	+	-	+	+	+
Fraxinus excelsior L.	SE. FAIL	-	SE. FAIL	-	-	-	-
Olea europaea L.	+	+	+	+	+	+	+

Olea europaea L.	+	+	+	+	+	+	+
Olea europaea L.	+	+	+	+	+	+	+
Olea europaea L.	+	+	+	+	+	+	+
Olea europaea L.	+	+	+	+	+	+	+
Passiflora incarnata L.	+	M. NBCI	+	+	+	+	+
Passiflora incarnata L.	-	M. NBCI	-	-	-	-	-
Passiflora edulis Sims, 1818	+	M. NBCI	M. NBCI	+	+	-	+
Passiflora edulis Sims, 1819	+	M. NBCI	SE. FAIL	+	+	-	+
Punica granatum L.	+	+	+	+	+	+	+
Punica granatum L.	+	+	+	+	+	+	+
Punica granatum L.	+	+	+	+	+	+	+
Punica granatum L.	+	SE. FAIL	+	+	+	+	+
Ziziphus jujuba Mill.	+	+	+	+	+	+	+
Ziziphus jujuba Mill.	+	+	+	+	+	+	+
Ziziphus jujuba Mill.	-	-	SE. FAIL	-	-	-	-
Aesculus hippocastaneum L.	-	-	+	-	+	+	+
Aesculus hippocastaneum L.	-	-	+	-	+	+	+
Aesculus hippocastaneum L.	SE. FAIL	-	-	-	-	-	-
Aesculus indica (Wall. ex Camb.) Hook.f.)	M. NBCI	+	M. NBCI	+	-	+	+
Aesculus indica (Wall. ex Camb.) Hook.f.)	M. NBCI	+	M. NBCI	+	-	+	+
Aesculus indica (Wall. ex Camb.) Hook.f.)	M. NBCI	SE. FAIL	M. NBCI	-	-	-	-
% of success of the sequences obtained	32/74 (43%)	22/60 (37%)	40/62 (65%)				
% of success of initial dataset	32/89 (36%)	22/91 (24%)	40/89 (45%)	38/89 (43%)	50/91 (55%)	49/89 (55%)	55/91 (60%)

Table 9

+	species correctly identified by BLAST
-	species incorrectly identified by BLAST
M.NBCI	sequence of the species made with requested marker does not exist in the NCBI database
SE. FAIL	sequence of the species is missing due to failed in amplification/sequencing
	FAILED to be identified in with neither of the markers

NCBI identification success at genus level

Species	*rbcl*	*MatK*	*trnh-psbA*
Pistacia terebinthus L.	-	M. NBCI	M. NBCI
Pistacia terebinthus L.	+	M. NBCI	M. NBCI
Ilex aquifolium L.	+	+	+
Ilex aquifolium L.	+	+	+
Ilex aquifolium L.	+	+	+
Ilex aquifolium Loud. var. Marginata	+	+	+
Ilex latifolia Thunb.	+	+	+
Berberis vulgaris L.	-	SE. FAIL	-
Berberis vulgaris L.	-	SE. FAIL	SE. FAIL
Berberis vulgaris L.	+	SE. FAIL	+
Berberis aristata DC.	-	SE. FAIL	-
Betula pendula Roth.	+	+	+
Betula pendula Roth.	SE. FAIL	+	SE. FAIL
Humulus lupulus L.	+	+	+
Humulus lupulus L.	+	+	+
Sambucus nigra L.	+	+	+
Sambucus nigra L.	+	+	+
Sambucus nigra L.	+	+	+
Sambucus nigra L.	+	+	+
Sambucus nigra L.	+	+	+
Sambucus ebulus L.	+	+	+
Sambucus ebulus L.	+	+	+
Sambucus racemosa L.	+	+	+
Ceratonia siliqua L.	+	+	M. NBCI
Ceratonia siliqua L.	+	+	M. NBCI
Ceratonia siliqua L.	+	+	M. NBCI
Cercis siliquastrum L.	+	SE. FAIL	SE. FAIL
Cercis siliquastrum L.	+	SE. FAIL	+
Cercis siliquastrum L.	+	SE. FAIL	+
Castanea sativa Mill.	+	+	+
Castanea sativa Mill.	+	+	+
Glycyrrhiza glabra L.	+	+	SE. FAIL
Glycyrrhiza echinata L.	+	+	+
Glycyrrhiza echinata L.	SE. FAIL	+	SE. FAIL

Ginkgo biloba L.	+	SE. FAIL	+
Ginkgo biloba L.	+	SE. FAIL	+
Lavandula angustifolia Mill.	+	+	+
Lavandula angustifolia Mill.	+	SE. FAIL	+
Lavandula angustifolia Mill.	+	+	+
Lavandula angustifolia Mill.	+	SE. FAIL	+
Lavandula dentata L.	+	-	+
Lavandula dentata L.	+	SE. FAIL	+
Lavandula latifolia Medik.	+	+	+
Lavandula latifolia Medik.	+	+	+
Lavandula stoechas L.	+	-	+
Lavandula stoechas L.	+	+	+
Vitex agnus-castus L.	+	+	+
Vitex agnus-castus L.	+	SE. FAIL	+
Vitex agnus-castus L.	+	SE. FAIL	+
Vitex glabrata R.Br.	+	SE. FAIL	+
Rosmarinus officinalis L.	-	SE. FAIL	+
Rosmarinus officinalis L.	-	+	+
Rosmarinus officinalis L.	-	+	+
Laurus nobilis L.	+	+	+
Laurus nobilis L.	+	+	+
Althaea officinalis L.	-	-	-
Althaea officinalis L.	-	+	-
Althaea officinalis L.	-	+	+
Althaea cannabina L.	-	SE. FAIL	+
Fraxinus ornus L.	+	-	+
Fraxinus ornus L.	+	-	+
Fraxinus ornus L.	+	-	+
Fraxinus ornus L.	+	-	+
Fraxinus ornus L.	+	-	+
Fraxinus angustifolia Vahl	+	-	+
Fraxinus angustifolia Vahl	+	-	+
Fraxinus angustifolia M.Bieb. ex Willd.	+	-	+
Fraxinus excelsior L.	+	-	+
Fraxinus excelsior L.	SE. FAIL	-	SE. FAIL
Olea europaea L.	+	+	+

Olea europaea L.	+	+	+
Olea europaea L.	+	+	+
Olea europaea L.	+	+	+
Olea europaea L.	+	+	+
Passiflora incarnata L.	+	+	+
Passiflora incarnata L.	+	+	+
Passiflora edulis Sims, 1818	+	+	+
Passiflora edulis Sims, 1819	+	+	SE. FAIL
Punica granatum L.	+	+	+
Punica granatum L.	+	+	+
Punica granatum L.	+	+	+
Punica granatum L.	+	SE. FAIL	+
Ziziphus jujuba Mill.	+	+	+
Ziziphus jujuba Mill.	+	+	+
Ziziphus jujuba Mill.	-	-	SE. FAIL
Aesculus hippocastaneum L.	+	+	+
Aesculus hippocastaneum L.	+	+	+
Aesculus hippocastaneum L.	SE. FAIL	+	+
Aesculus indica (Wall. ex Camb.) Hook.f.)	+	+	+
Aesculus indica (Wall. ex Camb.) Hook.f.)	+	+	+
Aesculus indica (Wall. ex Camb.) Hook.f.)	+	SE. FAIL	+

Table 10

+	**genera correctly identified by BLAST**
-	genera incorrectly identified by BLAST
M.NBCI	sequence of the species made with requested marker does not exist in the NCBI database
SE. FAIL	sequence of the species is missing due to failed in amplification/sequencing

IV. Discussion

1. Applications of DNA barcoding in medicinal and aromatic plants

DNA barcoding is an increasingly attractive tool for species identification in terms of cost, speed and objectivity and might enhance biodiversity inventories (Gaston & O'Neill 2004; Gotelli 2004). The cost for identifying a sample via barcoding, including the processes of DNA extraction, DNA amplification, purification of the PCR product and sequencing using capillary electrophoresis, has been estimated to range from 2,5$ to 8$ per sample, depending on laboratory facilities and consumable equipment (Hajibabaei et al. 2005; Cameron et al. 2006; Janzen et al.2005). Standardized barcodes provide transparent and comparable results that can be easily repeated by anyone, even by a non-taxonomist specialist. A technician in DNA barcoding could replace dozens of taxonomists for routine identification, allowing taxonomists to concentrate on identifying reference specimens for establishing reliable databases.

Additionally, it allows the analysis of poor fragment samples of different life stages and it is effective at species discovery. (Kerr et al. 2007; deWaard et al. 2009). For example, seeds or seedlings and fragments of plant material that do not bear the requisite morphological characters for identification as in the case of medicinal and aromatic plants. As it has been mentioned above, adulteration of MAPs and accidental misidentification from closely related species is growing as a global concern. In most of the cases this usually occurs as a result of: i) materials not having readily distinguishable morphological characters; ii) materials sharing similar common names, iii) the substitution of economically valuable materials with inexpensive specimens. For instance, Baker and Little used MatK DNA barcodes to highlight misidentified plant species in herbal supplements. Over a quarter of the commercially available herbal supplements of black Cohosh they tested did not contain the target North American species Actea raceomosa and instead contained Asian species of Actea as substitutes. As a result of all these reasons, there is an increasing demand for plant identification in various fields such as the one of international protected species in trade; about 29000 plant species protected by CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora; http://www.cites.org/eng/disc/species.shtml) developing effective methods to distinguish CITES-listed from non-CITES listed species, as well as in forensics, herbal medicines and commercial foodstuffs sectors.

2. BLAST identification

DNA barcoding in botany as a way to identify species has been suggested for widespread use (Pang et al. 2012; Zaya & Ashley 2012; Aubriot et al. 2013; Ghahramanzadeh et al. 2013; De Mattia et al. 2011). One of the aims of this research was to test the effectiveness of DNA barcoding as new tool for non-taxonomists to identify this high demanding plant group of medicinal and aromatic plants.

A necessary pre-requisite for the barcoding identification of unknown samples is a collection of reference sequences made publicly available for comparisons. Ideally, such a library should provide multiple samples

from unambiguously identified species, and cover intraspecific variability and closely related species to evaluate variation overlaps of similar barcodes. Therefore, one of the most important components of the Barcode Initiative is the construction of a public reference library of species identifiers which could be used to assign unknown specimens to known species. An important limitation DNA barcoding potential users may actually meet is related with a yet incomplete reference list in the database; the still short number of available species implies that a user could not be able to retrieve a species name or even assign a query to the wrong species. In this study, the lowest percentage of molecular data was retrieved surprisingly by MatK (37% of the species in GenBank), even if it is considered one of the most recommended core-barcodes with the highest discriminatory power. The majority of data were produced by trnH-psbA (65% of the species in GenBank) showed a good coverage, highlighting that this marker, beyond the core barcode, is becoming the most widely used plastid region. In my dataset the wrong discrimination at the genus level appeared to occur only in the cases of families with few genera present in GenBank (Pistacia and Ceratonia from the families Anacardiaceae and Fabaceae respectively). Overall wrong genera identification from different families didn't occur in any case of the three tested markers.

A further important step is the requirement for a query system allowing a direct match of an unknown DNA barcode identifier to the correct species in the reference list. GenBank uses the most common similarity methods as BLAST and megaBLAST to query sequence against a selected database; nevertheless these methods are prone to errors that may occur in barcoding. Highest BLAST score is usually used to evaluate if a query is correctly assigned, but it has been shown that the simple solution of choosing the top BLAST scores is not always reliable (Koski & Golding 2001). In fact a longer sequence with an imperfect match to the query may get a higher score than a shorter sequence with a perfect match. This becomes a particular problem for DNA barcoding studies because the length of the sequences depends on the primer design. My results revealed this limitation with seven rbcL sequences (Betula pendula Roth., Sambucus ebulus L. and Sambucus racemosa L., Glycyrrhiza glabra L., Althaea officinalis L., Fraxinus ornus L. and Fraxinus angustifolia Vahl.). Even though the samples from the GenBank were 100% similar to the queried ones, our samples resulted unidentified by NCBI for the differences of bp in the deposited sequences.

The best markers combination in this approach of the study resulted to be the rbcl + MatK + trnh-psbA (60% of identification success on the whole dataset of 241 samples), in contrast to the CBOL core barcode combination of rbcL+matK (43%), but in agreement with some other research groups (Hollingsworth et al. 2009). In addition the next best identification success (55%) was achieved by both primer combinations of rbcl + trnh-psbA and MatK + trnh-psbA. Based on these results, it is therefore assumed that trnh-psbA was the marker that demonstrated the most impressive discrimination success among the tested loci.

DNA barcodes are gaining popularity for authenticating medicinal and aromatic materials (Song et al. 2009; Zhang et al. 2009; Yao et al. 2009; Chen et al. 2010; Asahina et al. 2010). Therefore, there is an urgent need of establishing new databases exclusively providing DNA barcode sequences, basic information and key references of medicinal and aromatic plants. In this research with the Blast method approach, genera

presence and discrimination was average and not totally informative, highlighting the need for a better implementation of existing databases.

3. Universality and variability of DNA barcodes in the medicinal and aromatic plants of Mediterranean forests

Crucial characteristics for evaluating the performance of barcoding loci include universal applicability, ease of data retrieval (e.g. PCR conditions, sequencing and data analyses) and sufficient variability of the markers used (Kress & Erickson 2007; Fazekas et al. 2008). The key requirement of a DNA barcode is that it is variable enough to identify individual species but not too variable within species, as that would impede the definition between intra- and inter-specific diversities. Under these criteria, the most updated recommendation from the CBOL Plant working group is that rbcl + MatK is adopted as the core DNA barcoding region for land plants (CBOL PWG 2009), with trnh-psbA (the next best performing plastid locus) as a supplementary barcode option for difficult plant groups. Particularly, the latest locus has received much attention from other authors and is considered to be an effective and accurate DNA barcode for the species identification of medicinal and aromatic plants (Guo et al. 2011; Song et al. 2009; Yao et al. 2009; Ma et al. 2010)

In my dataset, this core 2-locus barcode showed different performances confirming previous studies: rbcL marker showed the highest amplification (100%) and sequencing success (98%), therefore being the most retrievable across the entire dataset, whereas matK showed a lower amplification (91%) and sequencing success (87%) respectively. In general, even if this finding is considered a quiet high amplification/sequencing result which agrees with the proposals both of CBOL Plant working group and of other authors (Lahaye et al. 2008), MatK_KIM (Kim, unpublished) failed in the discrimination of some certain plant groups. This limitation of its universality was highlighted, as already reported by Kress & Erickson (2007); Fazekas et al. (2008); Ford et al. (2009); De Mattia et al. (2012) and others. MatK_KIM, used in this study, failed in the amplification of the whole group of Berberis species, something that is already confirmed (Roy et al.2010) and of all *Cercis siliquastrum* L. specimens as well as in sequencing 11 more individuals including the whole group of *Ginkgo biloba* L. and almost the whole genus of Vitex. In addition, trnh-psbA, displayed 98% amplification success, as it failed only in the cases of *Althaea officinalis* L. (Botanical Garden of Padova) and *Cercis siliquastrum* L. (Abruzzo, Italy). Finally, I was able to obtain 82 sequences (94%) of this locus showing just some limitation to applicability in the Fabaceae family (*Glycyrrhiza echinata* L. from Botanical Garden of Viterbo and *Glycyrrhiza glabra* L. Botanical Garden of Padova).

Fabaceae is the third largest family of angiosperms worldwide, with approximately 650 genera and more than 18000 species (Lewis et al. 2005). The family includes many species that are used in medicinal applications. These species possess important medicinal properties and have been widely used as components of pharmaceutical products. For instance, *Glycyrrhiza glabra* L., which is generally used in

traditional medicines, has inhibitory effects on HIV replication in vitro and anti-Fas antibody-induced hepatitis (Okamoto, 2000; Watanabe et al., 1996). In this doctorate thesis, three genera of the mentioned family were tested.

Despite the discussed limitations to applicability above, Fabaceae family was perfectly discriminated. Regarding the results produced by tree-based analysis (Fig. 9), its genera and species grouped in one of the most supported clusters in the tree based reconstruction and they were separated by the similarity approach with 98% stringency. Remarkably, in the case of Glycyrrhiza genus, the two tested species were distinguished with 100% stringency.

On the other hand, regarding the results of Lamiaceae family, three different genera were tested. Both Vitex and Lavandula groups (the groups with the more than one analyzed species) in the multiregion matrice analysis, their resolving power was limited as it succeeded only in the genus level. In fact no DNA barcoding gap was detected for none of these species in any of the tested primers. However, despite the limited amplification power that MatK demonstrated in this family (failed in 4 samples of Lavandula group and in almost the whole group of Vitex), it was the only locus that managed to produce different haplotypes and succeeded in discriminating two (*L. dentata* L., *L. stoechas* L.) of the four tested species of Lavandula genus. In fact, other authors (Theodorisdis et al. 2012) confirm the sufficient use of MatK as a suitable individual barcode in the Lamiaceae family. Nevertheless, the relative low amplification and sequencing success, using more than one primer pair indicate that further primer development is needed.

There is no doubt that matK and trnH-psbA are the two most efficient chloroplast regions to cope with rbcl, at least in terms of variability. In this study, the resulting discriminatory power of the tested loci between sister species are in line with data from the literature which reported portions of coding genes such as rbcl as low variable regions below the genus level, and it seems that this locus is useful only for the assignation of the correct genus or family taxonomic rank. In agreement, trnH-psbA displayed the highest efficacy in this dataset, and failed to differentiate two species within Ilex genus, whole Lavandula and Vitex groups and two species of Fraxinus genus. However, Ash species for instance, are remarkable for their ability to intercross, and the consequent difficulty to be barcoded (Fernandez-Manjarres et al. 2006; Gerard et al. 2006). Such reticulate evolution has been shown in Oleaceae (Besnard et al. 2009; Yuan et al. 2010) and many other species (Rieseberg 1994), sometimes at a large scale in tree genera (Hamzeh 2004; Bouille 2011) and it could surely account for part of the shared polymorphisms observed, at least between closely related species. Nevertheless, an impressive exception of Fraxinus group was observed. *F. ornus* L. species (five tested individuals) was the only one that differentiated from the rest of the tested Fraxinus group, something that is already affirmed by other authors (Roy et al.2010), by producing different haplotypes in both matK and trnH-psbA markers while rbcl did not manage to discriminate any of the Fraxinus species. Here, this result is also confirmed by the values of matK barcoding gap where the minimum interspecific value was 0,002 and the maximum intraspecific divergence 0.

Regarding the results of Caprifoliaceae family, three different species of the only tested genus (Sambucus) were analyzed. TrnH-psbA was the only locus that managed to discriminate the Sambucus species producing 7 haplotypes in total, including one haplotype shared by two *S. nigra* L. individuals. Both MatK and rbcl primers failed in distinguishing *Sambucus nigra* L. from *Sambucus ebulus* L. In fact, the existence of barcoding gap in trnh-psba locus is evident where the minimum interspecific value was 0,026 and the maximum intraspecific divergence 0,018. It is therefore confirmed that trnh-psbA marker possesses attributes that are desirable in the plant barcoding system (unless we face Taxonomically Complex Groups, TCG's; Hollingsworth et al. 2011). The primary limitation for this locus depend on the quality of the obtained sequences, which can be negatively affected by the presence of a poly A/T structure with highly variable length in the different species (Armenise et al. 2012, Zhu et al. 2010). As a matter of fact, in some certain plant groups as the one of Sambucus for instance, the disturbance of the poly A/T appearance in the electropherograms was so severe that made it impossible for the sequences to be read. Thus, the entire sequence was completed from conjoining partial bidirectional reads (Kress&Erickson 2007).

In addition, when species monophyly was tested, Caprifoliaceae was perfectly separated from the other families by the similarity approach with 100% stringency. However, the Sambucus species within this family did not manage to be distinguished. This can probably be explained due to the indubitable occurrence of natural and commercial hybrids of these species which documents the underlying polyphyly of a genus.

The overall rate of resolution so far described (Fazekas et al. 2009) indicates a general limit (ca. 72%) to the precision of species discrimination when markers from a single genetic linkage group are used. Large data sets include floristic sampling in temperate locations of North America and South Africa, and in the tropical forests of meso- and South America (Fazekas et al. 2008; Lahaye et al. 2008; Gonzalez et al. 2009). This barcoding data, purposely dedicated to woody plants sampled (except a few species) in many different ecological zones, match this limit and are the first obtained from a newly inspected geographical region, with peculiar features of plant biodiversity. If I assume that the investigated species set conforms well with the taxonomic diversity of Italian-Greek dendroflora, then I may reasonably expect that the proposed limit of about 72% of species identification is likely to be confirmed (or even increased because of the taxonomic breadth existing between remaining taxa) in future studies. These results may open up significant perspectives for future barcoding campaigns of forest ecosystems, from local to continental levels.

It has been shown that woody plant lineages have consistently lower rates of molecular evolution as compared with herbaceous plant lineages (Smith and Dologhue 2008), suggesting that the application of DNA barcoding concepts should be more difficult for tree than for non-woody floras (Fazekas et al. 2009). In fact, in this study, it can be confirmed that except from the case of Lavandula and Vitex group, were the species resolution was not possible, probably, due to the hybridization occurrence in each one of these taxa, discrimination rate was achieved within the rest of the herbaceous plant groups. Moreover, the discrimination rate of plastid barcoding loci varies greatly among different plant lineages. In tree species, some characteristic examples of other authors using different barcode combinations showed no resolution in

12 Quercus (Piredda et al. 2011), 18 Betula and 26 Salix species (von Crautlein et al. 2011), whereas 63 and 100% were achieved in Alnus (26 species) and Compsoneura (8 species), respectively (Ren et al. 2010, Newmaster et al. 2008).

In addition, in the family of Aquifoliaceae, another wood plant tested species, Ilex the only representative of this genus with two analyzed species and one variety *I. aquifolium* L., *I. aquifolium* Loud. var. *Marginata* and *I. latifolia* Thunb. failed to be discriminated by any of the three candidate barcoding primers. Natural interspecific hybridization occurs with high frequency in section Ilex, both in wild populations and in cultivation. Most commercial hybrids are sterile or subfertile, but vegetative propagation enables them to persist. Complex hybrid populations may arise, and if they are subfertile, they may cross with parental or nonparental species. This situation leads to large genetic diversity and to several taxonomic problems, further complicated by polyploidy and vegetative propagation phenomena. These events may have generated the genetic differences detected by the three locus combination among different analyzed Holy trees. Based on these data it can therefore be concluded that the DNA barcoding approach cannot be considered as a good traceability tool for Ilex group, because it is not able to distinguish different hybrids and these from their parents. It should be considered that this is not a problem of DNA barcoding only, but a clear limitation of all the molecular approaches based on plastidial markers in plant kingdom (Bruni et al., 2010).

Finally, the genus of Berberis was decided to be excluded by this study, as the results indicate either probable morphological misidentification or a possible mistake during the elaboration of the laboratory analysis despite the great care taken to validate all specimens a priori using morphology and practicing in the DNA amplification. An empirical study in the genus Inga (Dexter et al. 2010), based on a field morphological identification and molecular fingerprinting, reported an error rate about 7% in morphological identification. The present rate of misidentification was low and did not affect the general findings of the study.

4. Contribution to conservation

Plants are a vital part of the world's biological diversity and an essential resource for human well-being. Besides the crop plants that provide our basic food and fibres, many thousands of wild plants have great economic and cultural importance and potential, providing food, medicine, fuel, clothing and shelter for vast numbers of people throughout the world. (Global Strategy for Plant Conservation 2002). Of particular concern is the fact that many species are in danger of extinction and threatened by habitat transformation, over-exploitation, alien and invasive species, pollution and climate change (Krupnick&Kress 2005). The ultimate and long-term objective of the Global strategy for Plant Conservation (ESPC) is to halt the current and on-going loss of plant diversity. More specifically, target 11 of the ESPC focuses of the biodiversity of wild flora endangered by international trade. Annually more than 400.000 tonnes of medicinal and aromatic plants are traded globally with 80% harvested from the wild (Planta Europa 2008).

Because of the great economic importance of many species of the analyzed families in this doctorate thesis, the reliable discrimination and identification of these species are critical. The cost and time effectiveness of DNA barcoding enables easy species identification (Frézal & Leblois 2008), even from small amounts of plant tissue. Therefore, DNA barcoding can be useful not only for taxonomy but also for the control of the tradable plant genetic resources contributing to the protection and maintenance of plant diversity.

In conclusion, this study highlights the utility of DNA barcoding as a promising tool in providing a practical and standardized identification of medicinal and aromatic plants thus contributing to the conservation and the trade control of these valuable plant resources. The next step in this research would be the establishment of a dedicated aromatic plant DNA barcoding database in which all species and cultivars are described under the morphological and molecular approaches (based on trnH-psbA alone or in combination with matK). Based on this large database it will be possible to better evaluate the discrimination power of different DNA barcoding markers and the support of proper bioinformatic tools (Casiraghi et al. 2007) will lead to the development of an innovative tool suitable for rapid spices identification during the industrial production process.

V. Bibliography

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Parallel Study

Geographical and seasonal variation of taxol content in the needles of *Taxus baccata* L. estimated by high-pressure liquid chromatography (HPLC)

Abstract

Yews have been known for their toxicity to humans and domesticated animals and have been making medicinal headlines, as they provide diterpenoid anticancer compounds such as paclitaxel. Paclitaxel or taxol (marketed in France as Taxol® since 1994) contributes to the treatment of various forms of cancer like ovarian and breast. It has been isolated both from the bark and needles of *Taxus* spp but is found only in traces. Thus the compound cannot be produced on an industrial scale without eventually destroying the species. Systematically, this research was to screen the different provenances of yews, more specifically, of *Taxus baccata* L. for the quantity of paclitaxel in relation to season variation. Needles were extracted with a solution of 96% EtOH, H_20 and AcOH and dried at reduced pressure. The solid was redissolved with a small amount of methanol and directly analyzed by HPLC. The objective of the present study was the elucidation of the effect of vegetative period, sex and provenance on the taxol content from the needles of yew trees. A variation has been found between the different European provenances (central-European and Mediterranean/ Macaronesian) as well as within the natural population of *T. baccata* trees growing in Italy. The highest quantity of taxol was found in Hungary while the lowest in an Italia

I. Introduction

Yews have been known since remote antiquity for their toxicity to humans and domesticated animals, and have been making medicinal headlines in the last decades: directly or indirectly, they provide diterpenoid anticancer compounds with a novel mechanism of action, which were named taxan in the late 1960s (Kingstone et al., 1990). The compounds called taxoids, isolated from different yew materials (Suffness and Cordell, 1985), attract much attention from scientific laboratories due to their powerful antimitotic and antitumour properties (Einzig et al., 1990; Thigpen et al., 1990). Paclitaxel is one of the taxoids most often examined and described in the literature for its anticancer activity. (Donehower et al., 1987; Grem et al., 1987; Rowinsky et al., 1990; Swenerton, 1994)

Paclitaxel (Taxol®, Yewtaxan®) is an important anticancer drug which was first isolated from the bark of Pacific yew, *Taxus brevifolia* Nutt. (Wani et al., 1971), a slow growing evergreen tree from western US and Canada. Since then, it is widely used in clinical practices against various types of cancer including ovarian, breast, lung, skin and other cancers (Donehower et al., 1987; Grem et al., 1987; Rowinsky et al., 1990; Swenerton, 1994). Subsequently, taxol and taxoid derivatives have been reported from foliage and bark of several other species of taxus. These include *T. baccata*, *T. cuspidata*, *T. canadensis* and *T* x *media*. (Georg et al., 1993; Witherup et al., 1990).

Because of the lack of the sustainable supply and a growing demand for the drug, various means to increase the supply of paclitaxel have been investigated. Several studies on the content of paclitaxel and related deterpenoids in different *Taxus* species have been reported (Appendino et al., 1992; Georg et al., 1993; Kelsey and Vance, 1992; Wheeler et al., 1992; Vance et al., 1994). They all pointed out that the content of taxanes is highly variable and dependent of the plant source; it varies with the season, the location, as well as with the particular specimen taken regarding to the age and sex of the taxa. However a study of *T. baccata* showed that significant variation in the paclitaxel content exists among and within populations and species. (Wheeler et al., 1992)

In order to maximize the production of taxines (paclitaxel in this case), it is therefore necessary to understand the various factors that control and influence their biosynthesis and accumulation in the paclitaxel content from the needles of yew trees. (Kelsey and Vance, 1992)

The objective of the present study was the elucidation of the effect of vegetative period, sex and provenance on the taxol content from the needles of *Taxus baccata* L. grown in Italy, in other European stands, and in two Macaronesian provenances considered almost extinct, using high-pressure liquid chromatography (HPLC).

II. Materials and Methods

1. Plant material

Needles from several *Taxus baccata* L. specimens were collected from 9 different European provenances: Italy, Greece, Azores, Madeira, Algeria, Hungary and Austria including three different locations through Italy (Carpineto and Pescopennataro, Central Italy; Sicily, Southern Italy). Due to the accessibility of Carpineto Romano stand (41°33'51" N; 13°06'19" E), five yew individuals were chosen, according to their variability in sex and location. These five individuals have been tested for taxol content each month for 12 months, during the years of 2010-2011. Concerning the rest of the provenances, three specimens of Madeira island were collected, two of them from two different wild populations and the third one from the botanical garden of Madeira. Moreover, five more yews were collected from different populations within Azores Islands, two from Austria (Wienerwald), one from Greece (Mt. Parnassus), Algeria (Chréa National Park), and Hungary (Budapest) respectively, and finally two more plant materials from Italy (Central Italy and Sicily). Fresh needle material after the collection of the above individuals was separated from the stems and dried for 3 h at 60ºC in an oven with forced air ventilation (van Rozendaal et al., 1999).

2. Extraction procedure

One gram of dried needles were accurately weighed and placed in a 20ml brown bottle. The 15ml EtOH: H_2O: HOAc (80:19:1) was added to the needles after which the bottle was closed and left standing for 5 days at room temperature (van Rozendaal et al., 1997). In the mean time the bottle was shaken in order to ensure the coverage of the needles with the above solution. Subsequently, the extracts were filtered, evaporated to dryness in vacuum (40ºC) in a rotary evaporator and weighed and the residues were dissolved in 4 ml of methanol. Finally, they were stored at the fridge until the HPLC analysis.

3. HPLC analysis

The methanolic solutions of total extracts were directly introduced in a HPLC (high-pressure liquid chromatography) system without any prepurification procedure. Paclitaxel was quantified under conditions of flow rate 1,5ml/min and UV detection at 220nm using an external standard calibration curve obtained with semi-synthetic paclitaxel (Sigma). A Hypersil GOLD PFP 5mm 150 x 4.6 mm column with a pre-column (Thermo Scientific) was used and the separation was carried out using two solutions: A= H20, B= MeOH:MeCN, 7/93 in a linear gradient: 0-7.5 min at 35% B then to 58% B at 25 min. ChromatoPlus® application was used for managing the data of chromatography.

III. Results and Discussion

As shown in figure 1, even if the chromatogram is rather complex, the operative conditions allow to separate the paclitaxel in a complex mixture as total extract in a relatively short time. Moreover, the lack of a purification step of total extracts makes the described method useful to perform screening of a large set of samples.

The study assess a variation in the mean value of taxol content of needles between central-European and Mediterranean/Macaronesian provenances, as well as within the natural population of *T. baccata* trees growing in Italy (Figure 2).

The highest concentration of taxol concerning the different provenances of yews was observed in a sample collected from Hungary in June while the lowest was found in an Italian stand during the same month. The concentration of paclitaxel content was 205.6 μg/g of dried needles in the Hungarian sample while the one from Pescopennataro was 54.572 μg/g. Generally it can be concluded that the mean value of paclitaxel concentration from the different provenances follows the results from Carpineto except from the analysis of the Hungarian and Austrian individuals (Table 1).

Substantially, the data in Figure 2 clearly show as the taxol content of needles markedly depends on the vegetative period of the plant, since it is higher in the winter months. This finding indicates that the needles harvested between September and February represent a better source of taxol than the needles harvested in any other period which is in general agreement with the seasonal variation reported by other authors (Németh-Kiss et al., 1996).

Regarding to the results of Carpineto Romano (Figure 3), it can be established that they indicate a seasonal fluctuation. September gave the highest yields while March gave the lowest. As a whole in this intra specific analysis, it can be assumed that the paclitaxel content is also substantially gradually higher during the winter months.

As it was mentioned before, *Taxus baccata* individuals from Carpineto, were also tested for their sex in paclitaxel. Results shown in Figs 4-5, do not indicate any significant difference in the paclitaxel concentration between the different sex. Generally, it can be assumed that the paclitaxel compound is slightly higher in the male yews than in the other two categories; however, the level of the taxol does not vary substantially. (Table 2).

Other important parameters which were tested were the mean values of air and ground temperature as well as the relative humidity of Carpineto in correlation to the quantity of paclitaxel as presented in the Figs 6-8. As it seems, there is not any evident correlation between the variation of paclitaxel and the climatic conditions ($P > 0.05$).

This study points out preliminary results about variation of taxol content according to ecoprovenances. However, the work is still in progress and further investigations, including a larger sampling, correlations with phenological traits, and additional climatic parameters will be presented in a coming paper.

Figure 1 – *Typical chromatogram of the total extract from Taxus baccata L.*

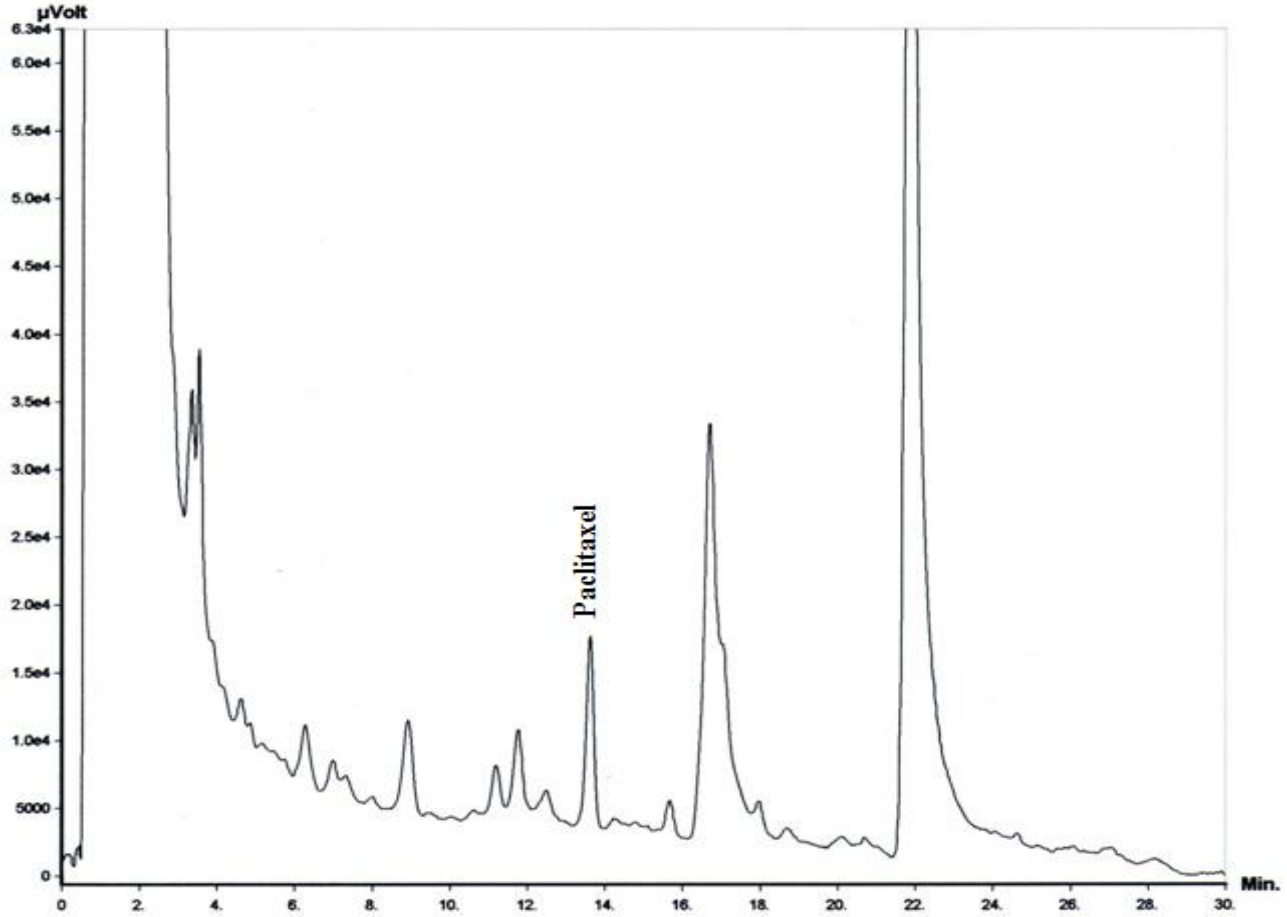


Figure 2 – *Monthly variation of taxol content in different provenances. Bars indicate standard variation.*

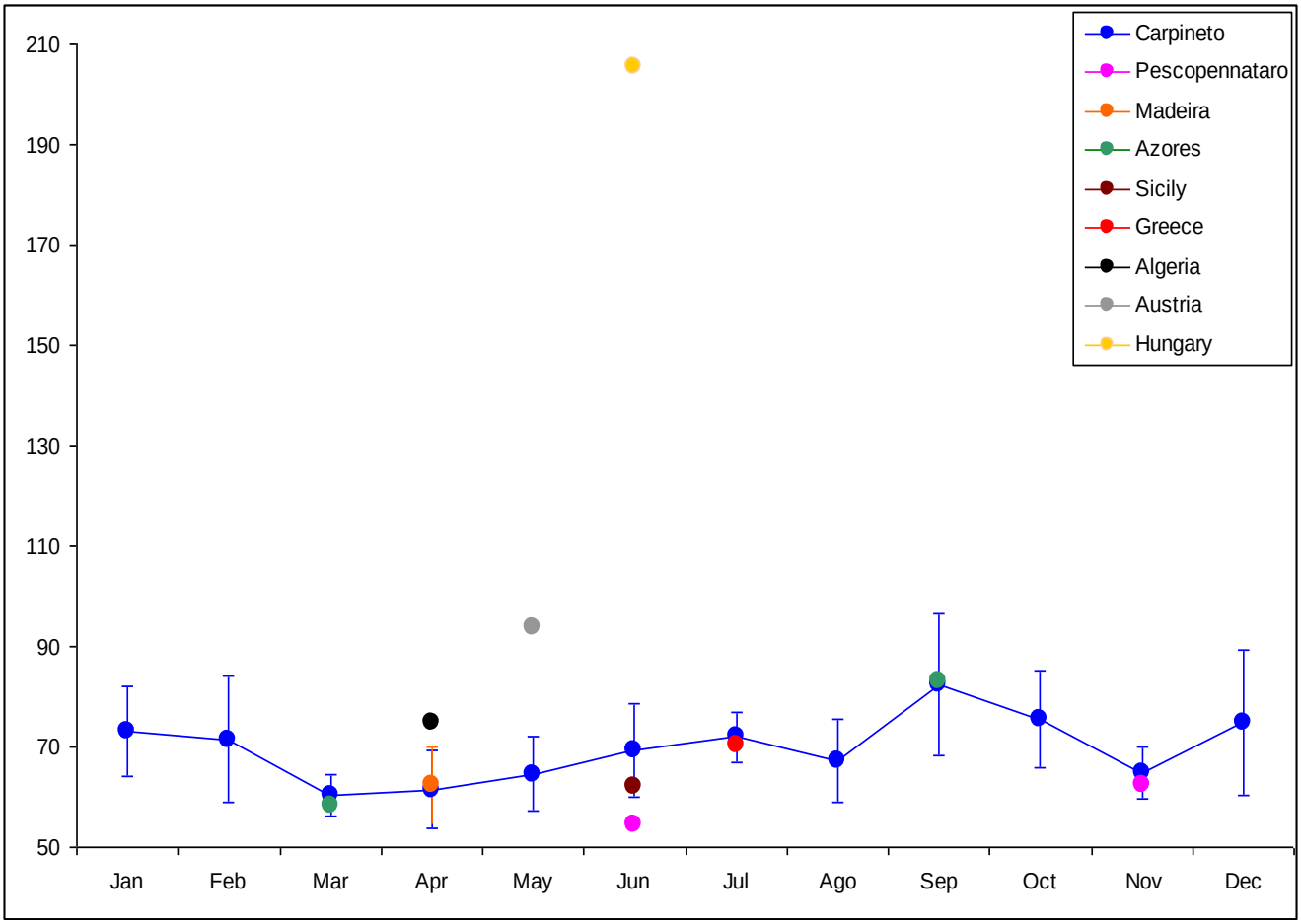


Figure 3 – *Monthly variation of taxol content at Carpineto Romano.*

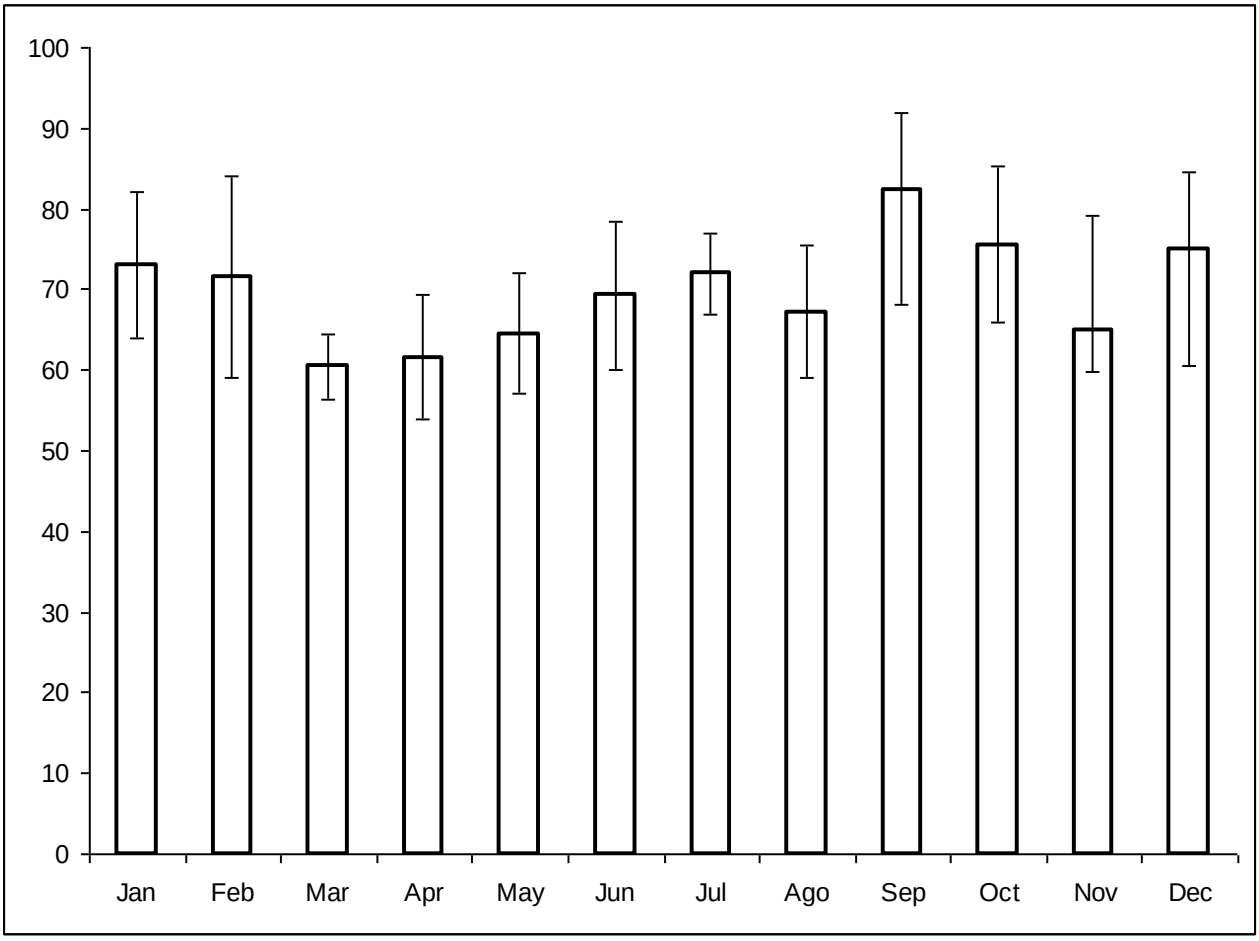


Figure 4 – *Mean monthly variation of Taxol content grouped by sex expression at Carpineto Romano.*

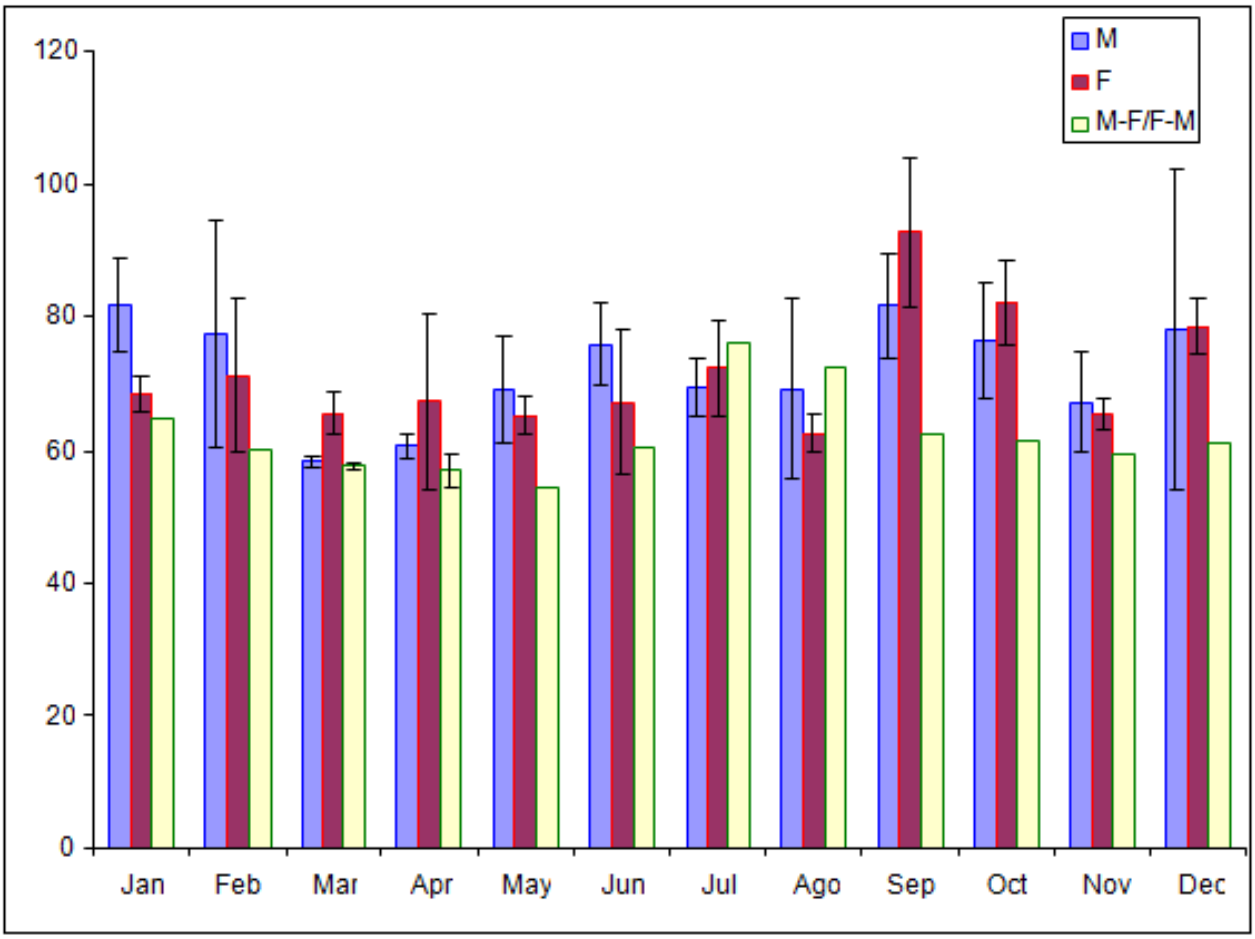


Figure 5 – *Mean monthly variation with standard deviation of Taxol content grouped by sex expression at Carpineto Romano.*

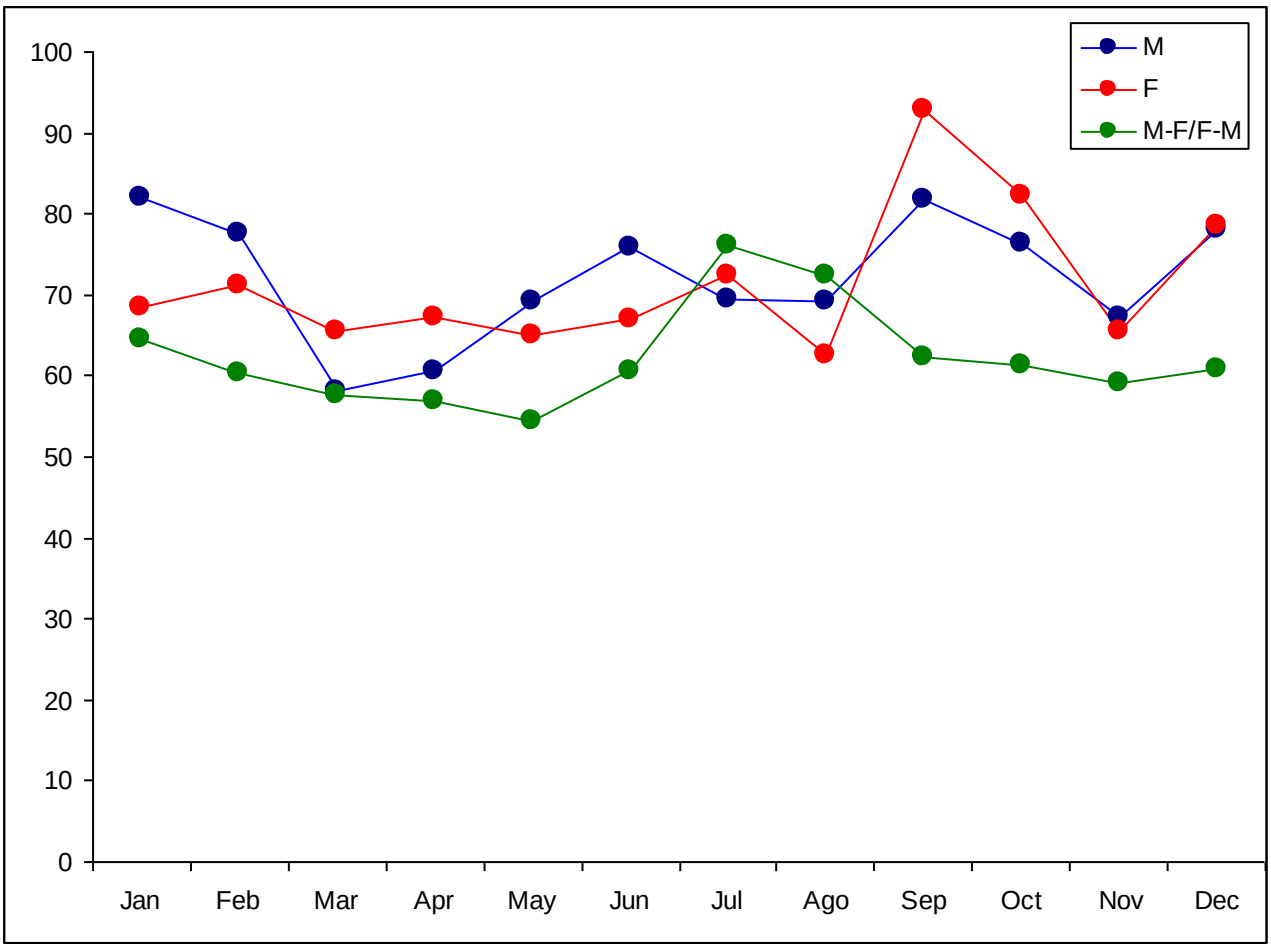


Table 1 – *Mean monthly Taxol content ± standard deviation in study provenances.*

	Carpineto	Pescop.	Sicily	Madeira	Azores	Greece	Algeria	Austria	Hungary
Jan	73.08 ± 9.06	-	-	-	-	-	-	-	-
Feb	71.54 ± 12.53	-	-	-	-	-	-	-	-
Mar	60.44 ± 4.14	-	-	-	58.38	-	-	-	-
Apr	61.549 ± 7.71	-	-	62.30 ± 7.70	-	-	74.92	-	-
May	64.57 ± 7.37	-	-	-	-	-	-	93.80 ± 7.98	-
Jun	69.31 ± 9.14	54.57	62.10	-	-	-	-	-	205.60
Jul	72.02 ± 5.04	-	-	-	-	70.44	-	-	-
Ago	67.17 ± 8.22	-	-	-	-	-	-	-	-
Sep	82.34 ± 14.18	-	-	-	83.02 ± 16.48	-	-	-	-
Oct	75.61 ± 9.68	-	-	-	-	-	-	-	-
Nov	64.87 ± 5.18	62.36	-	-	-	-	-	-	-
Dec	74.93 ± 12.53	-	-	-	-	-	-	-	-

Table 2 – *Mean monthly taxol content ± standard deviation at Carpineto Romano, grouped by sex expression.*

	M	F	M-F or F-M
Jan	81.91 ± 7.17	68.48 ± 2.69	64.63
Feb	77.51 ± 17.17	71.23 ± 11.55	60.23
Mar	58.22 ± 0.74	65.41 ± 3.23	57.68 ± 0.46
Apr	60.6 ± 1.98	67.25 ± 13.23	56.79 ± 2.42
May	69.11 ± 7.98	65.11 ± 2.98	54.44
Jun	75.93 ± 6.31	67.08 ± 10.95	60.54
Jul	69.49 ± 4.49	72.46 ± 7.14	76.19
Ago	69.18 ± 13.59	62.59 ± 2.99	72.34
Sep	81.79 ± 7.87	92.86 ± 11.15	62.42
Oct	76.46 ± 8.76	82.17 ± 6.13	61.38
Nov	67.12 ± 7.65	65.49 ± 2.30	59.14
Dec	78.06 ± 24.24	78.61 ± 4.28	60.92

Figure 6 – *Monthly variation of taxol content related to mean air temperature.*

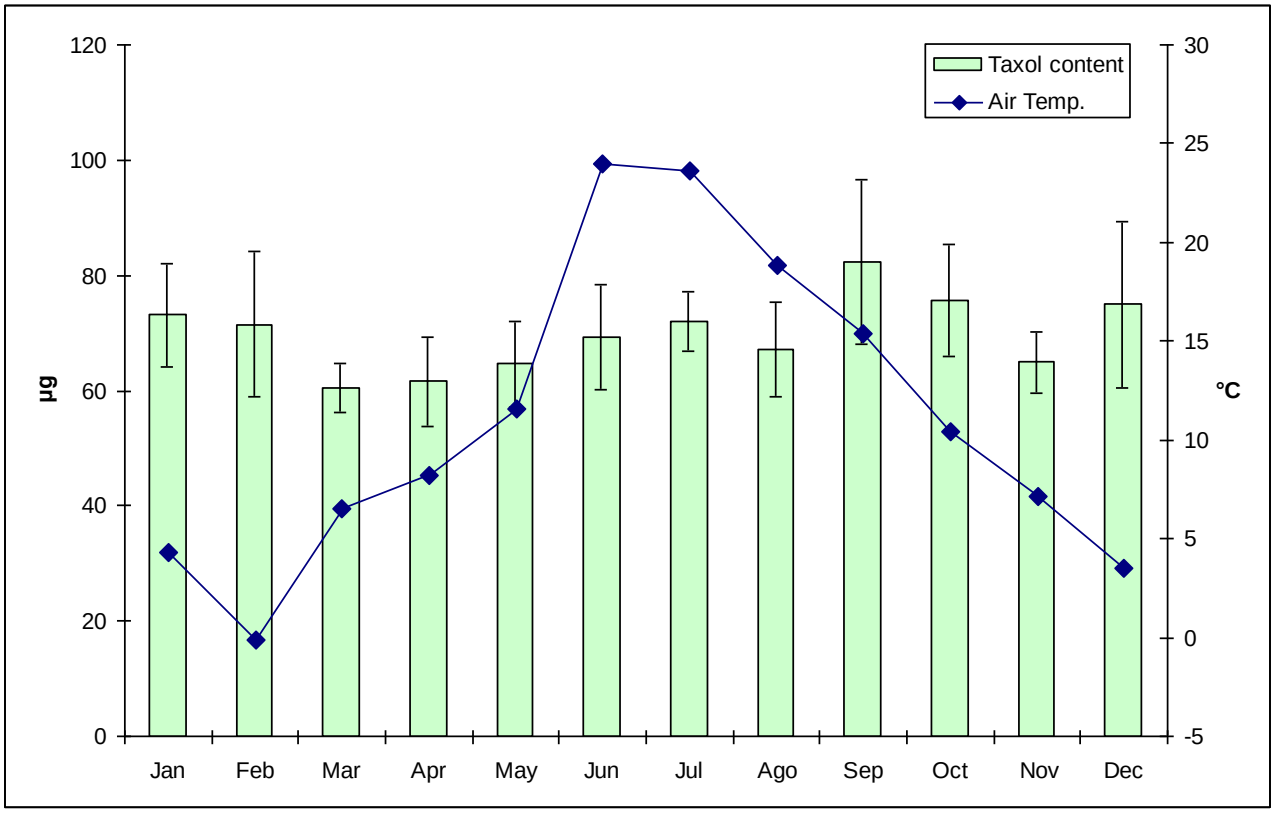


Figure 7 – *Monthly variation of taxol content related to mean ground temperature.*

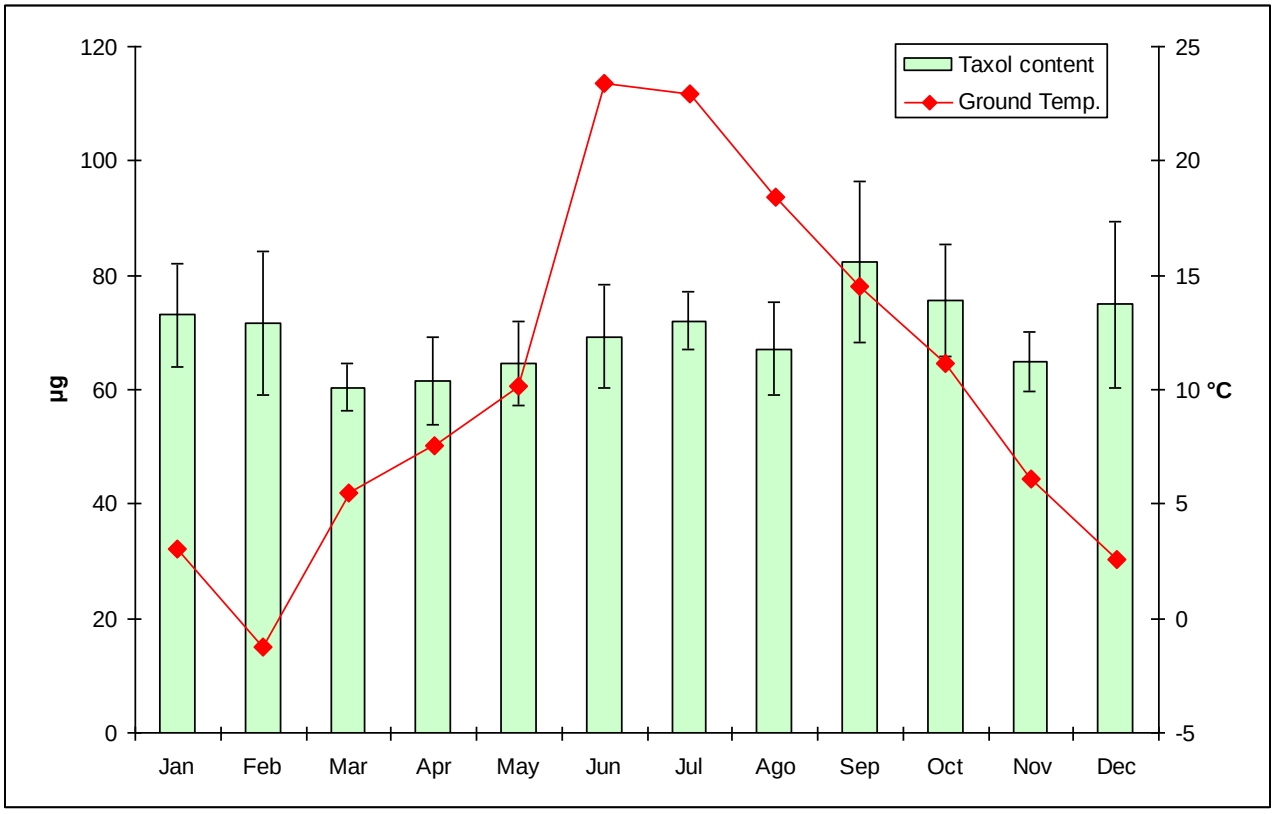
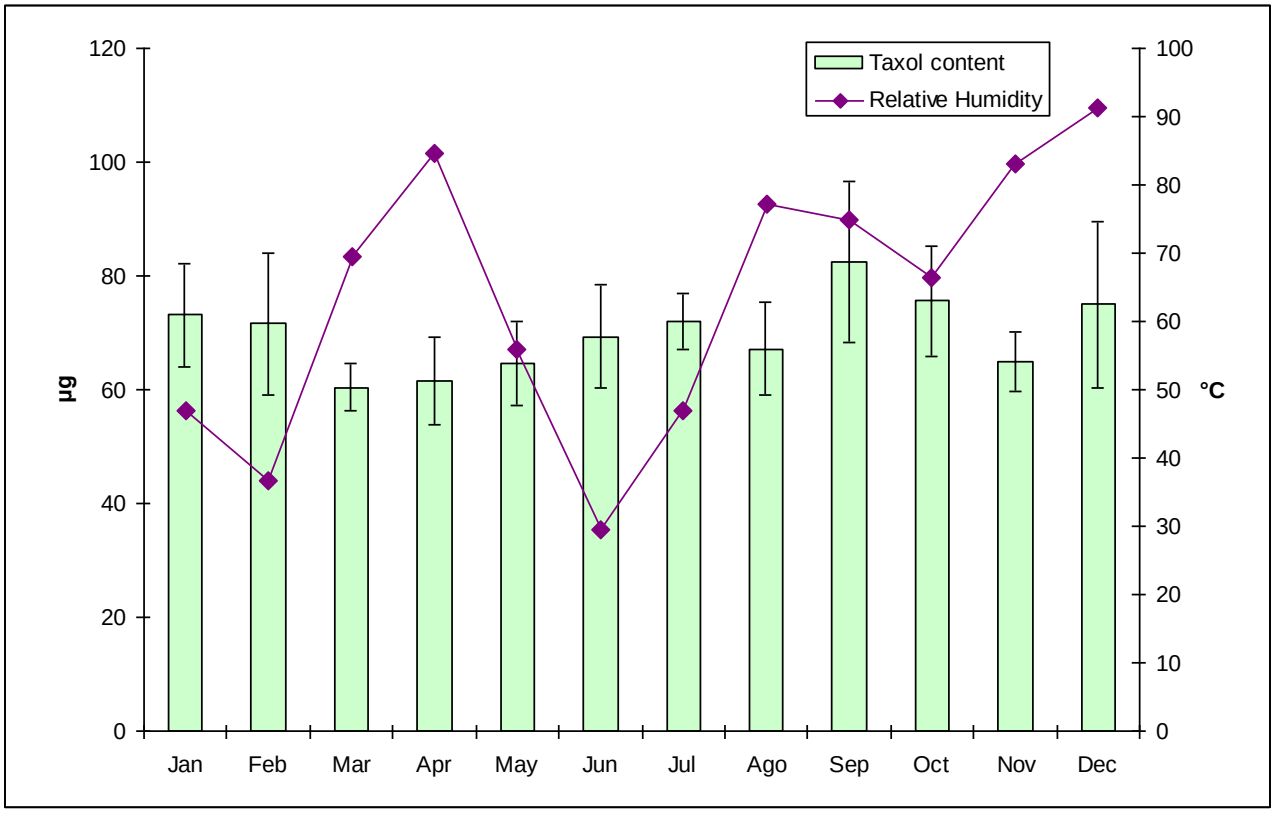


Figure 8 – *Monthly variation of taxol content related to relative humidity.*



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