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Genetic, chemical and agro-morphological evaluation of the medicinal plant *Origanum vulgare* L. for marker assisted improvement of pharmaceutical quality

Dissertation

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Ali Azizi

TABLE OF CONTENTS

I. INTRODUCTION

1.1. *Origanum vulgare* L. (oregano) as a medicinal and spice plant

Oregano is the common name for a general aroma and flavour primarily derived from more than 60 plant species used all over the world as a spice. Four main groups of plants commonly used for culinary purposes can be distinguished, i.e., Greek oregano (*Origanum vulgare ssp. hirtum* (Link) Ietswaart); Spanish oregano (*Coridohymus capitatus* (L.) Hoffmanns & Link); Turkish oregano (*Origanum onites* L.); and Mexican oregano (*Lippia graveolens* HBK) (Lawrence, 1984).

In Europe and, in general, all over the world, the most commonly found oregano species belong to the botanical genus *Origanum* so the commercial products of *O. vulgare* L. and *O. majorana* L. leaves are known as oregano and marjoram on the market (Olivier, 1997). Because of special compositions of essential oil, the leaves of *Origanum* plants are widely used as a very popular spice for food production. European oregano (*O. vulgare*) is used as flavour in meat and sausage products, salads, stews, sauces, and soups. Prior to the introduction of hops, oregano was used to flavour ale and beer (Kintzios 2002a).

Recently, this spice plant has drawn more attention of consumers due to the antimicrobial, antifungal, insecticidal and antioxidative effects of this herb on human healthy (Kokkini 1997, Kulisic et al. 2004, Bakkali et al. 2008). As a medicinal plant, European oregano has traditionally been used as a carminative, diaphoretic, expectorant, emmenagogue, stimulant, stomachic, and tonic. In addition, it has been used as a folk remedy against colic, coughs, headaches, nervousness, toothaches, and irregular menstrual cycles (Kintzios 2002a).

1.2. Botany

The genus *Origanum* belongs to the family *Lamiaceae* and comprises 43 species and 18 hybrids widely distributed in Eurasia and North Africa (Ietswaart 1980, Duman et al. 1998). The species *Origanum vulgare* L. is predominate in occurrence and the most variable species of the genus and the only one commonly known as `oregano' in the most European countries (Vokou et al. 1993, Tucker and Maciarello 1994).

Origanum vulgare L. is a beautiful plant, flowering in heady corymbs, with reddish bracts and purple corollas. The plant flowers in late summer, grows in spikes, and is purplish white. The plant height is 30–60 cm with comparable width (Kokkini 1997). One of the considerable morphological characteristics of the *Origanum* plants is the presence of glandular and nonglandular hairs (peltate hairs or glandular scales) covering the aerial organs. Both types of hairs originate from epidermal cells (Netolitzky 1932). The glandular hairs are numerous on the vegetative organs such as stems, leaves and bracts, while their density becomes reduced on the reproductive organs such as calyces and corollas (Bosabalidis and Tsekos 1984). The glandular hairs produce and secrete an essential oil with a

characteristic odour, mainly due to the monoterpenes being the major components of the oil (Scheffer et al. 1986).

The plants of *O. vulgare* have dense spikes, and tubular 5-toothed calyces, never becoming turbinate in fruit (Kokkini 1997). Ietswaart (1980) has recognised six subspecies within *O. vulgare* based on differences in indumentums, number of sessile glands on leaves, bracts and calyces, and in size and colour of bracts and flowers. These subspecies include: subsp. *hirtum* (Link) Ietswaart, subsp. *vulgare* L., subsp. *virens* (Hoffmannsegg et Link) Ietswaart, subsp. *viride* (Boissier) Hayek, subsp. *gracile* (Kock) Ietswaart and subsp. *glandulosum* (Desfontaines) Ietswaart. Kokkini (1997) confirms this distribution, but identifies subsp. *viride* of Ietswaart as subsp. *viridulum* (Martin-Donos).

In Greece, three geographically distinct subspecies have been recognized, namely *hirtum*, *vulgare* and *viridulum* (Kokkini et al. 1991). *Origanum vulgare* subsp. *hirtum* is mainly distributed on the islands and southern mainland and is characterized by relatively thick leaves with dense glandular hairs and numerous stomata (Bosabalidis and Kokkini1997). The other two subspecies (*vulgare* and *viridulum*) are localized in the northern parts of Greece in which lower temperatures predominate. The leaves of subsp. *vulgare* and subsp. *viridulum* are much thinner than those of subsp. *hirtum* and bear fewer glandular hairs and stomata (Bosabalidis and Kokkini 1997).

Based on these figures, the taxonomic difficulties with subspecies of *O. vulgare* seem to remain a considerable problem for breeding programmes and exploring its potential for utilization. DNA based molecular markers, which are not affected by environmental conditions; could be employed for the resolving the problem. Recently, Katsiotis et al. (2009) have carried out a study to clarify phylogenetic relationships and variations of Greek *O. vulgare* subsp. *hirtum* by RAPD markers and rDNA sequences. We have investigated the relationships between different subspecies of *O. vulgare* using two PCR-based marker approaches, Amplified Fragment Length Polymorphism (AFLP) and Selectively Amplified Microsatellite Polymorphic Loci (SAMPL), and we have also compared the relative efficiencies of these two marker systems (Azizi et al. 2009a).

1.3. Oregano essential oil

Oregano is the commercial name of those *Origanum* species that are rich in the phenolic monoterpenoids, mainly carvacrol and occasionally thymol (D'antuono et al. 2000). A number of chemically related compounds i.e. *p*-cymene; *γ*-terpinene, carvacrol methyl ethers, thymol methyl ethers, carvacrol acetates and thymol acetates; as well as *p*-cymenene, *p*-cymen-8-ol, *p*-cymen-7-ol, thymoquinone, and thymohydroquinone are present in the oil. The other chemical compounds, usually of less significance quantitatively, are present in *Origanum* are the acyclic monoterpenoids such as, geraniol, geranyl acetate, linalool, linalyl acetate and *β*-myrcene. Some sesquiterpenoids such as *β*-caryophyllene, *β*-bisabolene, *β*-bourbonene, germacrene-D, bicyclogermacrene, α-humulene, α-muurolene, *γ*-muurolene, *γ*-cadinene, *allo*-aromadendrene, α-cubebene, α-copaene, α-cadinol, *β*-caryophyllene oxide and germacrene-D-4-ol could also be present. In some of *Origanum* plants sabinyl compounds such as *cis*- and/or *trans*-sabinene hydrate, α-thujene, sabinene, *cis*- and *trans*-

sabinene hydrate acetates, *cis*- and *trans*-sabinol and sabina ketone can also be found (Skoula and Harborne 2002).

The essential oil compositions reported in the subspecies of *O. vulgare* are very variable:

1- Subspecies *hirtum* is most commonly carvacrol-rich and less commonly thymol-rich (Kokkini and Vokou 1989, Baser et al. 1994, Skoula et al. 1999).
2- Subspecies *glandulosum* Ietswaart is rich in cymyl compounds, mainly thymol and carvacrol and their methylethers (Houmani et al., 2002).
3- Subspecies *gracile* Ietswaart (syn. *O. tyttanthum* Gontsch.) and subspecies *viride* Hayek have been found either rich in acyclic compounds and sesquiterpenoids or carvacrol/- thymol rich (Sezik et al 1993, Baser et al 1997, Arnold et al 2000).
4- Subspecies *vulgare* and subspecies *virens* Ietswaart are rich in acyclic compounds and sesquiterpenoids (Alves-Pereira and Fernandes-Ferriera 1998, Sezik et al. 1993, Figuerdo et al. 2006b).
5- With the exception of subspecies *viride*, sabinene compounds are either absent or their presence is uncertain in other subspecies (Skoula and Harborne 2002, Azizi et al Manuscript).
6- Afsharypuor et al. (1997) report on essential oil composition of *O. vulgare* subsp. *viride*, that grows wild in northern parts of Iran (with linalyl acetate, sabinene, *β*-caryophyllene as main components) and differs substantially from the composition of essential oil of the same species, growing wild in the Balkan area (Bulgaria, Albania, Turkey, Greece, Yugoslavia) (carvacrol chemotypes) or cultivated in Israel (thymol chemotype).
7- Some authors reported linalool chemotypes in *O. vulgare* (D'antuono et al. 2000, Figuerdo et al. 2006a)

1.4. Pharmacology

Many of the studies confirmed the medicinal effects of oregano for human health. The *Origanum* species, which are rich in essential oils, have been used for thousands of years as spices and as local medicines in traditional medicine (Fleisher and Fleisher, 1988). Aerial flowering parts of *O. vulgare* subsp. *viride* are used in Iranian traditional medicine as diuretic, stomachic, antineuralgic, antitussive and expectorant (Afsharypuor et al. 1997). The antimicrobial test results showed that the essential oil of *O. vulgare* subsp. *hirtum* and also subsp. *vulgare* have great potential of antimicrobial activity against bacteria, fungi and yeast species and therefore can be used as a natural preservative ingredient in food and/or pharmaceutical industry (Biondi et al 1993, Sahin et al 2004).

Adam et al. (1998) report a valuable therapeutic potency of essential oil of subsp. *hirtum* against dermatophytosis (infection with fungi *Trichophyton rubrum*). It was found that the phenolic components in the essential oil, such as carvacrol and thymol have a strong antifungal potency (Farag et al 1989, Curtis et al 1996). According to the findings of Adam et al. (1998), carvacrol and thymol showed much higher antifungal activities against human pathogens than their biosynthetic precursor's *p*-cymene and *γ*-terpinene. Furthermore, *O. vulgare* has an antioxidant property and is applied in

human health. Cervato et al. (2000) prove that the antioxidant activities of extracts of oregano's leaves (both aqueous and methanolic extracts) can inhibit all phases of lipid peroxidative process.

The bioactivity of commercial essential oils of *O. vulgare* L. was studied In vitro for their antibacterial, antifungal, antioxidative and spasmolytic activities. Oregano was found to be strong antimicrobial agent and had a significant spasmolytic effect on smooth muscle (Lis-Balchin et al. 1996). The fumigant toxicity and insecticidal effect of oregano essential oils for storeroom insects has also been proved (Shaaya et al 1991, Baricevic et al 2001).

1.5. Cultivation of *Origanum vulgare* L.

Wild oregano (*O. vulgare*) is a herbaceous perennial, native in Asia, Europe and North Africa and it is quite tolerant to cold and dryness. During the winter period the aerial parts are destroyed, but the roots maintain their vitality for the revegetation in spring (Makri 2002). The possibility of the cultivation of *Origanum vulgare* in the Mediterranean region has been studied extensively in many years ago (Putievsky and Basker 1977). The yield and the essential oil content were examined during different seasons and stages of growth (Putievsky et al. 1988). Nevertheless the most of commercial oregano from the Mediterranean areas is collected from wild populations in the natural habitats for example in Greece and Turkey (Olivier 1997).

The climatic life zone for *Origanum vulgare* reported to be 5–28°C with an annual precipitation of 0.4–2.7 m and a range of 4.5–8.7 for soil pH is appropriate for its growth (Marzi, 1997). Oregano is good treated as an annual plant in cold climates where it will not over winter well. When it is grown as a perennial, the roots should be divided every 3 years for best growth and flavour (Marzi, 1997). The transplants can be established on fields with dry, well-drained soils that are somewhat alkaline (Kintzios 2002a). Cuttings (transplants) of new shoots (about 30 cm long) are removed in late spring once the leaves are firm enough to prevent wilting when placed in sand. Well-rooted plants are placed in the ground with a plant to plant distance about 30 cm or they planted outside in pots. If seeds are used, they should be sown in a seed box in spring and planted outside when seedlings are 7.5 cm tall (Makri 2002). Beside the soil preparation (ploughing), oregano cultivation demands fertilisation with ammonium phosphate and pest control. However, the most of savoury herbs like oregano are not especially subject to serious damage by diseases or insect pests. This may be due to inhibitory action of their essential oils (Makri 2002).

Harvesting the leaves and stem tips should start at the beginning of flowering stage. The flavour will start to improve after the formation of buds, just before flowering (Putievsky et al. 1988). To harvest a cutting height of the stems approximately at 10 cm from the ground should be used. After cutting, new tillers and shoots will grow and produce next crops (Marzi, 1997).

In central European countries, especially in Hungary, the cultivation of *O. majorana* has a long tradition, while oregano plants (*O. vulgare*) are partially collected from wild habitats even today. To avoid the disadvantages of exploiting oregano directly from the nature, efforts have been started in the area for domestication and cultivation of oregano (Bernáth, 1997).

1.6. Morphological, phytochemical and genetic diversity

Origanum vulgare L. is the species with the highest variability in the genus *Origanum*. Nevertheless, diversity, genetic resources and potential for utilization of *O. vulgare* have not yet been fully explored so that extended research on oregano germplasm is necessary (Mastro 1997). Most of genetic resources and diversity of the genus *Origanum* exists in collections of individual growers, which contain about 600 accessions (Kintzios 2002a). Several and different approaches (for example the Oregano Genetic Resources Network) have been undertaken for the conservation of oregano germplasm, and the activity of international organizations (such as IPGRI International Workshop on Oregano) could be mentioned (Padulosi 1997, Kintzios 2002a).

Taxonomic studies on the basis of morphological characters have shown a high level of diversity and presence of several subspecies for *O. vulgare* (Ietswaart 1980). Nevertheless, only *O. vulgare* L. subspecies *hirtum* has the leaf anatomy which corresponds to that of commercially marketed European oregano (Skoula and Harborne 2002). The large inter- and intraspecific diversities of *O. vulgare* could be observed for leaf and flower colour, trichome density, yield, leaf/stem ratio, or in general for very many morphological characters (Franz and Novak 2002).

The high variability of *O. vulgare* is not only true for morphology but also valid for chemical quality characters (the essential oil and its compounds). A remarkable phytochemical polymorphism with several chemotypes is also reported by several studies on this species that shows marked spatial segregation in nature (Fleisher and Sneer 1982, Chalchat and Pasquier 1998, D'antuono et al. 2000, Radušiene et al. 2005). Broad chemical variations have not only been observed between but also within populations and accessions. Investigations on single plants from a group of *O. vulgare* and their offspring resulted in an unexpected differentiation into different chemotypes including one with a marjoram-like profile (Marn et al 1999). The clearly discriminated chemical groups have also reported in a detailed assessment of several *O. vulgare* subsp. *hirtum* clones (Pasquier, 1997).

This wide heterogeneity could represent the individual genetic diversity, the morpho- and ontogenetic variability or modifications due to environment. Therefore it is important to separate genetic from any other sources of variation before conclusions on e.g. phytochemical breeding values (Franz and Novak 2002).

DNA based molecular markers, which are not affected by environment, have rarely employed for fingerprinting of *O. vulgare*. Katsiotis et al. (2009) have used RAPD markers and rDNA sequences to survey variations of Greek *O. vulgare* subsp. *hirtum*. An attempt on SSR (Simple Sequence Repeats) development on this commercial subspecies was also reported (Novak et al. 2008). A relative high level of genetic polymorphisms in *O. vulgare* which was analysed by Amplified Fragment Length Polymorphism (AFLP) and Selectively Amplified Microsatellite Polymorphic Loci (SAMPL) on intraspecific level were found in our own investigations (Azizi et al. 2009a).

1.7. Breeding of oregano: conventional and biotechnological approaches

Conventional and biotechnological plant-breeding techniques can be applied at the genetic level to improve yield and uniformity of medicinal herbs to bring them into cultivation, and also to modify pharmaceutical potency or toxicity (Canter et al. 2005). Exploitation of the genetic potential of these plants is still in its initial stage, and classical breeding approaches prevail due to the availability of high natural diversity. Nevertheless, the uses of biotechnological tools for example identify and localize genes that control secondary metabolite formation and their transformation are currently in progress (Pank 2007).

The chromosome numbers and the ploidy level of *O. vulgare* are previously reported to be 2n = 2x = 32 (Scheerer 1940). Male sterile (nucleo-cytoplasmic) and male fertile plants have been identified in several natural populations (Kheyr-Pour 1981). Broad accumulated diversity of carvacrol content in *O. vulgare* is one of the most important goals of previously breeding programs, resulting in numbers of cultivars of practical importance (Fleisher and Sneer 1982, Sezik et al. 1993, Skoula et al. 1999).

Genetic improvement is most necessary for oregano plants because of their high chemical and morphological heterogeneity. Taking into consideration both producers' and consumers' needs, efforts of any oregano breeding programme should be directed to the improvement of the following targets: 1st: yield-related parameters, e.g. growth habit, leaf/stem ratio, stress (salt, cold) tolerance and 2nd: quality-related parameters, e.g. high essential oil content and modified oil composition, (Makri 2002, Franz and Novak 2002). To achieve these goals, conventional breeding programs such as selection and hybridisation methods, combined with analytical controls on the variability in the material, can be the most appropriate tools for oregano crop improvement (Bernáth 2002).

Oregano, the world's commercially most valued spice could also be one of the novel targets for medicinal plant biotechnology (Kintzios 2002b). So far, several studies have been reported on the establishment of tissue cultures and the regeneration of plantlets from oregano plants. Different explants used for oregano callus cultures include hypocotyls and cotyledons (Matsubara et al. 1996), shoot apexes (Curtis and Shetty 1996, Shetty et al. 1996), nodal segments (Baricevic 1997), roots (Kumari and Pardha Saradhi 1992) and leaves (Alves-Pereira and Fernandes-Ferreira, 1998). However, the explants derived from *In vitro* grown seedlings, such as established clonal lines are often used by most researchers (Yang and Shetty, 1998).

Somatic embryogesis from cultured oregano tissues has never been reported in the literature. A big problem for oregano thissue culture is vitrification or hyperhydricity, which is a physiological malformation affecting plants regenerated via tissue culture. Some researchers could prevent vitrification in oregano shoot cultures by inoculating them with some soil bacteria, such as *Pseudomonas mucidolens* and *Beijerinkia indica* (Shetty et al. 1996, Bela et al. 1998, Perry et al. 1999).

However, cell, tissue and organ culture offer the opportunity to clonally micropropagate oregano lines with improved some important traits and these techniques can also be used for sustaining elite oregano clones and for conversation of its germplasm to inhibit the rapid genetic erosion of this species.

1.8. Marker Assisted Selection (MAS)

Developed genomics techniques have provided new tools for discovering and tagging novel genes. These tools can enhance the efficiency of classical breeding programs through their use in marker-assisted selection (MAS). In this way, the selection of target traits can be achieved indirectly using molecular markers that are concerned with the trait in question or that are closely linked to such genes (Xu and Crouch 2008). Justifications for the development and use of MAS in plant breeding are relevant to target traits that are difficult to manage through conventional phenotypic selection because they are expensive or time-consuming to measure, or have complex inheritance and also traits whose selection depends on specific environments or developmental stages that influence the expression of the target phenotype (Canter et al. 2005, Xu and Crouch 2008). Such traits, for example content of pharmaceutically important compounds, play an important role in improvement of medicinal and aromatic plants.

Nevertheless, there have been relatively few reports of molecular marker-based approaches to medicinal plant breeding, and not even the most skeletal of genetic maps is available for any of the important medicinal species (Canter et al. 2005). Identifying functional genes and useful DNA markers that can correlate DNA fingerprinting data with selected phytochemical compounds would have extensive applications in breeding of medicinal plants based on marker assisted selection (MAS). Such DNA markers can also be used for quality control of raw materials of medicinal herbs (Joshi et al. 2004).

An attempt has been made to study variations in chemical components and intraspecific variations using AFLP technique and the results proved that AFLP analysis has been found to be useful in predicting phytochemical markers in cultivated *Echinacea purpurea* germplasm and some related wild species (Baum et al. 2001).

1.9. Objectives

Knowledge of genetic diversity of wild and cultivated populations of *Origanum vulgare* is very important to clarify relationships between different subspecies of oregano. The knowledge of morphology, agronomic traits and phytochemical characters of these populations has an important impact on the improvement of oregano crop productivity as well as the conservation of genetic resources. In the perspective of the next breeding projects, more attention should be given to the genetic analysis of diverse genotype sets, which are particularly attractive for association analysis of qualitative traits such as essential oil compositions or special quality characteristics. Such genotype sets encompass a wide genetic and phenotypic diversity and association studies can potentially identify useful genes for use in breeding.

The objectives of the present study were:

1. To investigate genetic diversity in the *Origanum vulgare* germplasm using AFLP and SAMPL markers.
2. To estimate the phytochemical variability and to identify the chemotypes in germplasm.
3. To verify the capacity of the agro-morphological traits for discriminating between populations.
4. To elucidate any trait–trait correlation and marker–trait association using these molecular markers, quantitative phenotypic and chemotypic traits.
5. To clarify the response to soil moisture regime and nitrogen supply of three *O. vulgare* populations.

II. ARTICLES

Azizi A., Wagner C., Honermeier B., Friedt W.
Intraspecific diversity and relationships among subspecies of *Origanum vulgare* revealed by comparative AFLP and SAMPL marker analysis.
Plant Systematic and Evolution (2009) 281: 151–160

Azizi A., Yan F., Honermeier B.
Herbage yield, essential oil content and composition of three oregano (*Origanum vulgare* L.) populations as affected by soil moisture regimes and nitrogen supply. *Industrial crops and products* (2009) 29: 554–561

Azizi A., Hadian J, Honermeier B., Friedt W.
Associations between molecular markers, agro-morphological traits and chemical characteristics in a germplasm collection of the medicinal plant *Origanum vulgare* L. (Manuscript)

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ORIGINAL ARTICLE

Intraspecific diversity and relationship between subspecies of *Origanum vulgare* revealed by comparative AFLP and SAMPL marker analysis

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Abstract The genus *Origanum* is often referred to as an under-utilized taxon because of its complex taxonomy. *Origanum vulgare* L., the most variable species of the genus, is a spice and medicinal herb that is characterized by high morphological diversity (six subspecies). In this study, the relative efficiencies of two PCR-based marker approaches, amplified fragment length polymorphism (AFLP) and selectively amplified microsatellite polymorphic loci (SAMPL), were used for comparable genetic diversity surveys and subspecies discrimination among 42 oregano accessions. Seven assays each of AFLP and SAMPL markers were utilized. Effective multiplex ratio (EMR), average heterozygosity ($H_{av\text{-}p}$), marker index (MI), and resolving power (RP) of the primer combinations were calculated for the two marker systems. UPGMA and Structure analysis along with PCoA plots derived from the binary data matrices of the two markers depicted the genetic distinction of accessions. Our results indicate that both marker systems are suitable but SAMPL markers are slightly more efficient in differentiating accessions and subspecies than AFLPs.

Keywords Genetic diversity · AFLP · SAMPL · Medicinal herb · Origanum vulgare · Subspecies

Introduction

The genus *Origanum* is a member of the *Lamiaceae* family which is widely distributed in Mediterranean areas and Northern Africa (Ietswaart 1980; Kokkini 1997). This genus includes numerous species, subspecies, varieties, and hybrids that can be distinguished individually, but extensive variation still exists. Within the genus, *Origanum vulgare* L. (oregano) is an important commercial herb in the spice industry (Olivier 1997). Ietswaart (1980) distinguished six subspecies of *O. vulgare* on the basis of morphological characteristics: ssp. *hirtum* (Link) Ietswaart, ssp. *vulgare* L., ssp. *virens* (Hoffmannsegg et Link) Ietswaart, ssp. *viride* (Boissier) Hayek, ssp. *gracile* (Kock) Ietswaart and ssp. *glandulosum* (Desfontaines) Ietswaart. At present, most of the commercial oregano from the Mediterranean areas is collected from wild populations in Turkey and Greece (Olivier 1997) without focusing on specific subspecies. Recently, the antimicrobial, antifungal, insecticidal, and antioxidative effects of essential oil and extracts have created great pharmaceutical and industrial interest in oregano (Kulisic et al. 2004; Bakkali et al. 2008). The essential oil of oregano is composed of carvacrol and/or thymol as dominant components, followed by γ-terpinene, *p*-cymene, linalool, terpinen-4-ol, and sabinene hydrate (D'Antuono et al. 2000; Skoula and Harborne 2002). Genetic resources, variability and potential for utilization of *O. vulgare* have not yet been fully explored so that extended research on germplasm conservation is urgently needed (Putievsky et al. 1997; Mastro 1997). A number of studies have shown that variation among the populations of *O. vulgare* may occur with regard to morphological and phytochemical features (Chalchat and Pasquier 1998; D'Antuono et al. 2000). To optimally manage genetic resources for improvement of the cultivars, and to maintain and restore biodiversity, knowledge of genetic diversity within species is indispensable (Karp et al. 1997). DNA-based molecular markers, which are not affected by environmental conditions, have become increasingly important for surveying genetic diversity and

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genotype identification of medicinal plants (Nybom and Weising 2007). These markers can also be taxonomically useful, i.e. for phylogenetic studies to distinguish plant species and subspecies (Wollf and Morgan-Richards 1998; Khan et al. 2000; Raina et al. 2001; Monteleone et al. 2006).

Reports on DNA-based fingerprinting of *Origanum* species are rare (Kaufmann and Wink 1994; Klocke et al. 2002; Ayanoglu et al. 2006). Recently, Novak et al. (2007) carried out a study to identify SSRs (simple sequence repeats) derived from ESTs (expressed sequence tags) of epidermal glands of *O. vulgare* ssp. *hirtum*. Alternatively, amplified fragment length polymorphisms (AFLPs) have proven to be a powerful approach in plant genetics to analyze the relationships between natural and cultivated populations and are also suitable for molecular systematics, gene mapping and marker-assisted plant breeding (Mueller and Wolfenbarger 1999).

The selectively amplified microsatellite polymorphic loci (SAMPL) technique is a modification of the AFLP method (Vos et al. 1995), but it differs from AFLP in the selective amplification step by using one of the AFLP primers in combination with a SAMPL primer which is anchored to compound microsatellite motifs (Vogel and Scolnik 1998). Therefore, the SAMPL approach enables the amplification of microsatellite regions without prior cloning and characterization of specific microsatellite loci. SAMPL markers have been used with other molecular markers for studying DNA polymorphism and genetic diversity, and for analysis of relationships among and within populations of different plant species (Singh et al. 2002; Roy et al. 2004; Negi et al. 2006; Altintas et al. 2008; Sarwat et al. 2008). Until now, subspecies of *O. vulgare* could only be distinguished on the basis of morphological characteristics. Because of the pharmaceutical and economic importance of some subspecies, for example ssp. *hirtum*, it would, however, be highly useful to discriminate them precisely by use of DNA markers. Therefore, the purpose of this study was to compare the relative efficiencies of AFLP and SAMPL markers with regard to their applicability in genetic diversity surveys of oregano accessions and subspecies.

Materials and methods

Plant materials and DNA extraction

A total of 42 accessions of *O. vulgare* were investigated, 39 from the Gatersleben Genebank (IPK, Gatersleben, Germany) along with three cultivated types: "Heracleoticum" from the seed company Pharmasaat (Artern, Germany), and "Creticum" and "Samothrake" from the company Syringa (Hilzingen-Binningen, Germany) (Table 1).

Oregano plants were grown in a greenhouse (Institute of Crop Science and Plant Breeding I, Research Station Rauischholzhausen, Germany). Total genomic DNA was extracted from young leaves (100 mg per plant) of five-week-old plants following the CTAB procedure according to Doyle and Doyle (1990). After RNAse treatment, the DNA content was quantified by use of a NanoDrop ND-1000 UV–visible Spectrophotometer (Labtech International, Ringmer, UK). Genomic DNA of ten plants per accession was bulked and diluted to 25 ng/µl working solution.

AFLP and SAMPL analysis

The AFLP procedure used here is based on that developed by Vos et al. (1995) by using the Invitrogen AFLP Core Reagent Kit and following the manufacturer's instructions. Here, 125 ng genomic DNA (i.e., 5 µl working solution) were digested using *Eco*RI and *Mse*I restriction enzymes, and generated fragments were ligated with double-stranded site-specific adapters using T4-DNA ligase. Ligation was followed by two pre-amplifications using the following cycling conditions: 30 s at 94°C, 60 s at 56°C, and 60 s at 72°C (25 cycles) in a thermocycler (Perkin–Elmer, Waltham, MA, USA). The selective amplification mixture (total volume of 25 µl) consisted of 7.5–12.5 ng fluorescent dye-labeled *Eco*RI primer, 30 ng *Mse*I primer, 0.2 mM of each dNTPs, 2 µl PCR buffer, 0.5 U *Taq*-polymerase (Qiagen, Hilden, Germany), and 5 µl pre-amplified PCR-product in deionized distilled water. Details of the PCR reactions were described by Vos et al. (1995).

The SAMPL analysis was performed according to Vogel and Scolnik (1998). Pre-amplified AFLP library was used as template for selective amplification using fluorescent dye-labeled SAMPL and *Mse*I + three primers. Sequences of the two SAMPL primers were: 5′ C(AC)4(AG)4A 3′ (primer S2) and 5′ G(TG)4(AG)4A 3′ (primer S3). The conditions used for PCR reactions were as described by Singh et al. (2002). Twenty-four primer combinations were tested for both AFLP and SAMPL analysis. Seven of these were selected for each marker on the basis of their ability to generate informative data (Table 2). Selective amplification products were separated on 8% denaturing polyacrylamide gels using an LiCor 4,200 DNA analyzer. Fragment's size was estimated by comparison with a 50–750-bp labeled DNA ladder.

Scoring and analysis of data

AFLP and SAMPL fragments were detected using the RFLPscan 2.1 software package (Scan analytics, Fairfax, USA). The bands were scored for their presence (1) or absence (0) across 42 accessions for all the primer

Table 1 Sources, taxonomic identification and origin of the oregano accessions investigated

Accession no/name	Subspecies	Seed source	Country of origin
ORI 2	ssp. *vulgare*	Gatersleben Genebank	Germany
ORI 7	ssp. *vulgare*	Gatersleben Genebank	–[a]
ORI 8	ssp. *vulgare*	Gatersleben Genebank	–[a]
ORI 14	ssp. *vulgare*	Gatersleben Genebank	Georgia
ORI 15	ssp. *vulgare*	Gatersleben Genebank	Georgia
ORI 16	ssp. *vulgare*	Gatersleben Genebank	Italy
ORI 17	ssp. *vulgare*	Gatersleben Genebank	Italy
ORI 18	ssp. *vulgare*	Gatersleben Genebank	–[a]
ORI 19	ssp. *vulgare*	Gatersleben Genebank	–[a]
ORI 20	ssp. *vulgare*	Gatersleben Genebank	Georgia
ORI 21	ssp. *vulgare*	Gatersleben Genebank	–[a]
ORI 23	ssp. *vulgare*	Gatersleben Genebank	–[a]
ORI 24	ssp. *vulgare*	Gatersleben Genebank	Albania
ORI 26	ssp. *vulgare*	Gatersleben Genebank	–[a]
ORI 27	ssp. *vulgare*	Gatersleben Genebank	Italy
ORI 30	ssp. *vulgare*	Gatersleben Genebank	Italy
ORI 36	ssp. *vulgare*	Gatersleben Genebank	–[a]
ORI 39	ssp. *vulgare*	Gatersleben Genebank	–[a]
ORI 49	ssp. *vulgare*	Gatersleben Genebank	Germany
ORI 10	ssp. *viride*	Gatersleben Genebank	Italy
ORI 11	ssp. *viride*	Gatersleben Genebank	Italy
ORI 29	ssp. *viride*	Gatersleben Genebank	Italy
ORI 31	ssp. *viride*	Gatersleben Genebank	Albania
ORI 35	ssp. *viride*	Gatersleben Genebank	Italy
ORI 43	ssp. *viride*	Gatersleben Genebank	Albania
ORI 25	ssp. *virens*	Gatersleben Genebank	Albania
ORI 33	ssp. *virens*	Gatersleben Genebank	Spain
ORI 28	ssp. *hirtum*	Gatersleben Genebank	Albania
ORI 34	ssp. *hirtum*	Gatersleben Genebank	USA
ORI 42	ssp. *gracile*	Gatersleben Genebank	CSFR
ORI 6	–[b]	Gatersleben Genebank	Hungary
ORI 12	–[b]	Gatersleben Genebank	Italy
ORI 13	–[b]	Gatersleben Genebank	–[a]
ORI 37	–[b]	Gatersleben Genebank	Italy
ORI 40	–[b]	Gatersleben Genebank	Italy
ORI 41	–[b]	Gatersleben Genebank	Italy
ORI 45	–[b]	Gatersleben Genebank	–[a]
ORI 47	–[b]	Gatersleben Genebank	Germany
ORI 50	–[b]	Gatersleben Genebank	Germany
Heracleoticum	ssp. *hirtum*	Pharmasaat, Artern	Germany
Creticum	–[b]	Syringa, Hilzingen-Binningen	Germany
Samothrake	–[b]	Syringa, Hilzingen-Binningen	Germany

[a] Not determined

[b] Unknown

combinations employed to generate a binary data matrix. The total number of fragments scored, the number of polymorphic fragments, and the percentage of polymorphic fragments were determined for each primer pair used. Only polymorphic fragments were used for further data analysis. Genetic similarity based on the Dice coefficient (Dice 1945) was calculated by making a pairwise comparison between all oregano accessions using the Simqual module

Table 2 Total number (*n*) and number of polymorphic bands (np), percentage of polymorphism per assay (*%P*), main values of proportion of accessions containing band (mp), main values of band informativeness (mI_b) and resolving power (Rp) of primer combinations detected by SAMPL and AFLP markers among 42 accessions of *O. vulgare*

Primer combination	*n*	np	*%P*	mp	mI_B	Rp
AFLP						
E-AAC × M-CAA	65	38	58	0.25	0.49	18.8
E-CAT × M-CAT	103	77	75	0.19	0.39	29.9
E-CGA × M-CAT	55	26	49	0.20	0.41	10.6
E-ATG × M-CCC	69	55	80	0.22	0.44	24.4
E-ATG × M-CCG	76	65	86	0.22	0.44	28.3
E-AGT × M-CCC	122	98	79	0.14	0.28	27.1
E-CAG × M-CTC	106	72	68	0.24	0.47	34.1
Total	596	431				
Average	85^{MR}	62^{EMR}	71			24.7
SAMPL						
G(TG)4(AG)4A × M-ACG	99	92	93	0.17	0.33	30.6
G(TG)4(AG)4A × M-GTG	92	89	97	0.14	0.27	24.3
G(TG)4(AG)4A × M-CTC	58	55	95	0.16	0.32	17.8
C(AC)4(AG)4A × M-CAA	63	59	94	0.13	0.26	15.6
C(AC)4(AG)4A × M-CAT	70	64	91	0.11	0.22	14.1
C(AC)4(AG)4A × M-CAC	80	71	89	0.09	0.17	10.9
C(AC)4(AG)4A × M-CAG	96	82	85	0.15	0.30	24.8
Total	558	512				
Average	80^{MR}	73^{EMR}	92			19.7

MR multiplex ratio, *EMR* effective multiplex ratio

of NTSYS-pc software version 2.20e (Rohlf 2000). These similarity coefficients were used to construct dendrograms using the unweighted pair group method with arithmetic averages (UPGMA) employing the SAHN algorithm (sequential, agglomerative, hierarchical, and nested clustering) from this software package. The goodness of fit of the clustering compared with the basic data matrix was also tested by computing the co-phenetic correlation coefficient using the normalized Mantel statistics *Z* test (Mantel 1967) via the COPH and MXCOMP procedures of NTSYS-pc version 2.20e (Rohlf 2000).

Principal coordinate analysis (PCoA) was carried out on the basis of the pairwise genetic similarity matrix using the Dcenter and Eigen procedures of the NTSYS-pc software package (Rohlf 2000). All the statistical analyses were performed for the results of both marker systems.

In order to closely investigate the relationship between subspecies, the AFLP and SAMPL data were also analyzed by the computer program Structure, which clusters populations using a Markov chain Monte Carlo (MCMC) algorithm (Pritchard et al. 2000; Falush et al. 2007). This recent algorithm enables identification of discrete groups on the basis of the genotypes at multiple loci using a Bayesian approach. Structure version 2.3.1 (Hubisz et al. 2009) was used to obtain posterior probabilities of *K* for $K = 1$ through $K = 9$ clusters for each data set (AFLP and SAMPL) using the Admixture model, which allows for potential recombination between inferred clusters. We would expect the *K* clusters identified by Structure to correspond to subspecies. Each value of *K* was evaluated using ten independent MCMC replicates consisting of a burn-in of 10,000 iterations followed by a run of 50,000 iterations. We inferred the number of clusters according to Pritchard et al. (2000) with posterior probabilities of *K* calculated assuming uniform priors on *K* and using for each *K* the maximum value of the probability of the data given *K*, $\ln \Pr(X \mid K)$, obtained over MCMC replicates.

Calculation of effective multiplex ratio (EMR) and marker index (MI)

To obtain a measure of the usefulness of the marker systems, effective multiplex ratio (EMR) and marker index (MI) were calculated for both AFLP and SAMPL markers according to Powell et al. (1996). The multiplex ratio (MR) is calculated as the total number of loci detected per assay, while the effective multiplex ratio (EMR) is the number of polymorphic loci detected per assay. Polymorphic information content (PIC) or heterozygosity (H) for each marker is calculated using the formula of Roldan-Ruiz et al. (2000): $PIC = 2fi\ (1 - fi)$, where fi is the frequency of the amplified allele for a locus. Average heterozygosity for polymorphic bands ($H_{av\text{-}p}$) was estimated by taking the average of PIC values obtained for all markers.

Finally, marker index (MI) was calculated by multiplying the EMR by $H_{av\text{-}p}$ (Powell et al. 1996).

Calculation of resolving power (Rp)

The ability of the primer combinations to differentiate between accessions was assessed by calculating their resolving power (Rp) according to Prevost and Wilkinson (1999) using

$$Rp = \sum I_b$$

where I_b is the band informativeness with $I_b = 1 - [2 \times (0.5 - p)]$, where p is the proportion of accessions containing the band. The resolving power is based on the distribution of detected bands within the sampled accessions.

Results

Polymorphisms detected by AFLP and SAMPL

The seven selected AFLP primer combinations yielded a total of 596 scorable fragments, of which 431 (71%) were

found to be polymorphic. The number of polymorphic bands generated by each AFLP primer combination (Table 2) varied from 26 (E-CGA × M-CAT) to 98 (E-AGT × M-CCC). The level of polymorphism ranged from 49% (E-CGA × M-CAT) to 86% (E-ATG × M-CCG). In the case of SAMPL, as many as 558 scorable bands were visualized including 512 (92%) polymorphic bands. The percentage polymorphism across the 42 oregano accessions ranged from 85% for primer combination C(AC)4 (AG)4A × M-CAG to 97% for G(TG)4(AG)4A × M-GTG. The average polymorphism was 92%. A typical representative SAMPL profile generated by employing the primer combination G(TG)4(AG)4A × M-CTC with a total of 58 amplification products is shown as an example in Fig. 1. Table 2 gives the total number of fragments amplified and the percentage polymorphism per assay (calculated as polymorphic fragments divided by the total number of fragments) detected across all the oregano accessions.

Genetic relationships and cluster analysis

Genetic similarity (GS) matrices were calculated for both AFLP and SAMPL products. GS based on AFLP data across the 42 accessions investigated varied from 0.22 (ORI 28 vs. ORI 49) to 0.71 (ORI 28 vs. ORI 34), and based on SAMPL data from 0.02 (ORI 34 vs. ORI 23 and ORI 34 vs. ORI 41) to 0.67 (ORI 47 vs. ORI 49). The average values of GS shared by the 42 accessions were found to be 0.54 and 0.42 for AFLP and SAMPL, respectively. UPGMA cluster analysis based on genetic similarities obtained for AFLP and SAMPL revealed the genetic relatedness among the oregano accessions (phenetic dendrograms, Fig. 2). The phenograms generated from AFLP and SAMPL data revealed a consistent pattern of grouping. Basically, all oregano accessions of the same defined subspecies (based on Genebank catalog and characterized information) clustered in identical groups by AFLP and SAMPL analysis, except the accessions from ssp. *virens* and some members of ssp. *hirtum*, which are split into different subgroups (Fig. 2). The major difference between the two phenograms is represented by the difference in the first and second clusters and subclusters. The results of principal-coordinate analysis (PCoA) are shown in Fig. 3. The plots of PCoA obtained from AFLP and SAMPL data support the results of UPGMA cluster analysis. The PCoA using AFLP and SAMPL markers revealed that the first two axes explain 74 and 76% of the total variation, respectively. The cultivated accessions along with members of subspecies *hirtum* were clearly separated from other accessions by the first axes (PCoA I: 45.47%) for AFLP data (Fig. 3a), while in case of SAMPL analysis these accessions were separated by the second axes (PCoA II: 30.91%) (Fig. 3b). The Mantel test revealed that the Dice similarity matrices obtained with AFLP and SAMPL markers were always significantly correlated with the respective phenograms (goodness of fit: $Z = 0.87$, $P = 0.001$ and $Z = 0.85$, $P = 0.001$ for AFLP and SAMPL, respectively). In contrast, the correlations between the AFLP and SAMPL matrices were relatively low ($Z = 0.43$, $P = 0.001$) (Rohlf and Fisher 1968).

Structure analysis identified five clusters of accessions for each AFLP and SAMPL data set ($K = 5$ having a posterior probability of one relative to other evaluated values of K). The results of Structure analysis, log-likelihood estimation of cluster number, and assignments of cluster membership for each subspecies estimated for AFLP and SAMPL profiles are summarized in Table 3. The accessions belonging to the subspecies *hirtum* have a relatively high proportion of membership in related clusters inferred from the two marker systems, suggesting good genetic differentiation of this mostly cultivated subspecies from other, wild, subspecies. Based on the AFLP data set, accessions belonging to the subspecies *viride* and *virens* have higher membership in cluster five than other accessions, while these accessions have the highest membership in cluster one based on the SAMPL data set (Table 3). These relationships between subspecies resulting from Structure analysis are in agreement with the UPGMA clusters based on Dice similarity (Fig. 2). However, the Structure analysis also shows that both AFLP and SAMPL methods give different clustering patterns in separating some groups of accessions or subspecies.

Marker index and primer resolving power

Marker index (MI) and resolving power (RP) were calculated to measure the ability of techniques and primer combinations to differentiate accessions and distinguish between subspecies. Effective multiplex ratios (EMR) were estimated at 62 and 73 for AFLP and SAMPL analysis, respectively, and average heterozygosity for polymorphic bands ($H_{av\text{-}p}$) was calculated at 0.22 and 0.20. Therefore, MI was slightly higher for SAMPL than AFLP (14.25 vs. 13.53) in the investigated populations.

For each primer combination, the main value of proportion of accessions containing band (mp), main value of band informativeness (mI_b) and resolving power (Rp) are presented in Table 2. The resolving power (RP) of the different primer combinations ranged between 10.6 (E-CGA × M-CAT) and 34.1 (E-CAG × M-CTC) for AFLP and from 10.9 (C(AC)4(AG)4A × M-CAC) to 30.6 (G(TG)4(AG)4A × M-ACG) for SAMPL primers (Table 2). Average resolving power values of 24.7 and 19.7 were obtained for AFLP and SAMPL primer combinations, respectively.

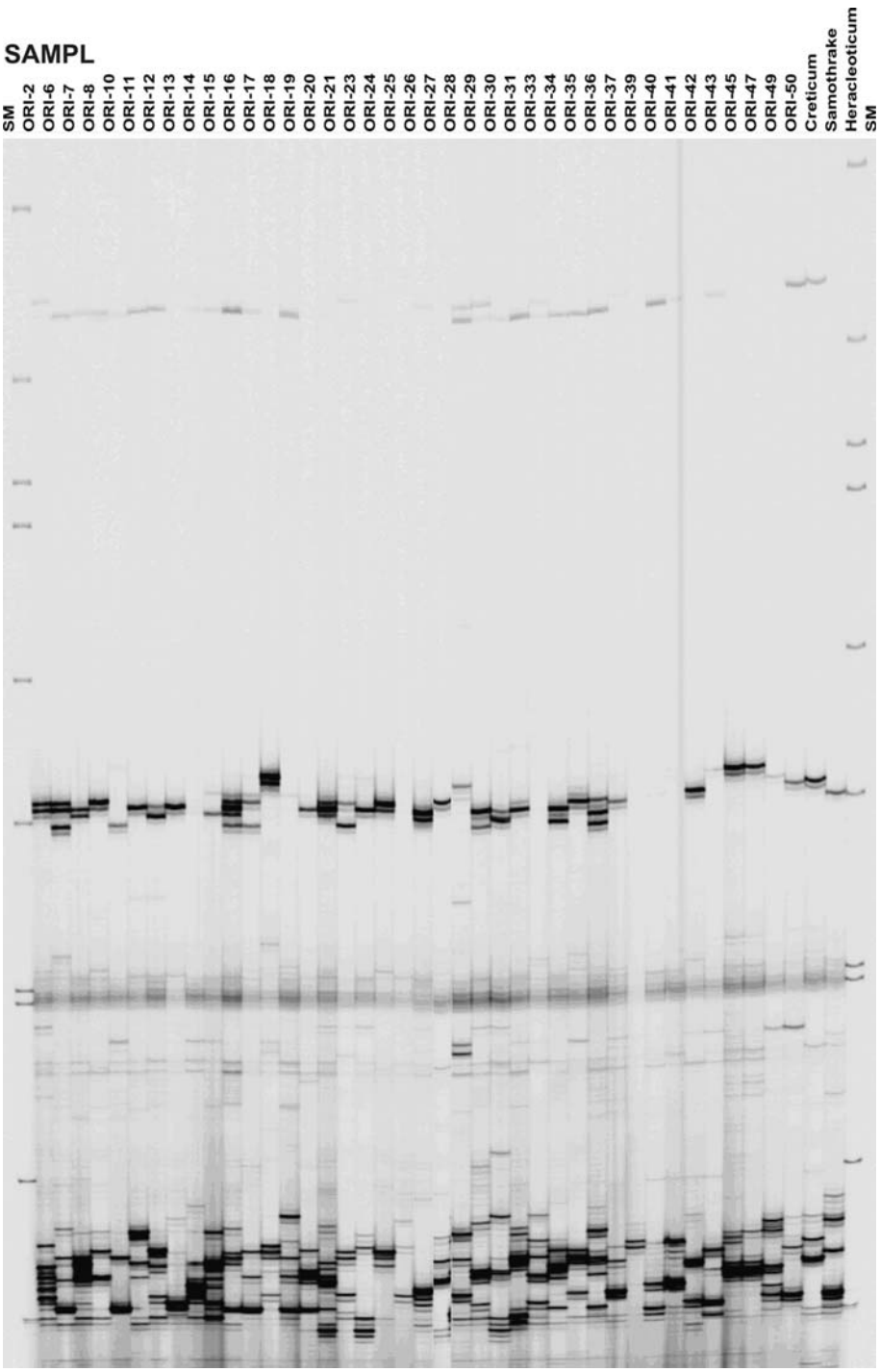

Fig. 1 SAMPL fingerprint of 42 oregano accessions generated with the primer combination G(TG)4(AG)4A × M-CTC, *SM* size marker

Discussion

DNA polymorphisms

This is the first study using AFLP and SAMPL markers to investigate the genetic diversity and subspecies differentiation in *O. vulgare*. In several earlier studies of other species it has been reported that SAMPL is an efficient marker system compared with AFLP. Negi et al. (2006) analyzed the efficiency of SAMPL and AFLP techniques in assessing the levels of genetic diversity among accessions of *Withania somnifera* and *W. coagulans.* In another study, AFLP and SAMPL were used to measure the intra-population genetic variation of *Azadirachta indica* (Singh et al. 2002). Both of these comparative studies confirmed higher effectiveness of SAMPL compared with AFLP.

It is of interest to note that SAMPL is a technique similar to ISSR (inter-simple sequence repeats) but it uses the AFLP

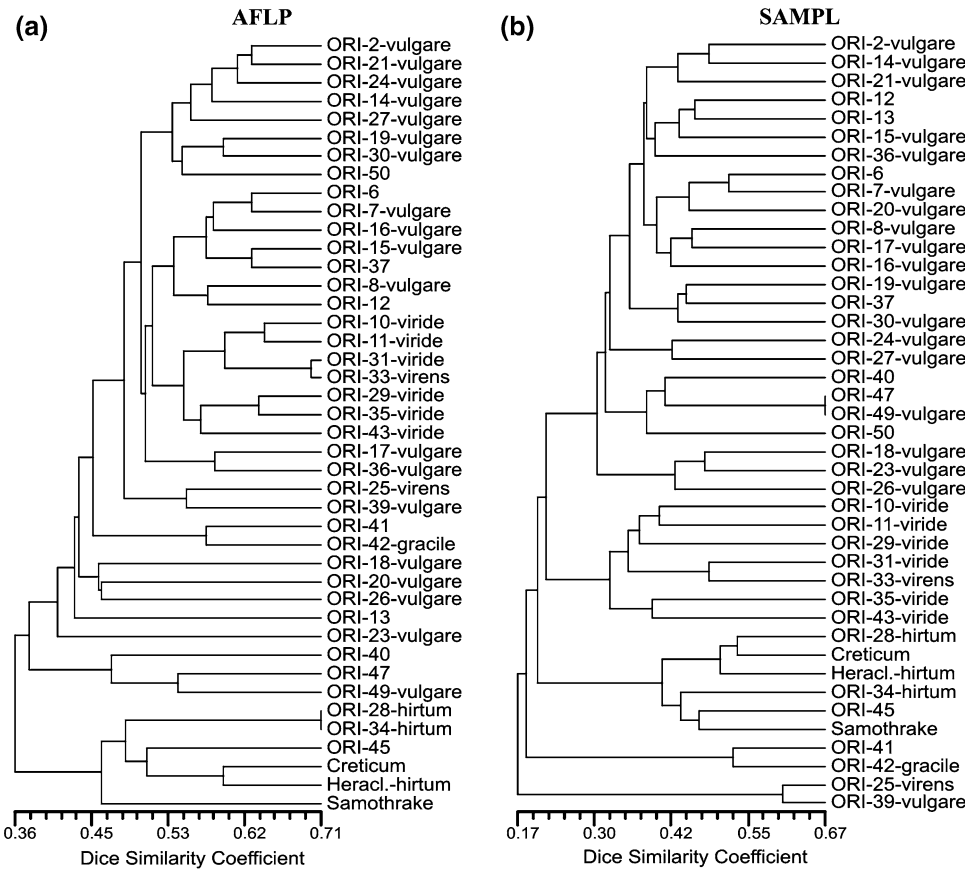

Fig. 2 UPGMA dendrogram showing relationships between 42 *O. vulgare* accessions based on 431AFLP markers (**a**) and 512 SAMPL markers (**b**)

procedure as a starting point to find SSR loci within AFLP-generated fragments. The ISSR technology is based on the amplification of regions (100–3,000 bp) between inversely oriented closely spaced microsatellites. Indeed, ISSR regions can be targeted within the AFLP-generated fragments by the SAMPL procedure (Rakoczy-Trojanowska and Bolibok 2004).

Although SAMPL is technically more demanding than ISSR, findings of some studies were shown to be more suitable for revealing genomic differences than ISSR markers (Bolibok et al. 2005; Sarwat et al. 2008).

In this study, comparison of AFLP and SAMPL marker efficiency in terms of multiplex ratio (MR) or the average number of fragments amplified per assay revealed that MR generated by AFLP was higher than those by SAMPL (Table 1). However, a key observation made on comparing effective multiplex ratios (EMR) of the two marker systems is that SAMPL detected more polymorphic fragments per assay (Table 1), and the data analysis of the 42 *O. vulgare* accessions studied revealed that SAMPL enabled the detection of a higher degree of polymorphism than AFLP analysis.

The marker index (MI) is a convenient estimate for marker efficiency (Milbourne et al. 1997). SAMPL analysis yielded a slightly higher MI than AFLP within oregano accessions (14.25 for SAMPL vs. 13.53 for AFLP). This is because of the EMR component, which was shown to be higher for the SAMPL assay. This result is also attributed to the lower proportion of polymorphic bands obtained by AFLP markers, even though the AFLP system generated a higher number of loci per assay. This result corroborates those from other studies of *Vigna* (Tosti and Negri 2002), *Secale* (Bolibok et al. 2005), and *Tribulus* (Sarwat et al. 2008) in which the MI value for the SAMPL markers was shown to be higher than that for the AFLP markers. According to Negi et al. (2006), among different species of *Withania*, the MI detected by AFLP markers was higher than that obtained by SAMPL. However, if a single species is considered the MI detected by SAMPL markers may be higher than that obtained by AFLP. In a study of genetic variation among Eurasian *Isatis tinctoria* populations Spataro et al. (2007) found that AFLP estimated a slightly higher genetic diversity than SAMPL. In contrast, the results of this study of oregano indicate that SAMPL markers were slightly more efficient at detecting DNA polymorphism among accessions. This is considered to be because of targeting of hyper-variable microsatellite loci in the genome by SAMPL analysis.

However, the number of scorable bands for AFLP is quite high in comparison to SAMPL which may be an

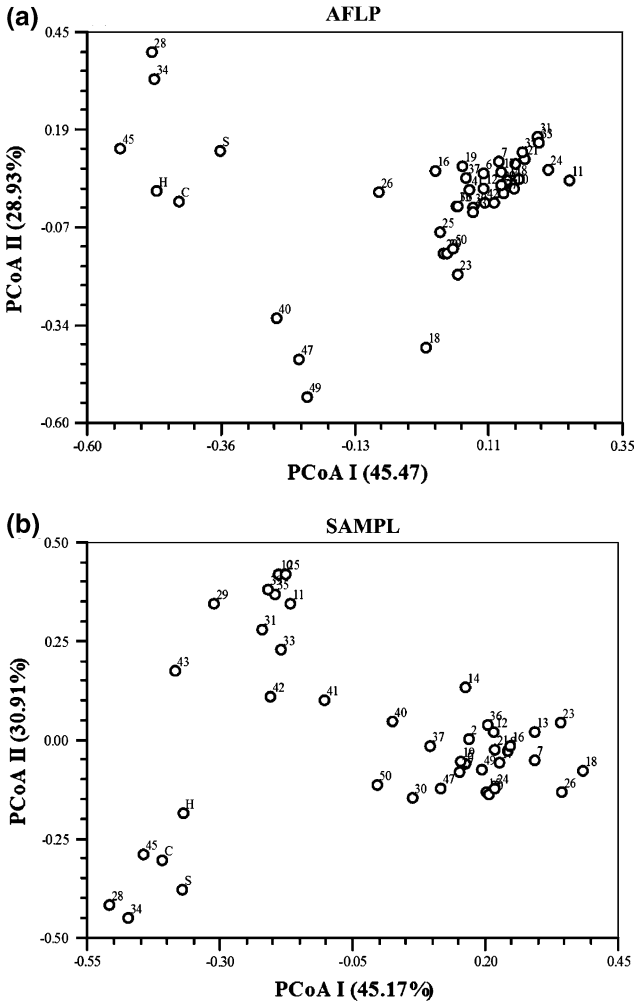

Fig. 3 Principal coordinate analysis (PCoA) plots obtained from AFLP (**a**) and SAMPL (**b**) markers showing the relationships among 42 accessions of *Origanum vulgare* L. The *numbers* represent the accession number and *C*, *H* and *S* represent "Creticum", "Heracleoticum", and "Samothrake", respectively

additional advantage of AFLP, thus increasing its power to detect polymorphisms (Roy et al. 2004). In our study we used seven informative primer combinations to compare the two techniques and detected unequal numbers of scorable polymorphic bands for the techniques. In case of a higher number of scorable bands (maybe by a higher number of primer combination sets), the effect observed could be reduced.

For the resolving power, p (proportion of accessions containing band) is a considerable factor; p factors are calculated for each locus containing polymorphic bands. Nevertheless, the number of polymorphic bands is also an important property. For example the main values of p and I_b for primer combination E-AGT × M-CCC were relatively low (0.14 and 0.28, respectively) but the resolving power of this primer combination was calculated to be relatively high (27.1; Table 2). This effect is because of the large number of scorable bands (98 polymorphic bands).

Discrimination of subspecies

According to the Genebank catalogue (IPK-Gatersleben, http://gbis.ipk-gatersleben.de/gbis_i/) the 42 accessions evaluated in this study belong to different subspecies including 19 from ssp. *vulgare*, six from ssp. *viride*, three from ssp. *hirtum*, two from ssp. *virens*, one from ssp. *gracile*, and 11 from undefined subspecies (Table 1). Earlier studies of oregano plants in Greece showed that the pharmaceutical properties of subspecies can be different (Kokkini and Vokou 1989). For example, the subspecies *hirtum* contained a large amount of essential oil (8%) with carvacrol as dominant component (95%).

In recent years, molecular and phytochemical markers have been used for authentication and interpretation of medicinal plant phylogeny at different taxonomic levels. For example, RFLP and RAPD markers were successfully used to distinguish subspecies of *Plantago* (Wollf and Morgan-Richards 1998), and AFLP markers have been used to discriminate species of *Erothroxylum* (Johnson et al. 2005) and *Plectranthus* (Passinho-Soares et al. 2006). In our study, two clustering patterns, UPGMA and Structure analysis, with PCoA based on AFLP and SAMPL data revealed clear separation of the subspecies, demonstrating their high genetic differentiation at the DNA level. However, the relationship between subspecies as observed in this study by AFLP and SAMPL markers was somewhat different. According to the RP values of all primer combinations, the AFLP primers discriminated the *O. vulgare* accessions better than the SAMPL primers.

In general, our results of AFLP and SAMPL analysis confirm the morphological classification.

In conclusion, the SAMPL approach seems to be a suitable tool to strengthen the resolution of the AFLP technique. It has been shown here to be powerful for taxonomic investigation of *O. vulgare* and identification of subspecies of oregano. This may also be true for other plant taxa. The lack of correlations between similarity and cophenetic matrices obtained with the data from the two marker systems suggests, however, that different marker systems should be used simultaneously for a genetic diversity study to best estimate the level of genetic diversity and delineate the genetic relatedness. Obviously, different molecular markers survey different regions of the genome and detect different kinds of polymorphism, leading to higher genetic resolution in combination compared to single analyses alone.

On the basis of this combined AFLP and SAMPL analysis it can be concluded that substantial genetic diversity exists among oregano populations. This finding is

Table 3 Results of Structure analysis: log-likelihood estimation of cluster number, and assignments of cluster membership for each subspecies for AFLP and SAMPL profiles

Estimate for AFLP data	*K* (no. clusters)					
	1	2	3	4	5	6
Highest ln Pr($X \mid K$)	−8686.1	−8275.1	−8246.3	−8148.7	−8071.7	−8196.3
Δ ln-likelihood	−614.4	−203.4	−174.6	−77	0	−124.6
Posterior Pr(K)	2×10^{-267}	6×10^{-89}	2×10^{-76}	3×10^{-34}	1	1×10^{-54}

Proportion of membership of subspecies in inferred clusters		Cluster membership				
Subspecies	No. accessions	1	2	3	4	5
Vulgare	19	0.023	0.429	0.308	0.133	0.107
Viride	6	0.005	0.022	0.115	0.055	0.803
Virens	2	0.01	0.256	0.183	0.075	0.476
Hirtum	3	0.761	0.009	0.009	0.215	0.005
Gracile	1	0.005	0.584	0.04	0.29	0.08
Unknown	11	0.091	0.212	0.23	0.401	0.066

Estimate for SAMPL data	*K* (no. clusters)					
	1	2	3	4	5	6
Highest ln Pr($X \mid K$)	−8915.6	−8545.4	−8494.3	−8311.2	−8258.6	−8283.8
Δ ln-likelihood	−657	−286.8	−235.7	−52.6	0	−25.2
Posterior Pr(K)	4×10^{-286}	2×10^{-125}	3×10^{-103}	9×10^{-24}	1	1×10^{-11}

Proportion of membership of subspecies in inferred clusters		Cluster membership				
Subspecies	No. accessions	1	2	3	4	5
Vulgare	19	0.059	0.259	0.029	0.021	0.633
Viride	6	0.715	0.140	0.004	0.100	0.041
Virens	2	0.672	0.195	0.005	0.036	0.092
Hirtum	3	0.030	0.040	0.003	0.911	0.015
Gracile	1	0.115	0.367	0.003	0.417	0.098
Unknown	11	0.030	0.177	0.092	0.308	0.393

$K = 5$ clusters is favored for AFLP and SAMPL data sets

very important for management of its genetic resources and for domestication and breeding programs in oregano.

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Herbage yield, essential oil content and composition of three oregano (*Origanum vulgare* L.) populations as affected by soil moisture regimes and nitrogen supply

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ABSTRACT

To compare the response of oregano (*Origanum vulgare* L.) populations to soil moisture regimes and nitrogen fertilization, a greenhouse experiment with three populations of oregano cultivated in Germany (*O. vulgare* var. *creticum*, *O. vulgare* ssp. *hirtum*, *O. vulgare* var. *samothrake*) was conducted during 2006–2007 at the research station Rauischholzhausen of Justus Liebig University in Germany. A completely randomized experimental design with three soil moisture regimes (optimal, consistent water deficiency and water deficiency from the beginning of flowering) and two nitrogen fertilization levels with six replications was realized. Dry matter production of population *O. vulgare* var. *samothrake* was stable for two experiment years, whereas those of the populations *O. vulgare* var. *creticum* and *O. vulgare* ssp. *hirtum* were higher in 2007 than in 2006. Among tested populations *O. vulgare* var. *samothrake* showed the highest essential oil content in both experiment years. Consistent water deficiency caused reduction of dry matter, but not essential oil content. Water deficiency in flowering stage reduced also dry matter production, but increased essential oil content, resulting in the highest essential oil yield in 2006 and a comparable essential oil yield as control in 2007. Higher nitrogen levels caused an increase in dry matter production of oregano for both experiment years and a decrease in essential oil content in 2007, which can be explained in terms of dilution effect. Totally, 42 compounds were identified in essential oils of three populations by means of GC–MS. Carvacrol was the dominant compound (70.0–77.4%) for all essential oil samples, followed by γ-terpinene (8.1–9.5%) and *p*-cymene (4.5–5.3%). The composition of essential oil of oregano populations was independent of cultivation conditions. In conclusion, the population of *O. vulgare* var. *samothrake* showed a stable dry matter yield with higher essential oil content than the populations of *O. vulgare* var. *creticum* and *O. vulgare* ssp. *hirtum*. Water deficiency after beginning of blooming (folded flowers) can induce an increase in essential oil content and thus result in higher quality of oregano herbage and higher water use efficiency of oregano plants.

1. Introduction

The genus *Origanum* belongs to the family of *Labiatae* and includes many species that are commonly found as wild plants in the Mediterranean areas (Skoula and Harborne, 2002). Because of special compositions of essential oil the leaves of *Origanum* plants are widely used as a very popular spice for food production. Recently, this spice plant has drawn

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more attention of consumers due to the antimicrobial, antifungal, insecticidal and antioxidative effects of this herb on human healthy (Kokkini, 1997; Kulisic et al., 2004; Bakkali et al., 2008). The commercial products of *Origanum* leaves are known as oregano or marjoram on the market (Olivier, 1997).

The essential oil of oregano is composed of carvacrol and/or thymol as dominant components, followed by γ-terpinene, *p*-cymene, linalool, terpinen-4-ol and sabinene hydrate (Kokkini et al., 1997; D'antuono et al., 2000; Skoula and Harborne, 2002). Results of various studies indicated that the antioxidant effects of oregano might be related to the dominant components, carvacrol and thymol, of the essential oil (Lagouri et al., 1993; Aeschbach et al., 1994; Yanishlieva et al., 1999).

The quality of oregano is determined mainly by the essential oil content and the composition of the essential oil. Both parameters may vary considerably depending on genotypes, climate conditions and nutrient supply during the cultivation (D'antuono et al., 2000; Novak et al., 2003). In addition, the components of oregano essential oil seem to be determined to a greater extent by genotype, while environment conditions account only for smaller variation in the components of the essential oil (Novak et al., 2003). Studies on oregano plants in Greece showed that the subspecies *hirtum* (*O. vulgare* ssp. *hirtum* (Link) Ietswaart, syn.: *Origanum heracleoticum*) contained a high amount of essential oil. The content of essential oil as high as 8% with carvacrol as dominant component (95%) was reported for this subspecies (Kokkini and Vokou, 1989). Because of its high essential oil content with high percentage of carvacrol, this subspecies was systematically and widely cultivated in Greece and known as "Greek oregano" (Chatzopoulou et al., 2004).

In Mediterranean countries, *O. vulgare* var. *creticum* (syn: *Origanum creticum*) was found to contain essential oil with a wide range of carvacrol percentage from 3% to 68% (Bernáth, 1997). In Germany, *O. vulgare* var. *samothrake* (syn.: *Origanum samothrake*) was widely cultivated and the products of leaves are known as pepper oregano. So far, there is no report on the yield and composition of the essential oil of this cultivated population in literature.

Besides genotype, cultivation conditions may also influence the essential oil content of oregano leaves and the composition of the essential oil. For example, nitrogen fertilization affected the composition of the essential oils by increasing the percentage of thymol and carvacrol with a simultaneous decrease of γ-terpinene and *p*-cymene in *Origanum syriacum* (Omer, 1999). Furthermore, in the flower head of *Chrysanthemum coronarium*, the composition of essential oil was affected by fertilization. In comparison with control (no fertilization), NPK fertilization caused an increase in camphor with a simultaneous decrease of germacrene D (Alvarez-Castellanos and Pascual-Villalobos, 2003). In the similar way, fertilization significantly changed geranial and citronellal in essential oil of *Leptospermum petersonii* (Diatloff, 1990).

In addition, the water supply is one of the most determinative cultivation conditions which significantly affect the yield and essential oil content of various spices and herb crops (Singh et al., 2000, 2002; Zehtabi-Salmasi et al., 2001; Delfine et al., 2005). In most cases *Origanum* plants must be irrigated during the cultivation period to obtain a good yield. For example during cultivation of *Origanum dictamnus* in Crete (Greece), irrigation was necessary for two harvests in 1 year (Skoula and Kamenopoulos, 1997). Practically, the time at which the plants are irrigated is important for the efficiency of irrigation. For example, appropriate irrigation strategies showed a great potential for improvement of the yield of monoterpenes in field-grown spearmint and rosemary (Delfine et al., 2005).

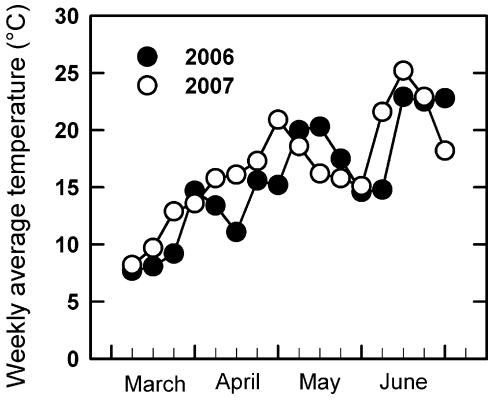

Fig. 1 – Weekly average temperature of greenhouse for the two growing seasons of oregano in 2006 and 2007.

According to Putievsky et al. (1988) essential oil content of oregano was higher in full bloom stage than in the stage of start flowering. Therefore irrigation schedule for oregano plants based on developmental stages may provide an approach for optimizing irrigation efficiency in the cultivation regions where water resources are limited.

The objective of this study was to evaluate the responses of three cultivated populations of oregano to different water supply regimes during developmental stages and to nitrogen fertilization.

2. Materials and methods

2.1. *Experiment design*

2.1.1. *Plant and soil materials*

The experiment was conducted in the greenhouse of the research station Rauischholzhausen of Justus Liebig University, Germany from 2006 to 2007. The weekly mean temperatures of greenhouse during the experiment period are shown in Fig. 1. Three populations of oregano were used in this study. The seeds of *O. vulgare* ssp. *hirtum* (syn.: *O. heracleoticum*) were provided by the company Pharmasaat (Artern, Germany) and the seeds of *O. vulgare* var. *creticum* (syn.: *O. creticum*) and *O. vulgare* var. *samothrake* (syn.: *O. samothrake*) were obtained from the company Syringa (Hilzingen-Binningen, Germany).

The soil used in this experiment was a loess soil from the research station Rauischholzhausen. The soil material was taken from the layer of 0–20 cm. The soil contained 7.8 mg P/100 g, 14.9 mg K/100 g and 1.42 mg N/100 g. The sieved soil (<2 mm) was homogenized, air-dried and mixed with sand (soil: sand = 1:2 w/w) and fertilized with P, K, Mg and $CaCO_3$

(P: 0.3 g as $Ca(H_2PO_4)2H_2O$, K: 1.5 g as K_2SO_4, Mg: 0.1 g as $MgSO_4 \cdot 7H_20$ and $CaCO_3$: 3.0 g per pot) to warrant optimal nutrient supply (except for nitrogen) for plant growth. The mixed soil showed pH 6.7 (in H_2O).

In March 2006, 12-day-old plant seedlings with four leaves were transplanted from seedling bed into Mitscherlich pots with a volume of 6 l (5 plants per pots). Two harvests were carried out at the full flowering stage in 2006 and 2007. The date of harvests for the populations *O. vulgare* ssp. *hirtum* and *O. vulgare* var. *creticum* were 27th June 2006 and 22nd June 2007, and for *O. vulgare* var. *samothrake* 3rd July 2006 and 29th June 2007, respectively. For harvesting the plant material was cut 5 cm above soil surface. After determination of fresh matter, plant samples were dried in an air-circulating oven at 40 °C for 4 days and then the dry matter was determined. Between two growing seasons the plant growth was minimal. To warrant a good comparison, plants were uniformly cut shortly before the beginning of the second growing season.

2.1.2. *Water supply regimes*

Three water supply regimes were scheduled based on developmental stages and were applied to the oregano plants 2 weeks after transplanting:

1. *Control (W1)*: In this treatment soil water content was maintained at 60% of maximal water-holding capacity during the seedling development stage. During the stem elongation and flowering (at the beginning of flowering) development stages soil water content was maintained at 70% of maximal water-holding capacity. Soil water content was controlled by daily weighing. To prevent nutrient leaching, a dish was placed under each pot and the leachet collected in the dish was given back to soil before watering.
2. *Water deficiency (W2)*: In this treatment the soil water content was constantly maintained at 50% of the maximal water-holding capacity throughout the whole cultivation period.
3. *Late water deficiency (W3)*: In this treatment the soil water content was maintained at 60% and 70% of the maximal water-holding capacity during the seedling and stem elongation development stages, respectively. During the flowering stage the water content was then reduced to 50% of maximal water-holding capacity.

2.1.3. *Nitrogen fertilization*

Two nitrogen fertilization levels were applied. In the experiment 2006, 0.5 g (N1) and 1 g N (N2) per pot were applied. In the following year (2007), the amount of nitrogen supply was changed to 0.5 and 1.5 g per pot for the treatment N1 and N2, respectively. Nitrogen fertilizer (NH_4NO_3) was thoroughly mixed with soil before transplanting in 2006. For the second year (2007) fertilization was carried out in March of 2007. The fertilizer was dissolved with water and top-dressed to the soil.

2.2. *Essential oil extraction*

Samples of at least 20 g of dried leaves and inflorescences were hydro-distilled for 3 h using a Clevenger-type apparatus (Europäische Arzneibuch, 1997). The essential oil content was gravimetrically quantified. Each sample was analyzed two times and the average content of essential oil was used for further statistic evaluation. The essential oil obtained was kept at 4 °C until further analysis.

2.3. *GC and GC–MS analyses*

For determination of the composition, essential oil samples were diluted by 100 times with hexane. The identification of the components of the essential oil was realized by gas chromatography-mass spectrometry (GC–MS). A Varian 3900 GC coupled with a Varian Saturn 2100T ion trap mass detector was employed for the identification. A capillary column VF-5ms (30 m × 0.25 mm i.d. and 0.25 μm coating thickness) was used for separation of the components. Helium was used as carrier gas with a flow rate of 1.1 ml/min. Temperature program for oven and injector was the same as for the GC (see below). The samples were injected through an autosampler (Varian CP-8400). Ionization was realized by electron impact at 70 eV, electron multiplier 2200 V, ion source temperature 230 °C and transfer line temperature 240 °C. Mass spectral data were acquired in the scan mode in the *m*/*z* range 35–450.

The identification of components of the essential oil was based on comparison of Kovat's retention indices and mass spectra in corresponding data libraries (Adams, 1995; Figuérédo et al., 2006) and mass spectra libraries (Weily 90 and NIST 98). Kovat's retention indices were calculated from the gas chromatogram by linear interpolation between bracketing *n*-alkanes. The *n*-alkanes (C_8–C_{24}; Alfa Aesar Karlsruhe, Germany) were used as standards. The main components carvacrol, γ-terpinene and *p*-cymene were further identified by co-injection of authentic standards (Roth, Karlsruhe, Germany).

For quantification of individual components, the essential oil was analyzed using a Varian CP-3800 gas chromatograph equipped with flame ionization detector (GC-FID). A capillary column DB-5 (30 m × 0.25 mm i.d. and 0.25 μm coating thickness) was used for the separation of individual components of the essential oil. Helium was employed as the carrier gas with a flow rate of 1.1 ml/min. Temperature was programmed from 60 (5 min), to 250 °C with a ramp rate of 5 °C/min, with a final hold time of 10 min. The injector and detector were maintained at 260 and 280 °C, respectively. The sample (1 μl) was injected with 1:50 split ratio by an autosampler (Varian 8200CX).

The percentage of individual components was computed from peak areas. Response factors of detector and FID normalization were considered for the data processing.

2.4. *Statistical analyses*

A completely randomized experimental design was conducted as a three-factorial pot experiment with six replications. The tested factors were oregano populations, water supply and nitrogen fertilization. Variance analysis for three-factorial experiment design was carried out using the SPSS program for windows version 15. LSD values were calculated and used for the comparison of means of different treatments within one factor.

3. Results

3.1. Differences in dry matter production, the content and yield of essential oil among oregano populations

Variance analysis showed there was significant difference for each factor, but no significant interactions between them. Within factor of population, significant differences in dry matter production and essential oil content were observed during an experiment period of 2 years with two harvests. During the experiment period of 2006, an average dry matter of 36 g/pot was determined for *O. vulgare* var. *creticum* and *O. vulgare* var. *samothrake*, while *O. vulgare* ssp. *hirtum* showed a significantly higher dry matter of 40 g/pot (Fig. 2a). During the experiment period of 2007 (second year), a comparable dry matter of 45 g/pot was determined for both *O. vulgare* var. *creticum* and *O. vulgare* ssp. *hirtum*, while a significantly lower dry matter of 35 g/pot was determined for *O. vulgare* var. *samothrake* (Fig. 2a). It was also evident that in comparison with the experiment period 2006, the dry matter of oregano in 2007 was higher for *O. vulgare* var. *creticum* and *O. vulgare* ssp. *hirtum*, but not for *O. vulgare* var. *samothrake*. It seems that the yield potential of *O. vulgare* var. *samothrake* is significantly stable but lower than that of *O. vulgare* var. *creticum* and *O. vulgare* ssp. *hirtum*. However, for both harvests this population showed a significantly higher essential oil content than the other two populations (Fig. 2b). In addition, the essential oil content of oregano harvested in 2007 was higher than that of oregano harvested in 2006 for *O. vulgare* var. *creticum* and *O. vulgare* var. *samothrake*, but not for *O. vulgare* ssp. *hirtum*. The essential oil yield of oregano was calculated by multiplying dry matter with essential oil content and expressed as g/pot. In general, the essential oil yield was higher for 2007 than for 2006 (Fig. 2c). The essential oil yield was same for all three tested oregano populations in 2006. However, in 2007, the population *O. vulgare* var. *creticum* and *O. vulgare* var. *samothrake* showed significantly higher essential oil yield than *O. vulgare* ssp. *hirtum* (Fig. 2c).

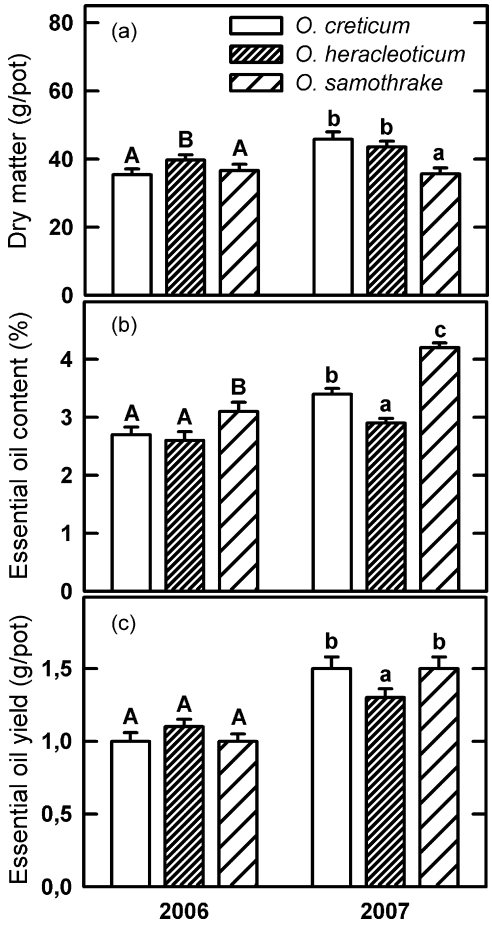

Fig. 2 – Differences in dry matter (a), essential oil content based on dry matter (b) and essential oil yield (c) of oregano populations. Values represent means ± S.E. Significant differences among populations were measured by the least significant difference (LSD) at $P < 0.05$ and indicated by different letters.

3.2. Effects of water supply regimes on the dry matter production, the content and yield of essential oil

Water deficiency significantly decreased dry matter production for two harvests in 2006 and 2007 (Fig. 3a). In comparison with control, a dry matter decrease of 17% and 11% was recorded for consistent and later water deficiency, respectively, during the experiment period of 2006. In the following year, an additional differentiation of the effect of water deficient treatments on dry matter was observed. In comparison with control (W1), dry matter of consistent water deficiency (W2) was reduced by 30%, while later water deficiency (W3) caused only 10% loss of dry matter production (Fig. 3a). Compared to the experiment period of 2006, the dry matter of oregano was higher in the experiment period of 2007 for control (W1) and later water deficiency (W3). However, the oregano dry matter from the treatment of consistent water deficiency (W2) was comparable between two harvests. In addition, later water deficiency significantly increased essential oil content of oregano for both harvests (Fig. 3b). Conversely, consistent water deficiency (W2) showed no effect on essential oil content. Compared to the experiment period of 2006, the essential oil content of oregano in 2007 was higher for control (W1) and for consistent water deficiency (W2), but not for later water deficiency (W3). In 2006, essential oil yield of oregano was comparable for control (W1) and consistent water deficiency (W2), while a significantly higher essential oil yield was measured for later water deficiency (W3) (Fig. 3c). In 2007, both control (W1) and later water deficiency (W3) showed a comparable essential oil yield, which was significantly higher than that of consistent water deficiency (W2) (Fig. 3c).

3.3. Effects of nitrogen supply on the dry matter production, the content and yield of essential oil

In comparison with low nitrogen level (N1: 0.5 g N/pot), higher nitrogen levels (N2: 1 g N/pot for 2006 and 1.5 g N/pot for 2007, respectively) significantly increased the dry matter production of oregano (Fig. 4a). In addition, the increment was 9% and 26% for the harvest in 2006 and 2007, respectively. Since the dry matter of low nitrogen level (N1) was comparable between two harvests, the result indicates a nitrogen dose dependency of

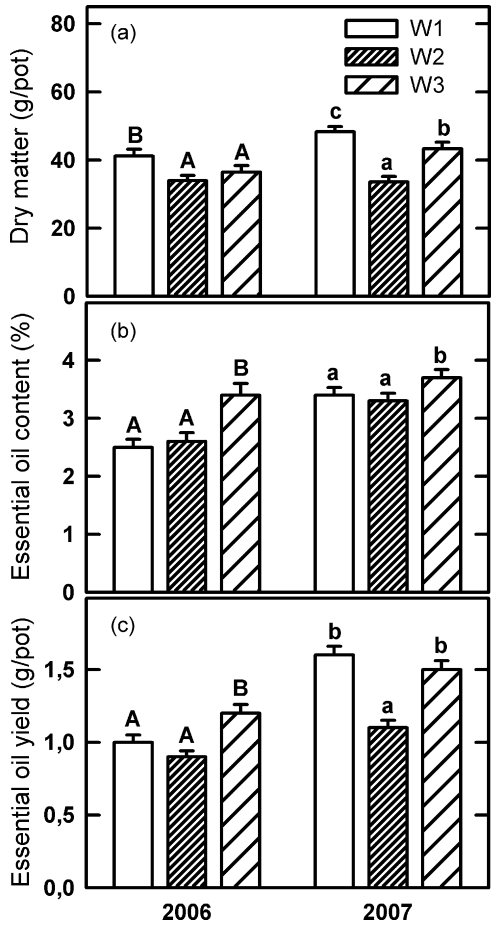

Fig. 3 – Effects of water supply regimes on the dry matter (a), essential oil content based on dry matter (b) and essential oil yield (c) of oregano. W1: 60% for seedling stage and 70% water-holding capacity for stem elongation and flowering stages; W2: 50% water-holding capacity throughout whole growth period; W3: 60% water-holding capacity for seedling stage, 70% water-holding capacity for stem elongation stage and 50% water-holding capacity for the stage after flowering. Values represent means ± S.E. Significant differences among different water supply regimes were measured by the least significant difference (LSD) at $P < 0.05$ and indicated by different letters.

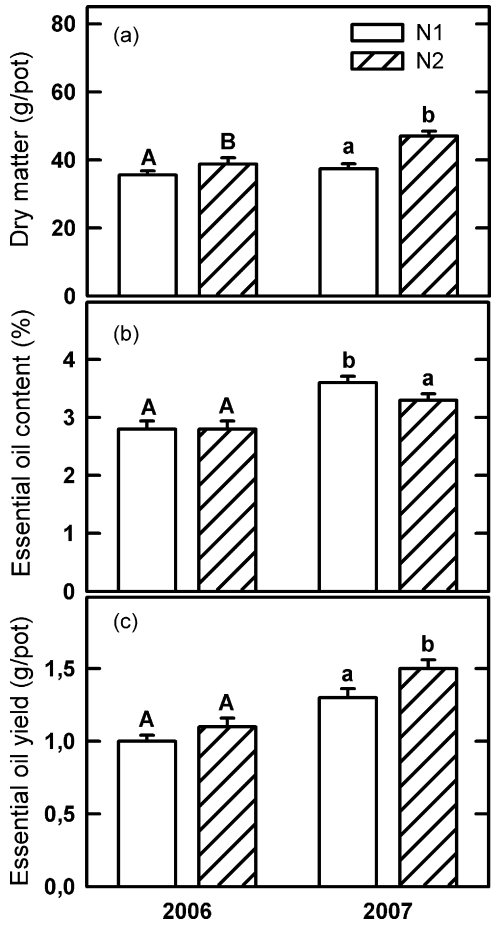

Fig. 4 – Effects of nitrogen supply levels on the dry matter (a), essential oil content based on dry matter (b) and essential oil yield (c) of oregano. N1: 0.5 g N/pot; N2: 1.0 and 1.5 g N/pot for the experiment 2006 and 2007, respectively. Values are means ± S.E. Significant differences between nitrogen supply levels were measured by the least significant difference (LSD) at $P < 0.05$ and indicated by different letters.

dry weight of oregano. The essential oil content of oregano was not affected by the nitrogen levels in the experiment period of 2006. However, significantly lower essential oil content was caused by the higher nitrogen level in 2007 (Fig. 4b). Compared to the harvest in 2006, the essential oil of oregano in 2007 was higher for both nitrogen levels. The essential oil yield was not affected by different nitrogen fertilization levels in 2006 (Fig. 4c). On the contrary, a significantly higher essential oil yield was recorded for the higher nitrogen fertilization level (N2) in 2007 (Fig. 4c).

3.4. *Essential oil composition of oregano*

Totally, 42 constituents were identified for the oregano essential oil: 36 constituents for *O. vulgare* ssp. *hirtum*, 30 constituents for *O. vulgare* var. *creticum* and 27 constituents for *O. vulgare* var. *samothrake* (Table 1). Carvacrol content was the dominant constituent of the essential oil for all three populations tested, ranging from 70.0% to 77.4%. The second major constituent was γ-terpinene (ranging from 8.1% to 9.5%) and the third one was *p*-cymene (ranging from 4.5% to 5.3%). The other main constituents were trans-sabinene hydrate (2.8%) for *O. vulgare* ssp. *hirtum*, thymol (3.7%) for *O. vulgare* var. *creticum*, and β-caryophyllen (2.8–3.1%) for all three populations.

The differences in three major constituents of essential oil of oregano were studied further. Among three populations tested, the carvacrol percentage of essential oil was significantly lower for *O. vulgare* var. *samothrake* as compared with *O. vulgare* var. *creticum* or *O. vulgare* ssp. *hirtum* during two successive growing seasons (Fig. 5a). Correspondingly, the percentage of γ-terpinene was significantly higher for *O. vulgare* var. *samothrake* as compared to *O. vulgare* var. *creticum* or *O. vulgare* ssp. *hirtum* (Fig. 5b). This relationship between carvacrol and

Table 1 – Essential oil composition[a] (%) of three populations of *Origanum vulgare* L. The plants were grown with optimal soil moisture regime (W1) and 0.5 g N/pot in 2007.

Compounds	RI[b]	*O. vulgare* ssp. *hirtum*	*O. vulgare* var. *creticum*	*O. vulgare* var. *samothrake*
α-Thujene	924	0.6	0.4	0.3
α-Pinene	931	0.3	0.2	0.2
Camphene	948	0.1	0.1	–
Sabinene	971	0.2	–	–
β-Pinene	976	0.1	–	–
1-Octen-3-ol	978	0.1	0.2	0.2
3-Octanone	984	0.1	–	–
Myrcene	988	1.3	0.8	0.7
α-Phellandrene	1006	0.2	0.1	–
δ-3-Carene	1008	0.1	–	–
α-Terpinen	1016	1.3	1.1	0.9
p-Cymene	1024	5.3	4.5	4.8
Limonene	1029	0.2	0.1	–
β-Phellandrene	1030	0.2	0.2	–
(Z)-β-Ocimene	1035	0.1	–	–
(E)-β-Ocimene	1046	0.1	–	–
γ-Terpinen	1058	8.1	8.2	9.5
cis-Sabinene hydrate	1070	0.8	0.6	0.5
Terpinolen	1085	0.1	–	–
trans-Sabinene hydrate	1101	2.8	0.3	0.3
Borneol	1173	0.2	0.6	0.4
Terpinene-4-ol	1181	0.5	0.4	0.3
α-Terpineol	1196	0.2	0.1	–
trans-para-mentha-2-one	1199	0.1	–	–
trans-Dihydrocarvone	1205	0.1	–	–
Carvacrol methylether	1239	0.1	0.1	0.7
Thymoquinon	1252	0.1	0.6	0.8
Thymol	1292	0.3	3.7	0.3
Carvacrol	1303	77.4	74.9	70.0
Carvacrylacetate	1365	0.3	0.2	0.2
β-Caryophyllen	1420	3.0	3.1	2.8
α-Humulene	1456	0.3	0.5	0.4
Allo-Aromadendrene	1461	–	0.2	0.2
α-Muurolol	1496	–	–	0.1
β-Bisabolene	1508	0.5	0.8	0.7
γ-Cadinene	1514	0.1	0.4	0.3
δ-Cadinene	1519	0.1	0.7	0.6
3-Methoxy-2,4,5-trimethyl-Phenol	1557	–	0.6	0.5
Spathulenol	1578	–	–	0.2
Caryophyllene oxide	1584	0.1	–	0.1
epi-α-Muurolol	1645	–	0.2	0.3
α-Eudesmol	1659	–	0.3	–

[a] Percentages obtained by FID peak-area normalization.
[b] Linear retention indices (DB-5 column).

γ-terpinene was not found for *p*-cymene. During two successive growing seasons, neither different water supply regimes nor different nitrogen fertilization levels caused significant change in the composition of the essential oil of oregano.

4. Discussion

The focus of the present study was to investigate the effects of nitrogen application levels and water deficiency on the dry matter production and the essential oil content of oregano. Nitrogen fertilization at high level (N2: 1.5 g N/pot in 2007) increased dry matter production, but decreased essential oil content (Fig. 4a and b). An early study showed that nitrogen fertilization with more than 2 g N/pot significantly decreased essential oil content of wild Egyptian oregano (*Origanum syriacum* L.) (Omer, 1999). A similar effect was also reported for other genera of the family *Labiatae*, *Rosmarinus officinalis* (Boyle et al., 1991) and *Thymus vulgaris* L. (Baranauskiene et al., 2003). According to Muzika (1993) nitrogen fertilization at high level reduced the content of phenolic compounds in *Abies grandis* seedlings and the carbon/nutrient balance hypothesis could not adequately explain this effect. In fact, nitrogen fertilization with higher level significantly increased the dry matter production and significantly higher essential oil yield in 2007 (Fig. 4a and c). These results indicate that the decreased essential oil content by high level nitrogen fertilization was attributed to a dilution effect (Fig. 4b). According to Bosabalidis and Tsekos (1984), the leaves of *Origanum* plants bear numerous glandular and non-glandular hairs on both

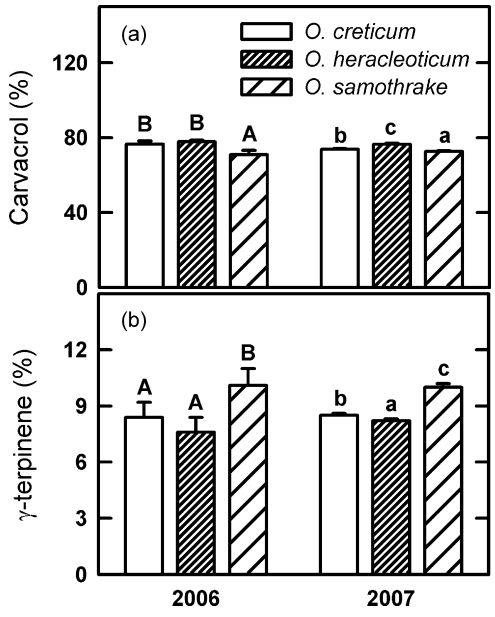

Fig. 5 – Differences in the major components of essential oil of oregano populations: carvacrol (a) and γ-terpinene (b). Values represent means ± S.E. Significant differences among populations were measured by the least significant difference (LSD) at $P < 0.05$ and indicated by different letters.

sides, in which essential oil is accumulated. The number of the glandular hairs seemed to be constant for mint and sweet basil leaves whose area changed under different environmental conditions (Sangwan et al., 2001). This may hold also true for oregano. In addition, in our study, essential oil composition of oregano was not affected by nitrogen fertilization levels, which is not in agreement with the results reported by Omer (1999).

In comparison with optimal water supply (W1), both water deficient treatments showed a significantly lower dry matter production of oregano (Fig. 3a). In addition, the consistent water deficiency caused higher degree of reduction of dry matter production than the water deficiency after flowering (Fig. 3a). It is interesting to note that in comparison with control, later water deficiency caused a significantly increase in essential oil content, whereas the consistent water deficiency failed to do it (Fig. 3b). These results confirm the conclusion that the amounts of essential oils produced under drought conditions were either maintained or enhanced, depending on the species and magnitude of the stress (Singh et al., 2000, 2002; Sangwan et al., 2001; Zehtabi-Salmasi et al., 2001; Delfine et al., 2005). In mints and sweet basil, it was found that higher essential oil content due to drought stress was related to higher density of oil glandular hairs (Sangwan et al., 2001). This may also be true for oregano. Later water deficiency significantly improved essential oil content and thus the quality of oregano herbage. In addition, this treatment reduces the water amount used for production and thus increases the efficiency of irrigation. This can significantly benefit the farm performance. Nevertheless, the effect of this treatment needs to be tested under field conditions in the future.

The composition of essential oil was not significantly affected by nitrogen fertilization levels or by water deficiency (data not shown). Also, the difference in the composition of essential oil was rather limited among tested oregano populations (Fig. 5a and b). Our findings are in good agreement with those of others (Novak et al., 2003). All tested populations show high carvacrol percentage and can be referred to carvacrol-rich chemotype (Skoula et al., 1999; Chatzopoulou et al., 2004).

5. Conclusions

Our study showed that dry matter production and essential oil content of *Origanum vulgare* L. can be significantly affected by environmental and agronomical conditions including nitrogen fertilization and soil moisture regime, whereas percentage of main compounds of essential oil such as carvacrol, γ-terpinene and *p*-cymene remained unaffected. Higher nitrogen level increases dry matter yield, but reduces essential oil content of oregano herbage. An optimal water supply during seedling development and stem elongation stages and a restriction of water supply after beginning of flowering may increase the content of essential oil and thus improve the quality of oregano herbage. These effects need to be tested under field conditions. The population *O. vulgare* var. *samothrake* showed a stable dry matter yield and higher essential oil content and may serve as good material for case studies with focus on breeding programs and farm performance in oregano production.

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Associations between molecular markers, agro-morphological traits and chemical characteristics in a germplasm collection of the medicinal plant *Origanum vulgare* L.

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Abstract

Forty-two accessions of *Origanum vulgare* L. mostly originating from Europe were evaluated to detect molecular, quantitative agro-morphological and chemotypic polymorphisms and to discover possible associations between them. Twelve traits related to agronomic and morphological characteristics were measured. Components in the essential oils were identified by GC-MS and 18 major compounds were investigated. A total of 477 molecular polymorphisms including 214 AFLP and 263 SAMPL (Selectively Amplified Microsatellite Polymorphic Loci) were used for genotyping. Euclidean distances of phenotypic and chemotypic data, and genetic distances (1-Dice's similarity) of molecular markers were compared by applying Mantel tests to ascertain the congruencies between them. A relatively high correlation between chemotypic patterns and genetic markers was identified while a lower correlation was found between the phenotypic and genetic matrices. Cluster analysis, population inference and principal component analysis (PCA) revealed a broad variation among accessions. Pair-wise analyses of correlation among all traits showed that stem diameter was correlated to essential oil yield and carvacrol content. Therefore, this morphological trait can be used for indirect selection regarding quality. Associations between traits of interest and genetic markers were tested using five methods including three general linear model (GLM) and two unified mixed linear model (MLM) approaches. Significant associations were found for 4 AFLP and 24 SAMPL with four key traits including drug fraction, essential oil yield, carvacrol and thymol content. These associations can constitute a useful starting point for marker-assisted selection. Therefore, the results provide the basis for molecular breeding of *Origanum vulgare* L for pharmaceutical purposes.

Keywords: *Origanum vulgare;* Quantitative traits; Essential oils; AFLP; SAMPL; Marker-Trait Associations

Introduction

Origanum vulgare L. is a perennial aromatic herb belonging to the family *Lamiaceae* used as medicinal plant because of the essential oils produced in the aerial parts (Skoula and Harborne 2002). The species is naturally distributed all over Europe, North Africa and western Asia (Kokkini 1997). Aromatic leaves and inflorescences of plants are widely used as a very popular spice in food industry (Olivier 1997). The biological activity of essential oils and herb extracts cause a high pharmaceutical and industrial interest in *O. vulgare*, since antimicrobial, antifungal, insecticidal and antioxidative effects have been reported (Kulisic et al. 2004; Bakkali et al. 2008).

O. vulgare is the most variable species of the genus *Origanum* and the only one commonly known as `oregano' in most European countries (Bernáth 1997). Taxonomic studies on the basis of morphological characters have led to the discrimination of several subspecies. Ietswaart (1980) distinguished six subspecies of *O. vulgare*, i.e. *hirtum*, *vulgare*, *virens*, *viride*, *gracile* and *glandulosum*. Only *O. vulgare* L. subspecies *hirtum* has the leaf anatomy which corresponds to that of commercially marketed European oregano (Skoula and Harborne 2002).

This species has also a high phytochemical polymorphism with several chemotypes which shows marked spatial segregation in nature (Fleisher and Sneer 1982; Chalchat and Pasquier 1998; D'antuono et al. 2000; Radušiene et al. 2005). The essential oil of oregano is composed of carvacrol and/or thymol as dominant components, followed by γ-terpinene, *p*-cymene, linalool and terpinen-4-ol (Skoula and Harborne 2002). The broad accumulated diversity of carvacrol content in oregano on the species level is one of the most important goals of breeding, which has resulted in a number of cultivars of practical importance (Fleisher and Sneer 1982; Sezik et al. 1993; Skoula et al. 1999). Nevertheless, genetic resources, diversity and potential for utilization of *O. vulgare* have not yet been fully explored so that extended research on germplasm is urgently needed (Novak et al. 2007). Characters most targeted in breeding of oregano include those related to spice productivity, i.e. leaf- and flower-fraction: drug fraction, and pharmaceutical properties such as essential oil yield and the content of two strong antimicrobial monoterpenes, carvacrol and thymol (Franz and Novak 2002; Makri 2002).

The chromosome number of *O. vulgare* was previously reported to be 2n = 2x = 32 (Scheerer 1940). Male sterile (nucleo-cytoplasmic) and male fertile plants have been identified in several natural populations (Kheyr-Pour 1981). Recently, Katsiotis et al. (2009) have carried out a study to clarify the phylogenetic relationships and variations of Greek *O. vulgare* subsp. *hirtum* by RAPD (random amplified polymorphic DNA) markers and rDNA sequences. Efforts to identify SSRs (Simple Sequence Repeats) derived from ESTs (Expressed Sequence Tags) of epidermal glands on this commercial subspecies were also reported (Novak et al. 2007). In a previous work, we have investigated the relationships between different subspecies of *O. vulgare* using two PCR-based marker approaches, Amplified Fragment Length Polymorphism (AFLP) and Selectively Amplified Microsatellite Polymorphic Loci (SAMPL), and we have also compared the relative efficiencies of these two marker systems for surveying intraspecific genetic diversity (Azizi et al. 2009a).

In Europe, approximately 90% of the medicinal and aromatic plant species used commercially are collected from the wild (Vines 2004). Domestication is a viable alternative and offers the opportunity to solve the problem. By bringing medicinal herbs into cultivation, conventional and biotechnological plant breeding techniques can be applied to improve yield and uniformity, and to modify pharmaceutical properties (Canter et al. 2005). Selection assisted by genetic markers, hybridization, polyploidization and mutation are some effective strategies to improve medicinal and aromatic plants (Bernáth 2002). A very useful tool for improving the efficiency of breeding programmes is the identification of polymorphic markers associated with phenotypic variation for important traits (Moose and Mumm 2008).

The methodology of association and linkage disequilibrium analyses, perfectly suitable for bi-allelic codominant marker types, mainly SSRs and single nucleotide polymorphisms (SNPs), has been well developed and used in a number of plant species (Gupta et al. 2005).

The potential of dominant markers, such as AFLPs, is poorly explored for association studies. However, many underrepresented plant species such as most of the medicinal plants or other crops with limited genomic information largely rely on dominant marker types such as AFLPs (Li et al. 2007). The last authors have recently investigated the use of dominant markers for estimating linkage disequilibrium in diploid species and developed an appropriate algorithm. Now, there are a number of reports on the use of AFLP markers for genome-wide linkage disequilibrium analyses and association studies in plants (e.g. Hansen et al. 2001; Kraakman et al. 2004; Skøt et al. 2005; Achleitner et al. 2008; Saïdou et al. 2009).

Studies on dominant markers suggested that they can be successfully applied to quantify population structure and assigning individuals to subpopulations (Q matrix) using a Bayesian approach when a large number of loci are genotyped (Pritchard et al. 2000; Hollingsworth and Ennos 2004; Falush et al. 2007). Dominant markers can also be a useful tool to estimate the kinship coefficients between individuals within populations (Hardy 2003). Yu et al. (2006) incorporated the outcome of population structure (Q matrix) with the estimation of relatedness between individuals obtained through the marker-based kinship matrix (K) into a unified mixed linear model (MLM) approach. This approach effectively decreases Type I error rates (false positives) and increases the power of the marker-trait association tests (Yu et al. 2006).

The goals of the present study were: (1) to use combined AFLP and SAMPL analyses for surveying genome-wide diversity in *O. vulgare*, (2) to verify the capacity of the phenotypic and chemotypic traits for discriminating between accessions, and (3) to elucidate any trait–trait correlation and marker–trait association using these molecular markers together with quantitative phenotypic and chemotypic traits.

Materials and Methods

Plant Material

A total of 42 accessions of *O. vulgare* L. were investigated, 39 accessions from the Gatersleben Genebank (IPK Gatersleben, Germany) along with three cultivated types: 'Heracleoticum' from the seed company Pharmasaat (Artern, Germany), 'Creticum' and 'Samothrake' from the company Syringa (Hilzingen-Binningen, Germany) (Table 1).

All accessions were grown during 2007 and 2008 at the research station Rauischholzhausen of Justus-Liebig-University, Germany. In March, 10 individual plants of each accession (12-day-old, 4-leaves stage) were transplanted from the seedling bed into Mitscherlich pots (6L). The soil mixture used in this experiment was based on a loess soil from the research station Rauischholzhausen/Ebsdorfergrund (Germany). The soil contained 7.8mg P/100 g, 14.9mg K/100 g and 1.42mg N/100 g. The sieved soil was mixed with sand (soil: sand = 1:2 w/w) and fertilized with N, P, K, Mg and CaCO3 to warrant optimal nutrient supply for plant growth. The mixed soil showed pH 6.7 (in H_2O). Plants were watered approximately twice a week by a controlled drip irrigation system. Finally, plants were harvested individually at the full flowering stage in July.

Phenotypic evaluation

Phenotypic data were recorded on 12 quantitative traits related to the agronomic and morphological characters and averaged across ten individual plants of each accession. These evaluations comprised plant height (PH, cm), the number of branches (NB), branch length (BL, cm), stem diameter (SD, mm), the number of nodes per stem (NN), distance of internodes (DI, cm), the number of leaves per node (NL), leaf length (LL, cm), leaf width (LW, cm), dry mass (DM, g/plant, air dried mass at 40°C), drug fraction (DF, g/plant, leaf- and flower-fraction as determined by separating it from the stem manually) and essential oil yield (EOY, %). To measure essential oil content of each accession, hydro-distillation method (see below, essential oil extraction) was performed.

To access trait variability and significant differences between accessions, analysis of variance (ANOVA) was performed using the SPSS version 16 (SPSS, Chicago, Illinois, USA). The character means were compared using the least significant differences (LSD) test at the 5% probability level. Furthermore, Pearson correlation coefficients were calculated using all investigated traits by SPSS.

Phytochemical Assessment

Essential oil extraction. Samples of at least 20 g of dried leaves and inflorescences were hydro-distilled for 3h using a Clevenger-type apparatus (Europäische Arzneibuch 1997). The essential oil yields were gravimetrically (w/w) quantified. Each sample was analyzed three times and the average yield of essential oil was used for statistic evaluation. The essential oil obtained was kept at 4 °C until further analysis.

The identification of individual components of the essential oils was realized by gas chromatography–mass spectrometry (GC–MS). For quantification purposes, percent values of peak areas were determined by gas chromatography–flame ionization detector (GC–FID). A Varian 3900 GC coupled with a Varian Saturn 2100T ion trap mass detector and a Varian CP-3800 GC–FID were employed. The chromatographic procedures have been previously described by Azizi et al. (2009b). The identification of components of the essential oil was achieved on the basis of comparison of Kovat's retention indices (KI) with those of literature data (Adams 1995; Figuérédo et al. 2006) and mass spectrometry by the comparing mass spectra of the unknown peaks with those stored in the Wiley 90 and NIST 98 mass libraries. Kovat's retention indices were calculated from the gas chromatogram by linear interpolation between bracketing n-alkanes (Lubeck and Sutton 1983). The contents of all constituents identified and quantified in the essential oils was subjected to an ANOVA, applying a least significant differences (LSD) test, using the SPSS, to test differences between accessions.

Genotyping by AFLP and SAMPL analyses

Total genomic DNA was extracted from young leaves (100 mg per plant) of 5-week-old plants using a modified CTAB (cetyltrimethyl ammonium bromide) procedure according to Doyle and Doyle (1990). After RNAse treatment, DNA content was quantified using NanoDrop ND-1000 UV-Vis Spectrophotometer (Labtech International, Ringmer, United Kingdom). Genomic DNA of 10 plants per accession was bulked and diluted to 25 ng/µl working solution.

The AFLP analysis (Vos et al. 1995) was conducted as described by Azizi et al. (2009a). The SAMPL procedure used here is according to Vogel and Scolnik (1998). Pre-amplified AFLP library was used as template for selective amplification using fluorescent dye-labelled SAMPL and MseI+3 primers. The parameters for PCR reactions have been previously described by Singh et al. (2002). Twenty four primer combinations were tested for both AFLP and SAMPL analysis. Out of them, three were selected (Table 2) for each marker on the basis of their ability to generate informative data and values of resolving power (see Azizi et al. 2009a). Selective amplification products were separated on 8% denaturing polyacrylamide gels using a LiCor 4200 DNA Analyzer. Fragment's size was estimated in comparison to a 50-750bp labelled DNA-ladder. AFLP and SAMPL fragments were detected using the RFLPscan 2.1 software package (Scan analytics, Fairfax, USA). The bands were scored for their presence (1) or absence (0) across 42 accessions.

Cluster analysis and principal components analysis

All polymorphic AFLP and SAMPL markers were combined and employed to calculate genetic similarities using the DICE coefficient (Dice 1945) in software NTSYS pc 2.20e (Rohlf 2000). From the similarity data, genetic distance were calculated for each pair of accessions (distance = 1 – similarity) and used for UPGMA clustering in NTSYS-pc. Cluster reliability was measured by bootstrap analysis with 1,000 random re-sampling using the Winboot software (Yap and Nelson

1996). The goodness of fit of the clustering compared to the basic data matrix was also tested by computing the co-phenetic correlation coefficient using normalized Mantel statistics Z test (Mantel 1967) via the COPH and MXCOMP procedures of NTSYS-pc.

In order to provide an overall distance measure between the accessions based on 12 phenotypic traits and 18 major chemical components, principal component analysis (PCA) was conducted on the accession means for each observed character using the NTSYS-pc. The selected variables corresponded to the major chemical components representing more than 10% of the total composition of the essential oil in at least one accession. All traits were standardised by subtracting the mean value and dividing by the standard deviation; this allows reducing the effects of different scales.

Comparison of distance matrixes

Euclidean distances were computed between accessions based on the quantitative phenotypic traits and major chemotypic characters (major components in essential oils). In order to investigate the congruencies between phenotypic, chemotypic and genomic distances, the genetic distance matrix based on combined dataset of AFLP and SAMPL, the Euclidean phenotypic distance matrix and Euclidean phytochemical distance matrix were compared using Mantel tests (Mantel 1967) by the MAXCOMP routine of NTSYS-pc. The normalized Mantel statistic Z was used to determine the level of association between the three matrices. Significance of Z was determined by comparing the observed Z values with a critical Z value obtained by calculating Z for one matrix with 1000 permuted variants of the second matrix.

Population inference

In order to infer population structure among accessions, the AFLP and SAMPL polymorphic markers were analyzed by dominant-marker model of the computer program STRUCTURE (Pritchard et al. 2000; Falush et al. 2007). STRUCTURE version 2.3.1 was used to assign accessions into subpopulations (K). Posterior probabilities of K ($Pr(X \mid K)$) were obtained for K=1 through K=10 clusters using the Admixture model, which allows for potential recombination between inferred clusters. Five runs were completed for each K, with 100,000 iterations, following a burn-in period of 50,000 iterations to find the optimal number of subpopulations and membership of each accession. We inferred the number of subpopulations according to Pritchard et al. (2000) with posterior probabilities of K calculated assuming uniform priors on K and using for each K the maximum value of the probability of the data given K obtained over replicates.

Association analyses

Association tests between quantitative traits and polymorphic AFLP and SAMPL markers were carried out across all accessions using the software TASSEL, version 3.0 (released April 2009, Bradbury et al. 2007). This software determines association between genomic sites and phenotypes,

while accounting for population structure and relative kinship. The mean values of 12 phenotypic traits and 6 major components of essential oils (see figure 1) were included in the analyses. Five different approaches were used to control for false-positive results in association tests (Table 3). First, a general linear model (GLM) was tested to detect single marker effects on quantitative traits. This model does not account for population structure as a potential cause of the genotype–phenotype relationship. In the second GLM model, Principal components (PC) 1 through 3 (PC_{1-3}) were used as quantitative covariates. For third GLM model estimates of the population structure obtained from the program STRUCTURE were incorporated into the model by using covariates that indicate percent contribution to each accession by a specific subpopulation (Q-matrix).

A fourth and fifth model were tested using a unified mixed linear model (MLM) following Yu et al. (2006). One contained the relative kinship matrix estimated from molecular marker data among all accessions, and the second contained the kinship matrix (K) plus the population structure (Q). P values for association tests were obtained from the F value of effects of each marker locus on trait values. Significance of F values was confirmed by 1000 permutations for each marker. The trait was considered to be significantly associated with a marker locus when both the P value from the F test and the experiment wise P value from the permutation test were <0.01. Phenotypic variance values (partial R^2) were computed for the fixed marker effects.

Results

Polymorphisms and Cluster analysis

The six selected AFLP and SAMPL primer combinations yielded a total of 572 scorable fragments across 42 accessions, of which 477 were found to be polymorphic (Table 2) ranging in size from 50 to 470 bp. Dice genetic similarity (GSD) for all accessions under investigation varied from 0.17 (accessions ORI23 vs. Samothrake) to 0.68 (ORI47 vs. ORI49 both from Germany) with an overall mean of 0.39 (data not presented).

The mean values of all investigated accessions for each of the 12 measured quantitative traits related to agronomic and morphological characters are presented in Table S1. The results from the analysis of variance (ANOVA) revealed that the examined accessions of *O. vulgare* were highly variable in all evaluated phenotypic characters (P<0.05). A total of 62 volatile compounds were detected in essential oils by GC and GC/MS analyses. The mean values of relative percentage amounts of the 18 main compounds are shown in Table S2.

The chromatographic fingerprints showed the presence of high intraspecific diversity of chemical constituents in the essential oils from the accessions of *O. vulgare*. Dominant components in essential oils, that determine different chemotypes, were four monoterpenes including carvacrol, thymol, *p*-cymene and *γ*-terpinene and two sesquiterpenes including *β*-caryophyllene and germacrene-D. In comparison to other reports on *O. vulgare* (e.g., Sezik et al. 1993; Skoula et al. 1999; D'antuono et al.

2000; Skoula and Harborne 2002), relatively high content of germacrene-D was found in essential oils investigated in our study.

The UPGMA cluster tree based of genetic distances (1 – Dice's similarity) estimated for 477 polymorphic markers is shown in Fig. 1. In general, the main clusters of oregano genotypes were supported by high bootstrap values, indicating the reliability and stability of the relationships as well as the robustness of the molecular marker dataset. The high co-phenetic correlation coefficient obtained (r = 0.89) confirmed also this trend. The dendrogram revealed two main clusters (Fig. 1). The first node separated the cultivated types of accessions along ORI28, ORI34 and ORI45 from another ones that split afterwards into three subclusters.

The relative expression of the oregano accessions for twelve phenotypic traits and six chemotypic characters is shown as a heat map in greyscales in Fig. 1. Dark colours indicate high-numerical values for the trait, and light colours indicate low values. For example, cultivated types of accessions, 'Heracleoticum', 'Creticum' and 'Samothrake' can be identified by their high values for CAC, carvacrol content (dark in the first column of [c]) and EOY essential oil yield (dark in the second last column of [d]). The accessions ORI25, ORI29 and ORI 37 shows a high expression of THC, thymol content (dark in the second column of [c]).

Inferred population structure

Analysis of 42 accessions with 477 AFLP and SAMPL markers identified six distinct subpopulations. The value of $Pr(X|K)$ was optimized at K = 6 for most numerical solutions. The membership of accessions in K = 6 subpopulations (Table S2) was highly consistent across multiple solutions. The resulting subpopulation numbers based on maximum proportion of membership of accessions in the subpopulations are shown in Fig. 1b. These inferred subpopulations are in close agreement with the major clusters in Fig. 1a.

Principal components analysis and correlation tests

In order to define phenotypic and chemotypic relationships among the accessions, main values of phenotypic traits (Table S1) and essential oil data (Table S2) were elaborated to conduct a principal components analysis (Fig. 2). The subpopulation numbers (k 1 through 6) based on maximum proportion of membership estimated by STRUCTURE analysis of genetic markers (Table S3) were also presented in Fig. 2 to gain further insight of congruencies among DNA, chemical and phenotypic variations. The first two principal components (PC) justified 19.5 and 14% of the total variation among 18 main chemical characters and 25.9 and 17.5% of the total variation among 12 phenotypic traits (Fig. 2b), respectively. PCA revealed the existence of a high morphological, agronomic and phytochemical variations among *O. vulgare* accessions so that along the first two principal components, all the accessions are clearly differentiated from each other on the scatter diagrams (Fig. 2a,b). The PC-II based on chemical compounds were able to separate the accessions containing the

desirable monoterpenes, carvacrol, thymol and their precursor, *p*-cymene, γ-terpinene from the other accessions containing the undesirable sesquiterpene such as β-caryophyllene and germacrene-D and etc. (Fig. 2a).

The distribution of the 42 accessions on the chemotypic plot (Fig. 2a) was notably different from that on the phenotypic plot (Fig. 2b). The plots also illustrate that the grouping patterns based on 477 AFLP and SAMPL markers (accessions with the same K subpopulation numbers) have more congruency with chemotypic characters than with quantitative agronomic and morphological traits.

The Mantel tests also showed that there was a relatively high, significant correlation between two distance matrices based on the genetic markers (AFLP and SAMPL) and the chemical compounds of essential oils (r = 0.65), confirming the congruence of interaccession relationships in the *O. vulgare* revealed by the chemical and molecular markers. The matrices obtained by phenotypic data (quantitative morphological and agronomic traits) and molecular marker data analyses revealed a moderate but significant correlation (r = 0.27). The chemical variability found between the analysed accessions was also significant but weakly correlated with the phenotypic attributes (r = 0.14).

Pearson correlations (Pearson coefficients: r) between phenotypic and chemotypic traits among 42 accessions of *O. vulgare* (Table S4) showed that there is significant positive correlation ($P<0.05$) between some agromorphological characters with dry mass and drug fraction. These traits included plant height (r = 0.350 and 0.291), number of branches (r = 0.494 and 0.403), branch length (r = 0.269 and 0.215) and number of nodes per stem (r = 0.327 and 0.295). For two economically important traits for oregano, essential oil yield and carvacrol content, only one morphological trait, the stem diameter found to be positively correlated to these traits (r = 0.585 and 0.553).

Marker-trait associations

A test of associations between 477 AFLP and SAMPL markers, 12 quantitative phenotypic traits and 6 chemotypic characters detected significant marker-trait associations for all traits using at least one of the simple or population structure controlling models. A total of 8,586 (477 markers × 18 traits) association tests were performed by each of the five models. Of these, 91, 42, 32, 74 and 68 were significant (results not shown) at the nominal threshold of P = 0.01 based on models 1, 2, 3, 4 and 5, respectively (table 3). The number of detected significant associations decreased in almost all traits when population structure was accounted for each GLM or MLM model. The number of significant associations varied also across traits, ranging from 4 to 19 for number of branches (NB) and GTC γ-terpinene content, respectively (results not shown).

Associations between 28 markers and four major economically important traits are presented in Table 4. These traits include drug fraction (DF), essential oil yield (EOY), carvacrol content (CAC) and thymol content (THC). The table also shows the proportion of phenotypic variance (R^2) of the traits explained by markers detected by significance based on the five models. The effects of AFLP-2_31 on two traits, EOY and CAC, were significant by all five tested models. Eleven markers were found to be

associated to THC, and association with the highest R^2 value for a trait was obtained for SAMPL-3_60 influencing THC (Table 4).

Discussion

Polymorphisms

This study represents a multidimensional approach that comprehensively investigates the genetic diversity within a collection of *O. vulgare* and assesses its structure based on two PCR-based molecular marker systems (AFLP and SAMPL), quantitative agronomic and morphological traits and chemical compounds of essential oils. Despite the drawback of being dominant markers, the major advantage of combined AFLP and SAMPL analysis is its capacity to cover the whole genome, as specified by restriction sites and microsatellite loci, to detect polymorphisms comparative to single analysis alone, making this strategy a very powerful tool for population studies. In the absence of SSR markers, as is still the case in *O. vulgare*, these two marker systems appear to be highly suitable for genetic diversity studies of oregano (Azizi et al. 2009a) and other medicinal plants (Sing et al. 2002; Sarwat et al. 2008).

In the present study, the UPGMA clustering, inferred population structure and principal component analysis based on genetic markers, quantitative agromorphological traits and chemotypic characters revealed a high level of polymorphisms. This finding is in good agreement with earlier reports by Chalchat and Pasquier (1998), D'antuono et al. (2000), Radušiene et al. (2005) and Katsiotis et al. (2009). However, commercial accessions investigated here showed a relatively low variance for their phenotypic and chemotypic characters.

In this study, the grouping patterns between *O. vulgare* accessions provided by three methods of diversity analysis, were rather different. For example, by AFLP and SAMPL markers accessions such as ORI47 and ORI49 with the highest similarity subgrouped together (Fig. 1a,b) while phenotypic analysis detected a high variation between them (Fig. 1d). This difference may be related to phenotypic plasticity of the plants in response to changes in the habitat environment (West-Eberhard 1989). This phenotypic plasticity could also be visually inspected in the heat map of the traits (Fig. 1d). As another example, the accessions ORI31 and ORI33 with a high similarity, as grouped by molecular markers in the same subpopulation, were very variable in many of phenotypic traits (Fig. 1). Population genetic structure is determined by joint effects of many factors including mating system, natural and artificial selection, mutation, migration and dispersal mechanism, drift, etc. (Hamrick and Godt 1989). The reproductive system is one of the important life-history characteristics that strongly influences genetic variability (Clegg et al. 1992). In *O. vulgare*, the mating system is mostly cross pollination (Kheyr-Pour 1981) which can cause a high level of genetic polymorphism and this variation may eventually led to differences in the genetic control of accumulation of monoterpenes such as carvacrol, thymol, *p*-cymene and *γ*-terpinene in the essential oils (Gershenzon And Croteau

1990). This wide variation in essential oil composition presumably has ecological advantages in protecting plants against different pests (Hough-Golstein and Hahn 1992).

In applied breeding for medicinal plants' improvement, chemotypic and genetic distances between genotypes are expected to provide predictors for high heterosis effects on pharmaceutical qualities and yield performance of their hybrids. In the present study, a high expression of germacrene-D (an undesirable compound) was observed in many wild accessions (Fig. 1c). Nonetheless, among these accessions, based on thymol content, essential oil yield and drug fraction, ORI8, ORI25, ORI27, ORI29, and ORI37 showed promising performance that can be exploited in breeding programmes.

Distance matrix congruencies and trait-trait correlations

The mantel test showed that there was a relatively high significant correlation between the distance matrix based on the chemical compounds of essential oils (terpenes) and the distance matrix based on the combined data set of AFLP and SAMPL markers, confirming the congruence of interaccession relationships in *O. vulgare* revealed by the chemical and molecular markers. This congruence supports the RAPD - terpenes correlation in a previous study on *Juniperus* spp. (Adams 2000) and the ISSR - terpenes correlation reported for *Primula ovalifolia* (Nan et al. 2003). High correlation between genetic and terpenoid distance matrices was also obtained in other aromatic and medicinal plants belonging to the *Lamiaceae* family such as *Ocimum gratissimum* (Vieira et al. 2001) and *Thymus vulgaris* (Echeverrigaray et al. 2001).

Hannover (1992) has provided evidence that terpene chemotypes are strongly controlled by genetic factors but this author also reported instances of environmental variation in terpene expression under extreme habitat conditions. In this case, genetic adaptation to the specific environment of the growing site is a factor to be taken into account (Curado et al. 2006). Genetic control of the chemical characteristics of thyme plants belonging to the *Lamiaceae* has been proven, so the monoterpenes accumulated by the plant are controlled by a series of loci with epistatic relationships (Vernet et al. 1986). It is therefore necessary to analyse both Quantitative Trait Loci (QTL) and genetic markers to better explain the relationship between the two sets of variations.

The correlation between genetic variability and the phenotypic (agro-morphological) attributes of the analysed accessions was found to be only medium. It is clear that such correlation estimates would be more when there is an association between the loci controlling the targeted morphological traits (QTLs) and the scored bands and when a large number of morphological traits are evaluated (Schut et al. 1997; Lefebvre et al. 2001). However, the moderate correlation ($r = 0.27$) between AFLP and SAMPL markers and 12 agro-morphological traits detected in the present study is considerably higher than that between RAPD markers and 12 morphological characters ($r = 0.12$) estimated in wheat by Máric et al. (2004).

The trait-to-trait correlations between the major chemical components and morphological characters should be considered when selecting the parental chemo- and phenotypes from oregano populations for breeding of pharmaceutical qualities. Morphological traits as indicators of chemotypes to be

selected would be most useful. For example, morphological traits with a high correlation to essential oil yield and content of phenolic monoterpenes (such as carvacrol and thymol) could provide a useful tool for indirect selection in breeding of pharmaceutical value of *O. vulgare*, if the expression of essential oil-related traits cannot reliably be measured during a breeding programme.

It is interesting that in the present study there were many strong correlations between chemical, and agro-morphological traits (Table S4). Stem diameter could be considered as an indicator for indirect selection and breeding for two economically important traits in oregano, i.e. essential oil yield and carvacrol content. Stem diameter is also negatively correlated to the undesirable compound germacrene-D in essential oil ($r = -0.424$). Our findings underline the results of previous studies on other *Lamiaceae* plants such as *Mentha* spp. (Mirzaie-Nodoushan et al. 2001).

Marker-trait associations

The aim of marker-trait association analyses in the present work was to constitute a starting point for marker-assisted selection in *O. vulgare* using AFLP, SAMPL, chemical and phenotypic polymorphisms. The whole-genome association analyses by AFLP, SAMPL markers dispersed throughout the genome could lead to the identification of a number of markers with significant associations to some economically and pharmaceutically important traits. We have identified four AFLP and 24 SAMPL markers associated to traits like drug fraction, essential oil yield, carvacrol content and thymol content (Table 3). SAMPL markers seem to be more effective for association analyses as in our previous study, this marker system was found to be useful for studies on intraspecific diversity and relationships among *O. vulgare* subspecies (Azizi et al. 2009a).

Considering that mapping data for the AFLP and SAMPL markers were not available, we were not able to examine the extent of disequilibrium among associated markers, so that it is not possible to speculate on the degree of disequilibrium between identified markers. However, the identified novel allelic variation for these important traits should be of considerable interest for breeding purposes, since the gene-linked SSRs and locus-specific SNPs are still not developed for *O. vulgare*.

The significant associations resulting from an unrecognized population structure are considered to be false positives. Therefore, we have used five different approaches for marker-trait association analysing to correct the effect of population structure. In the five models performed in the present study, using inferred population structure ‘Q’ (proportion of membership of accessions to sub-populations) or kinship matrix ‘K’ (general similarity based on shared kinship) for GLM or MLM models, decreased the number of detected significant associations and also reduced R^2 values (proportion of phenotypic variance explained) for most of the traits. In agreement with Achleitner et al. (2008), who have analysed associations of AFLP markers with quantitative traits among oat varieties, the lowest R^2 values were obtained when the combination of both ‘Q’ and ‘K’ was used in MLM (Yu et al. 2006) model (Table 4, fifth numeral).

Marker-trait associations found in the present study were supported by 1,000-times permutated P-values of the GLM and MLM models. However, it is still possible that some of the marker-trait

associations identified in our study are false positives, therefore further validation is required. Further mapping studies in segregating populations will help to confirm whether the associated markers are linked to QTLs influencing the traits and to confirm whether any of these QTL effects are caused by orthologs of known genetic factors. The validation of QTLs for the traits of interest can also be assisted by functional genomic studies.

Studies on QTLs linked to synthesis pathways of different monoterpenes in aromatic plants of the *Lamiaceae* family are very rare. Although there is no information on the mode of inheritance of carvacrol and thymol contents in *O. vulgare*, it has been reported that biosynthesis of these two phenolic monoterpene in *Thymus vulgaris* is governed by an epistatic series of several biosynthetic loci (Vernet et al. 1986). However, with regard to broad variation in the essential oil of *O. vulgare*, the biosynthetic pathway of carvacrol and thymol seems to be different and more complicated. This makes it difficult to detect QTLs for this pathway and also to identify individual genes because specific pathway branches control the synthesis of different monoterpenes.

However, the markers showing strongest effects on four economically and pharmaceutically important traits investigated in this study, could be starting points for further studies, marker assisted selection and practical breeding. Among the markers listed in table 3, based on the size of R^2 values, and the coassociation with more than one trait, there are three markers that are most interesting candidates for further work: I) Marker AFLP-2_31 may be used in breeding for pharmaceutical quality because it was co-associated with two key traits, essential oil yield and carvacrol content; however the marker effects (R^2 values) were relatively low (Table 3). II) Marker SAMPL-1_18 which is related to the drug fraction would be another considerable candidate to follow. III) The relatively strong effect of SAMPL-3_60 on thymol content (R^2 values: 0.23, 0.19, 0.19, 0.17 and 0.09) could make it useful for marker assisted selection of this very important antimicrobial compound (Table 3). The markers we identified can potentially help to improve the polygenic, complex quantitative traits related to pharmaceutical quality of *O. vulgare*.

Conclusions

A broad variation was found among oregano accessions by AFLP, SAMPL, chemical and phenotypic analyses. Significant correlation between two distance matrices based on the genetic marker systems AFLP and SAMPL and the chemical compounds of essential oils indicated the congruence of inter-accession relationships in *O. vulgare* revealed by the chemical and molecular markers while the phenotypic data and molecular markers revealed a moderate correlation. Analyses of trait-trait correlations and marker-trait associations showed that stem diameter, an easily measurable morphological trait, could be considered for indirect selection. Finally, three molecular markers, AFLP-2_31, SAMPL-1_18 and SAMPL-3_60 may be included in marker-assisted programmes to improve breeding efficiency of pharmaceutical properties.

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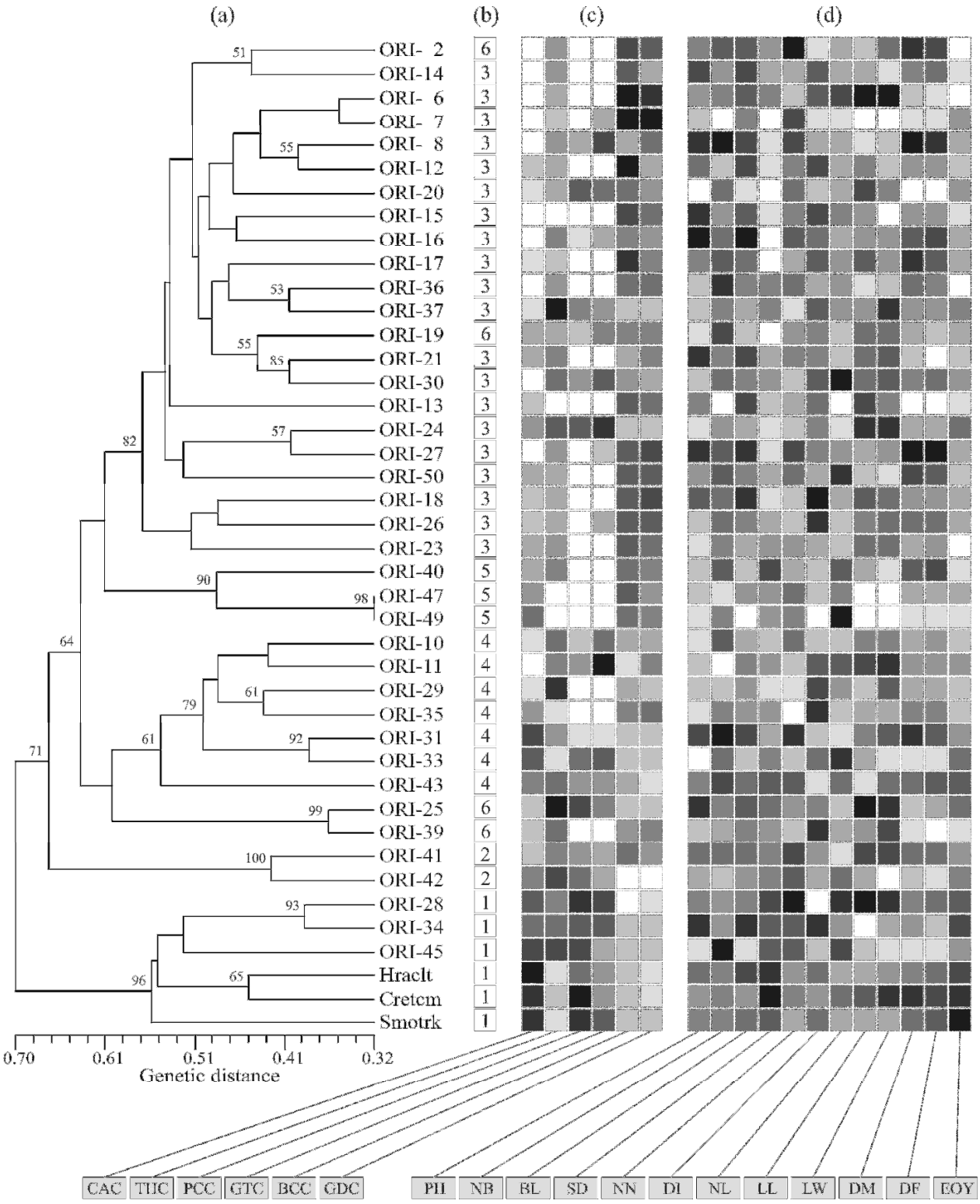
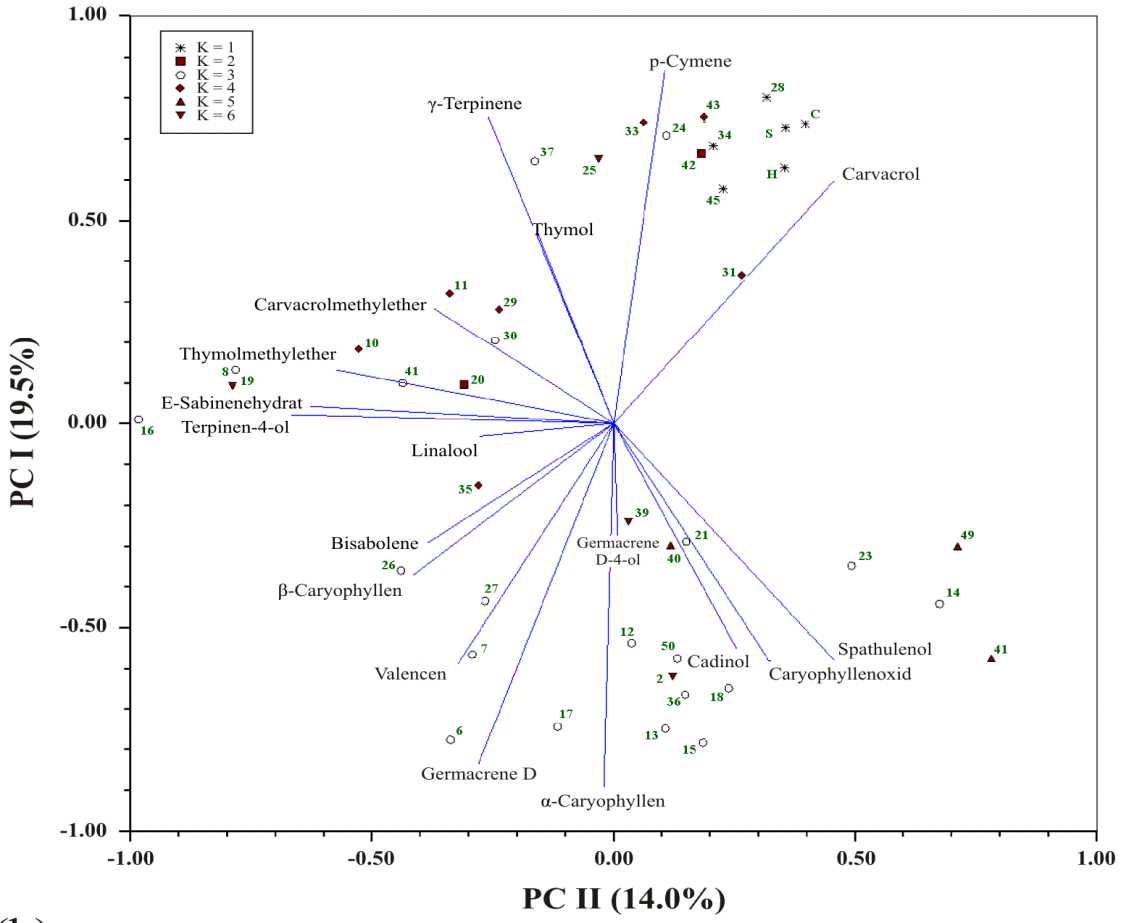


Fig. 1 (a): UPGMA dendrogram showing genetic relatedness between 42 oregano accessions based on 477 AFLP and SAMPL markers. Bootstrap values obtained from1,000 re-samplings higher than 50% are indicated at the branch nodes. (b): The subpopulation numbers based on maximum proportion of membership of accessions estimated by STRUCTURE analysis (see Table S3). (c) and (d) are the greyscale panels of six major chemotypic characters and twelve phenotypic traits, respectively. Dark colours indicate high numerical values for the trait, and light colours indicate low values. Trait abbreviations: PH plant height, NB number of branches, BL branch length, SD stem diameter, NN number of nodes per stem, DI distance of internodes, NL number of leaves per node, LL leaf length, LW leaf width, DM dry mass, DF drug fraction, EOY essential oil yield, CAC carvacrol content, THC thymol

content, PCC *p*-cymene content, GTC γ-terpinene content, BCC β-caryophylene content, GDC Germacrene-D content

(a)


K = 1
K = 2
K = 3
K = 4
K = 5
K = 6
p-Cymene
γ-Terpinene
Carvacrol
Thymol
Carvacrolmethylether
Thymolmethylether
E-Sabinenehydrat
Terpinen-4-ol
Linalool
Bisabolene
β-Caryophyllen
Germacrene D-4-ol
Spathulenol
Caryophyllenoxid
Cadinol
Valencen
Germacrene D
α-Caryophyllen
1.00
0.50
0.00
-0.50
-1.00
PC I (19.5%)
PC II (14.0%)

(b)

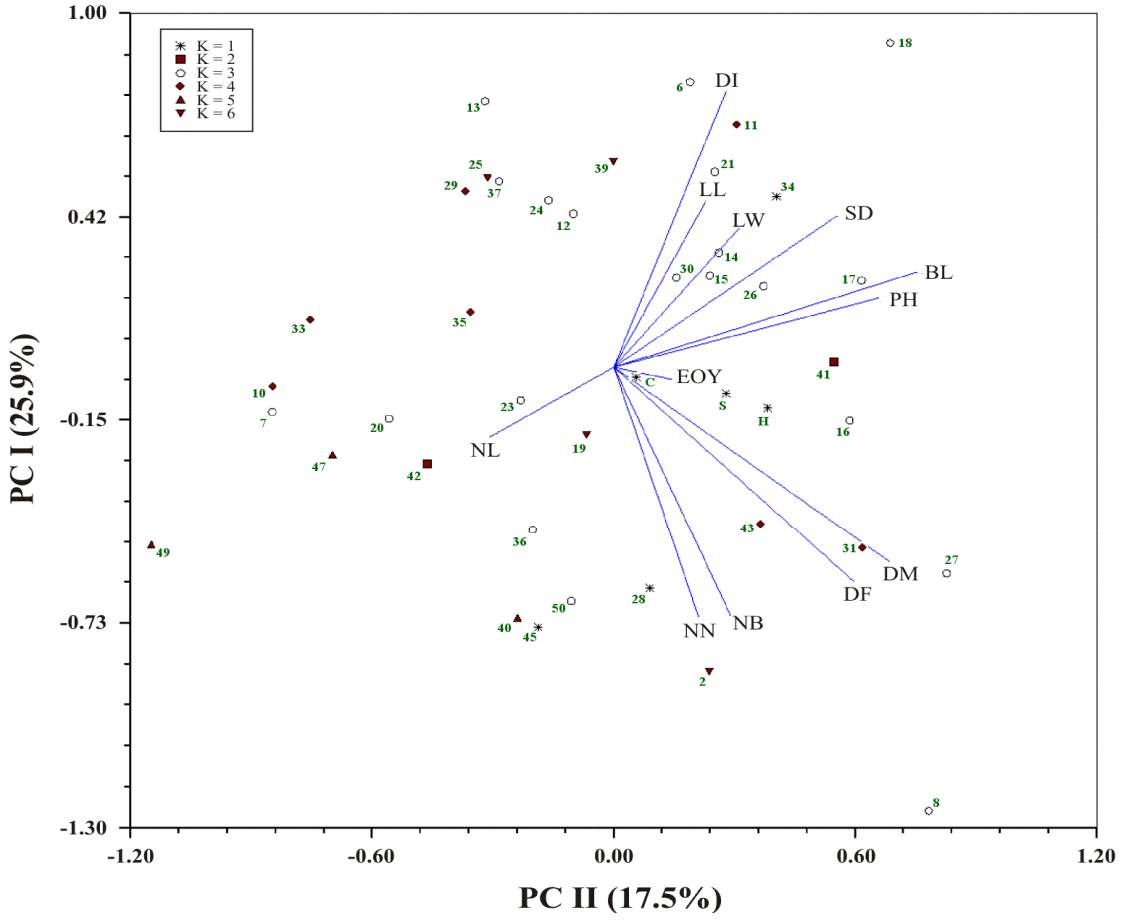

K = 1
K = 2
K = 3
K = 4
K = 5
K = 6
DI
LL
LW
SD
BL
PH
EOY
NL
DM
DF
NN
NB
1.00
0.42
-0.15
-0.73
-1.30
-1.20
-0.60
0.00
0.60
1.20
PC I (25.9%)
PC II (17.5%)

Fig. 2 Diagram showing the relationships among 42 accessions of *Origanum vulgare* based on Principal Component Analysis (PCA) using chemotypic (a) and phenotypic (b) data. K1-6 represent the subpopulations estimated by STRUCTURE analysis of 477 AFLP and SAMPL markers. The numbers represent the accession number and C, H and S are cultivated types ‘Creticum’, ‘Heracleoticum’, and ‘Samothrake’, respectively.

Tables

Table 1. Sources and origin of the oregano accessions investigated

Accession no/name	Seed source	Country of origin
ORI 2	Gatersleben Genebank	Germany
ORI 6	Gatersleben Genebank	Hungary
ORI 7	Gatersleben Genebank	*
ORI 8	Gatersleben Genebank	*
ORI 10	Gatersleben Genebank	Italy
ORI 11	Gatersleben Genebank	Italy
ORI 12	Gatersleben Genebank	Italy
ORI 13	Gatersleben Genebank	*
ORI 14	Gatersleben Genebank	Georgia
ORI 15	Gatersleben Genebank	Georgia
ORI 16	Gatersleben Genebank	Italy
ORI 17	Gatersleben Genebank	Italy
ORI 18	Gatersleben Genebank	*
ORI 19	Gatersleben Genebank	*
ORI 20	Gatersleben Genebank	Georgia
ORI 21	Gatersleben Genebank	*
ORI 23	Gatersleben Genebank	*
ORI 24	Gatersleben Genebank	Albania
ORI 25	Gatersleben Genebank	Albania
ORI 26	Gatersleben Genebank	*
ORI 27	Gatersleben Genebank	Italy
ORI 28	Gatersleben Genebank	Albania
ORI 29	Gatersleben Genebank	Italy
ORI 30	Gatersleben Genebank	Italy
ORI 31	Gatersleben Genebank	Albania
ORI 33	Gatersleben Genebank	Spain
ORI 34	Gatersleben Genebank	USA
ORI 35	Gatersleben Genebank	Italy
ORI 36	Gatersleben Genebank	*
ORI 37	Gatersleben Genebank	Italy
ORI 39	Gatersleben Genebank	*
ORI 40	Gatersleben Genebank	Italy
ORI 41	Gatersleben Genebank	Italy
ORI 42	Gatersleben Genebank	CSFR
ORI 43	Gatersleben Genebank	Albania
ORI 45	Gatersleben Genebank	*
ORI 47	Gatersleben Genebank	Germany
ORI 49	Gatersleben Genebank	Germany
ORI 50	Gatersleben Genebank	Germany
Heracleoticum	Pharmasaat, Artern	Germany
Creticum	Syringa, Hilzingen-Binningen	Germany
Samothrake	Syringa, Hilzingen-Binningen	Germany

*) unknown

Table 2 SAMPL and AFLP primer combinations used for genotyping42 accessions of *O. vulgare* and total number (n), number of polymorphic bands (np) and resolving power (Rp) of primer combinations

Primer combination		**Rp**	**n**	**np**
AFLP-1	E-CAT × M-CAT	29,9	103	77
AFLP-2	E-ATG × M-CCG	28,3	76	65
AFLP-3	E-CAG × M-CTC	34,1	106	72
SAMPL-1	G(TG)4(AG)4A × M-ACG	30,6	99	92
SAMPL-2	G(TG)4(AG)4A × M-GTG	24,3	92	89
SAMPL-3	C(AC)4(AG)4A × M-CAG	24,8	96	82
Total			572	477

Table 3 Methods performed for association analyses between molecular markers and quantitative traits (phenotypic and chemotypic) of *Origanum vulgare* L.

Model	**Data sets used in the Analyses**
1- GLM	(Phenotype, Chemotype) + (AFLP, SAMPL)
2- GLM	(Phenotype, Chemotype) + (AFLP, SAMPL) + (Covariates: PC_{1-3})
3- GLM	(Phenotype, Chemotype) + (AFLP, SAMPL) + (Covariates: Q_{1-5})
4- MLM	(Phenotype, Chemotype) + (AFLP, SAMPL) + (K)
5- MLM	(Phenotype, Chemotype) + (AFLP, SAMPL) + (K) + (Covariates: Q_{1-5})

Abbreviations: GLM general linear model, MLM mixed linear model, PC principal components,

'Q' (population structure that results from the existence of subpopulations)

'K', Kinship matrix (general similarity in genetic background arising from shared kinship).

Table 4 Proportion of phenotypic variance (R^2) of four key traits explained by AFLP and SAMPL markers detected by significance of five models of association analysis: First numeral through fifth are R^2 based Model 1 through 5 (see Table 3)

Locus	DF					EOY					CAC					THC				
AFLP-2_31						0,10	0,08	0,05	0,06	0,02	0,13	0,10	0,08	0,09	0,03					
AFLP-2_44											—	0,15	0,10	0,09	0,02	—	0,17	0,17	0,13	0,03
AFLP-3_6																0,19	0,12	—	0,10	0,10
AFLP-3_44	0,16	0,13	0,16	0,16	0,04															
SAMPL-1_3						—	0,26	0,11	—	—	—	—	—	0,10	—	0,14	—	—	0,04	—
SAMPL-1_18	0,18	0,15	0,15	0,18	0,09															
SAMPL-1_25						0,23	0,13	—	0,03	—										
SAMPL-1_54						0,28	—	—	0,02	—	0,20	—	—	—	—	0,18	—	—	0,14	—
SAMPL-1_56						—	—	—	0,05	—	—	0,06	—	0,11	—					
SAMPL-1_65						—	0,05	—	0,08	—	—	0,05	—	0,08	—					
SAMPL-1_68						0,43	—	0,04	—	0,04	0,32	—	—	—	0,09					
SAMPL-1_81											0,16	—	0,07	0,06	0,02					
SAMPL-2_2	0,12	—	0,20	0,09	0,01															
SAMPL-2_14						0,24	0,09	—	0,03	0,04										
SAMPL-2_21	0,12	—	0,20	0,09	0,01															
SAMPL-2_29						0,32	—	—	0,04	—	0,22	—	—	—	—					
SAMPL-2_82						—	0,07	—	0,04	—	—	0,10	—	0,09	—	0,15	—	—	—	—
SAMPL-3_9	0,14	0,12	0,13	0,14	—															
SAMPL-3_43																0,15	0,16	0,14	0,10	0,07
SAMPL-3_49																0,10	0,16	0,12	0,08	0,05
SAMPL-3_54						0,34	0,09	—	0,04	—	—	0,17	—	—	—	0,19	—	—	0,07	—
SAMPL-3_55																0,18	0,14	0,13	0,08	0,07
SAMPL-3_60																0,23	0,19	0,19	0,17	0,09
SAMPL-3_66						—	—	0,05	—	0,05						—	0,13	0,13	0,05	0,10
SAMPL-3_71						0,18	—	—	—	—	0,20	—	—	0,01	—					
SAMPL-3_73						—	0,09	—	0,04	—	—	0,20	0,07	—	0,06	0,24	0,12	0,09	0,05	0,02
SAMPL-3_78						0,17	—	—	—	—	0,20	—	—	—	—					
SAMPL-3_79						—	—	—	0,04	—	—	—	—	0,08	—					

The R^2 values shown correspond to the significance of marker at $P < 0.01$and (—) indicates that the model represented by the numeral in this position did not meet the $P < 0.01$ criterion. Trait abbreviations: *DF* drug fraction, *EOY* essential oil yield, *CAC* carvacrol content *THC* thymol content.

Supplementary Materials:

Associations between molecular markers, agro-morphological traits and chemical characteristics in a germplasm collection of the medicinal plant *Origanum vulgare* L.

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Supplementary Materials:

Table S1. Mean values for 42 accessions of *Origanum vulgare* L. for 12 quantitative agronomic and morphologic traits

Accs.	PH	NB	BL	SD	NN	DI	NL	LL	LW	DM	DF	EOY
ORI-2	59,7	32,9	51,4	3,2	26,4	2,1	6,9	23,1	20,1	70,0	42,2	0,06
ORI-6	56,0	22,0	51,6	3,6	14,2	4,0	13,6	37,6	25,4	36,2	22,6	0,04
ORI-7	50,3	7,7	46,2	2,5	22,7	2,1	5,8	21,2	16,1	33,0	23,4	0,34
ORI-8	71,0	46,7	55,3	2,6	23,6	2,5	7,6	21,7	17,8	77,2	45,2	0,22
ORI-10	44,2	29,2	39,2	2,8	19,6	2,4	6,4	27,8	19,8	41,7	28,6	0,13
ORI-11	51,1	8,1	47,6	3,2	13,1	3,9	11,5	31,8	23,0	44,5	29,3	0,44
ORI-12	63,5	15,4	56,6	2,6	17,8	4,1	7,0	27,1	17,5	45,7	28,9	0,14
ORI-13	61,4	6,8	56,5	2,8	15,8	3,7	4,6	31,5	19,8	26,6	19,1	0,08
ORI-14	65,8	17,5	56,3	2,9	15,8	3,9	7,1	23,8	17,1	52,6	34,6	0,07
ORI-15	71,3	18,4	53,5	2,7	17,0	4,1	8,7	25,9	16,3	48,6	28,8	0,11
ORI-16	75,9	26,4	66,4	2,5	21,2	3,5	6,8	25,1	18,9	59,6	40,1	0,23
ORI-17	62,2	31,4	49,8	2,4	15,4	4,0	8,2	30,5	19,0	67,6	35,9	0,20
ORI-18	64,0	24,2	61,1	2,6	13,4	5,3	7,0	30,7	20,4	47,9	28,6	0,23
ORI-19	46,4	34,8	36,6	2,2	15,9	3,1	6,0	28,3	20,1	40,1	24,3	0,26
ORI 20	38,0	24,2	32,5	2,3	18,7	2,3	7,0	31,5	19,9	27,5	18,0	0,35
ORI 21	69,6	22,1	57,9	2,9	17,9	3,1	6,6	28,1	21,4	35,1	21,3	0,14
ORI 23	46,5	22,3	38,6	3,3	16,5	2,5	6,2	28,6	20,2	40,6	28,6	0,06
ORI 24	46,4	17,8	38,5	2,7	13,5	3,1	5,5	36,1	23,1	39,8	27,0	1,04
ORI 25	68,0	20,0	52,2	3,9	16,6	3,2	6,5	36,7	24,3	36,7	26,6	1,18
ORI 26	51,6	27,2	45,1	3,0	14,4	4,5	6,2	27,9	20,3	55,6	33,6	0,19
ORI 27	71,7	34,5	61,3	2,7	22,8	3,1	7,9	23,4	19,5	74,5	48,9	0,22
ORI 28	59,8	17,7	47,4	4,5	26,8	2,0	15,3	38,9	24,3	50,2	33,5	2,41
ORI 29	49,8	15,8	42,5	2,7	12,2	4,1	8,0	23,2	21,6	39,6	25,7	0,15
ORI 30	51,1	25,1	46,5	3,2	14,0	4,0	20,2	29,3	21,9	53,1	34,9	0,41
ORI 31	65,9	46,8	56,0	3,0	25,7	2,3	5,6	27,6	21,5	65,4	36,5	0,38
ORI 33	39,2	25,4	35,8	3,7	13,7	3,6	16,1	23,4	17,1	32,8	22,4	1,12
ORI 34	70,1	18,6	60,7	4,2	20,2	4,4	8,3	20,8	18,3	44,0	24,4	2,86
ORI 35	45,6	19,7	40,0	3,3	10,2	4,4	6,4	24,1	18,5	51,3	32,4	0,13
ORI 36	49,3	43,9	44,5	3,5	19,6	2,6	8,1	21,9	17,4	56,0	30,7	0,06
ORI 37	49,3	20,0	39,5	3,5	11,8	3,9	8,1	25,7	23,4	46,0	28,5	0,77
ORI 39	51,9	16,6	47,1	3,4	14,6	4,4	7,3	25,6	22,4	33,2	21,8	0,08
ORI 40	48,9	31,6	36,8	4,5	15,6	2,3	10,3	23,8	17,2	61,2	39,4	0,10
ORI 41	63,0	24,4	50,3	3,6	22,5	2,9	5,5	33,7	22,8	55,6	35,0	0,31
ORI 42	54,0	15,7	40,8	3,7	20,7	2,7	9,3	23,8	16,2	35,7	22,9	0,67
ORI 43	61,2	37,0	48,0	4,3	21,7	2,1	9,3	22,3	20,6	54,6	36,1	2,41
ORI 45	44,0	48,0	34,9	4,2	20,2	2,3	12,7	23,1	17,1	32,2	23,0	0,49
ORI 47	51,2	13,4	42,8	3,4	19,5	2,4	8,7	20,8	16,0	40,1	26,9	0,19
ORI 49	45,3	23,0	29,3	3,4	17,6	1,9	21,2	21,3	16,3	31,0	22,2	0,07
ORI 50	56,3	32,5	47,1	2,9	19,7	2,6	16,4	23,3	17,2	64,1	37,9	0,16
ORI-H	63,4	20,0	57,3	4,7	16,6	3,8	7,6	23,8	18,9	49,6	33,7	2,90
ORI-C	57,8	22,7	40,8	5,1	17,4	2,9	9,5	30,5	23,1	69,9	42,7	3,24
ORI-S	59,8	22,6	48,4	4,1	15,8	3,6	7,7	24,1	18,5	55,2	36,1	3,85
SE	0,61	0,64	0,56	0,05	0,26	0,06	0,23	0,29	0,18	0,83	0,49	0,06
LSD (0.05)	4,9	5,2	4,8	0,4	2,2	0,6	1,7	2,5	2,1	6,3	4,6	0,16

PH: plant height (cm), NB: number of branchs, BL: branch length; SD: stem diameter (mm), NN: number of nodes per stem,

DI: distance of Internodes (cm), NL: number of Leaves per Node, LL: leaf length (mm), LW: leaf width (mm),DM: dry mass (g plant-1),

DF: drug fraction (g plant-1), EOY: essential oil yield (w/w)

Table S2. Mean values for 42 accessions of *Origanum vulgare* L. for 18 main compounds (10 % of total content of essential oil in at least one accession).

Accs.	p-Cymene	γ-Terpinene	Thymol	Carvacrol	β-Caryophyllene	Germacrene D	Linalool	E-Sabinene hydrate	Terpinene-4-ol	thymol meth.ether	Carvacrol meth.ether	α-Caryophyllene	Valencen	β-Bisabolene	Germacrene D-4-ol	Spathulenol	Caryophyllene oxide	α-Cadinol
ORI-2	0,0	0,0	11,3	0,0	22,6	29,9	0,0	0,0	0,0	0,0	0,0	0,0	3,6	5,8	0,0	9,9	13,1	0,0
ORI-6	0,0	0,0	4,3	0,0	25,1	36,2	0,0	0,0	0,0	3,1	0,0	4,7	2,0	5,5	2,4	0,0	11,3	2,6
ORI-7	0,0	3,9	2,3	0,0	25,1	37,2	0,0	2,9	6,7	0,0	0,0	2,9	2,0	1,3	0,0	4,4	2,6	2,9
ORI-8	1,5	9,4	12,2	0,0	6,3	24,6	0,0	9,1	9,4	1,3	1,4	0,7	3,3	3,6	0,0	1,1	0,1	0,0
ORI-10	0,9	7,1	35,4	0,8	5,7	12,5	4,6	0,0	1,5	4,4	4,6	1,0	3,9	3,6	0,0	1,7	0,0	1,2
ORI-11	2,0	11,1	21,2	0,0	1,9	21,6	3,6	0,0	0,6	5,3	2,3	0,0	3,4	0,0	2,9	0,0	0,0	1,9
ORI-12	0,0	0,0	7,8	3,6	25,5	11,9	0,0	0,0	0,0	0,0	4,0	4,1	0,0	4,3	0,0	4,6	32,6	0,0
ORI-13	0,0	0,0	0,0	2,8	15,6	25,6	0,0	0,0	0,0	0,0	0,0	2,8	5,5	3,5	0,0	8,3	12,4	5,0
ORI-14	0,0	0,0	12,2	0,0	15,5	9,7	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	16,2	46,3	0,0
ORI-15	0,0	0,0	0,0	0,0	22,8	23,7	0,0	0,0	0,0	0,0	0,0	0,0	8,0	3,6	0,0	10,1	9,8	4,0
ORI-16	0,6	3,0	24,2	0,0	9,4	15,9	0,0	7,4	12,6	3,4	3,1	1,3	3,2	4,9	0,0	1,1	1,0	0,0
ORI-17	0,0	0,0	3,4	1,8	24,6	19,8	7,8	0,0	0,0	0,0	0,0	1,4	4,8	3,8	9,1	0,0	8,2	4,5
ORI-18	0,0	0,0	6,9	5,0	15,8	34,9	0,0	0,0	0,0	0,0	0,0	0,0	4,6	0,0	13,6	0,0	6,8	5,0
ORI-19	1,0	6,1	4,2	14,6	9,9	21,3	0,0	13,2	10,2	0,6	2,7	1,4	2,2	1,3	0,0	0,9	1,4	0,0
ORI 20	3,6	7,5	7,4	6,4	12,9	14,8	2,7	3,8	5,2	1,3	3,0	2,1	2,4	3,0	0,0	4,0	9,7	0,0
ORI 21	0,0	0,0	19,3	15,7	5,7	21,5	0,0	0,0	0,0	0,0	0,0	0,0	5,4	4,1	0,0	3,8	1,6	4,0
ORI 23	0,0	0,0	11,1	9,7	15,2	24,5	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	8,1	31,5	0,0
ORI 24	3,5	10,3	47,2	20,5	2,4	1,1	0,0	0,1	0,6	0,8	0,4	0,4	0,5	1,3	0,2	0,0	0,0	0,3
ORI 25	3,8	6,4	67,4	3,3	2,6	0,9	0,1	0,2	0,4	2,3	0,2	0,4	0,3	2,6	0,0	0,0	0,0	0,0
ORI 26	0,0	3,6	6,2	7,6	15,2	28,3	0,0	3,7	5,4	1,5	3,3	2,0	4,2	3,1	0,0	5,1	5,8	1,5
ORI 27	0,0	2,4	13,8	0,0	14,8	34,7	0,0	0,0	0,0	4,3	0,0	2,0	4,4	3,5	0,0	6,0	6,7	0,0
ORI 28	4,4	9,8	19,5	55,1	1,3	0,2	0,0	0,2	0,3	0,0	0,4	0,2	0,0	1,4	0,0	0,0	0,0	0,0
ORI 29	0,0	0,0	62,5	0,9	4,9	1,4	0,0	0,0	1,2	4,1	3,7	0,8	0,5	3,5	0,0	0,7	0,0	0,8
ORI 30	2,4	8,5	28,1	0,0	8,7	11,3	3,8	0,0	2,6	1,7	2,4	0,9	2,6	2,7	0,0	2,8	0,9	2,4
ORI 31	0,5	0,7	12,8	65,5	3,3	1,0	1,6	0,0	0,0	0,4	2,0	0,4	0,7	2,6	0,0	0,7	0,3	0,0
ORI 33	3,3	8,8	0,8	61,7	2,4	1,0	0,0	0,4	0,4	0,0	9,5	0,4	0,4	3,1	0,0	0,2	0,0	0,0
ORI 34	3,6	8,2	36,1	35,7	3,0	1,5	0,0	0,4	0,5	0,1	0,7	0,3	0,4	2,0	0,0	0,1	0,1	0,0
ORI 35	0,0	0,0	1,5	22,5	9,8	24,8	1,8	0,0	4,0	0,0	9,7	1,7	1,8	3,9	0,0	2,7	3,3	1,7
ORI 36	0,0	0,0	13,0	0,0	12,9	25,1	0,0	0,0	0,0	0,0	0,0	0,0	6,3	6,9	0,0	16,4	19,3	0,0
ORI 37	2,6	5,4	66,6	0,8	2,7	0,9	0,5	0,2	0,5	3,3	4,5	0,3	0,3	1,5	0,0	0,3	0,1	0,0
ORI 39	0,0	0,0	33,0	7,6	8,6	19,7	0,0	0,0	0,0	0,0	0,0	0,0	4,3	6,1	0,0	4,8	7,4	0,0
ORI 40	0,0	0,0	21,2	12,4	12,4	20,7	0,0	0,0	0,0	0,0	0,0	2,4	0,0	4,6	0,0	5,4	6,8	2,0
ORI 41	2,2	3,3	22,4	4,5	11,1	13,0	3,4	0,0	0,0	4,5	3,0	1,6	1,4	3,8	0,0	3,1	5,7	0,0
ORI 42	3,3	3,5	52,2	30,0	1,1	0,0	0,0	0,2	0,5	0,4	2,0	0,1	0,0	2,0	0,0	0,0	0,0	0,0
ORI 43	4,6	10,7	38,7	28,5	4,2	0,3	0,6	0,0	0,3	0,2	0,0	0,4	0,2	1,6	0,0	0,2	0,3	0,0
ORI 45	3,9	2,9	1,5	69,4	2,7	2,7	0,2	0,2	0,8	0,0	4,1	1,0	0,3	3,1	0,0	0,0	0,3	0,0
ORI 47	0,0	0,0	0,0	21,0	18,8	13,5	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	21,2	6,6	9,7
ORI 49	0,0	0,0	0,0	40,1	12,0	17,1	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	20,5	6,3	4,0
ORI 50	0,0	0,0	7,1	11,7	16,7	32,1	0,0	0,0	0,0	0,0	0,0	1,8	4,4	2,5	0,0	7,0	8,9	2,3
ORI-H	3,3	5,7	1,4	74,3	2,6	0,5	0,0	1,5	0,5	0,0	0,2	0,3	0,4	1,5	0,0	0,0	0,1	0,0
ORI-C	5,7	5,5	2,7	72,2	2,5	0,6	0,0	0,3	0,4	0,0	0,1	0,5	0,3	1,8	0,0	0,0	0,1	0,0
ORI-S	4,5	8,5	1,4	71,0	2,7	0,9	0,0	0,3	0,3	0,0	0,7	0,4	0,4	1,7	0,0	0,2	0,1	0,0
SE	0,10	0,21	1,01	1,35	0,43	0,66	0,10	0,17	0,18	0,09	0,13	0,06	0,12	0,10	0,14	0,29	0,59	0,11
LSD (0.05)	0,12	0,16	0,31	0,30	0,30	0,30	0,07	0,08	0,09	0,09	0,11	0,16	0,17	0,17	0,06	0,16	0,27	0,12

Table S3. Proportion of membership of 42 accessions of *Origanum vulgare* in 6 inferred subpopulations (K=6) formed with 477 AFLP and SAMPL markers

	K					
	1	2	3	4	5	6
ORI-2	0,002	0,003	0,158	0,102	0,004	0,731
ORI-6	0,007	0,005	0,968	0,005	0,002	0,013
ORI-7	0,002	0,001	0,99	0,002	0,001	0,004
ORI-8	0,005	0,002	0,969	0,006	0,008	0,01
ORI-10	0,006	0,003	0,003	0,982	0,002	0,004
ORI-11	0,004	0,007	0,012	0,967	0,002	0,008
ORI-12	0,003	0,004	0,931	0,043	0,002	0,017
ORI-13	0,002	0,004	0,88	0,005	0,007	0,102
ORI-14	0,002	0,005	0,697	0,167	0,004	0,125
ORI-15	0,017	0,003	0,968	0,002	0,006	0,004
ORI-16	0,018	0,069	0,852	0,007	0,013	0,041
ORI-17	0,006	0,004	0,767	0,021	0,126	0,076
ORI-18	0,01	0,012	0,821	0,002	0,137	0,018
ORI-19	0,004	0,008	0,131	0,003	0,002	0,852
ORI 20	0,005	0,004	0,931	0,006	0,015	0,039
ORI 21	0,003	0,005	0,902	0,004	0,015	0,071
ORI 23	0,005	0,007	0,753	0,052	0,053	0,13
ORI 24	0,011	0,005	0,976	0,003	0,003	0,002
ORI 25	0,01	0,004	0,012	0,019	0,004	0,951
ORI 26	0,177	0,007	0,754	0,003	0,006	0,053
ORI 27	0,004	0,015	0,832	0,007	0,005	0,137
ORI 28	0,98	0,003	0,003	0,002	0,002	0,01
ORI 29	0,008	0,029	0,004	0,94	0,003	0,016
ORI 30	0,005	0,034	0,754	0,006	0,167	0,034
ORI 31	0,004	0,006	0,004	0,977	0,003	0,006
ORI 33	0,007	0,005	0,01	0,955	0,01	0,013
ORI 34	0,989	0,001	0,003	0,001	0,002	0,004
ORI 35	0,004	0,005	0,008	0,956	0,003	0,024
ORI 36	0,012	0,007	0,88	0,021	0,067	0,013
ORI 37	0,017	0,004	0,909	0,031	0,008	0,031
ORI 39	0,014	0,006	0,035	0,035	0,007	0,903
ORI 40	0,021	0,095	0,024	0,054	0,712	0,094
ORI 41	0,01	0,675	0,188	0,004	0,006	0,117
ORI 42	0,003	0,976	0,003	0,011	0,002	0,005
ORI 43	0,161	0,005	0,11	0,715	0,004	0,005
ORI 45	0,947	0,031	0,002	0,003	0,015	0,002
ORI 47	0,003	0,003	0,005	0,002	0,984	0,003
ORI 49	0,052	0,002	0,036	0,006	0,896	0,008
ORI 50	0,048	0,005	0,742	0,037	0,147	0,021
ORI-H	0,784	0,13	0,004	0,012	0,03	0,04
ORI-C	0,921	0,002	0,013	0,004	0,055	0,005
ORI-S	0,872	0,077	0,006	0,004	0,031	0,01

Table S4. Pearson correlations between phenotypic and chemotypic traits among 42 accessions of *O. vulgare*

	PH	**NB**	**BL**	**SD**	**NN**	**DI**	**NL**	**LL**	**LW**	**DM**	**DF**	**EOY**	**CAC**	**THC**	**PCC**	**GTC**	**BCC**	**GDC**
PH	1																	
NB	0,075	1																
BL	0,855**	-0,012	1															
SD	0,014	-0,048	-0,082	1														
NN	0,390**	0,354**	0,323**	0,023	1													
DI	0,168**	-0,327**	0,298**	-0,072	-0,553**	1												
NL	-0,193**	0,037	-0,198**	0,161**	-0,081	-0,051	1											
LL	-0,010	-0,172**	-0,003	-0,016	-0,135*	0,136*	-0,036	1										
LW	-0,031	-0,093	-0,009	0,080	-0,093	0,143*	-0,015	0,640**	1									
DM	0,350**	0,494**	0,269**	0,038	0,327**	-0,101	-0,001	-0,119*	-0,001	1								
DF	0,291**	0,403**	0,215**	0,077	0,295**	-0,142*	0,023	-0,102	0,025	0,913**	1							
EOY	0,139*	-0,107	0,065	0,585**	0,031	0,040	0,042	-0,012	0,110	0,086	0,118*	1						
CAC	-0,021	0,100	-0,099	0,553**	0,090	-0,120*	0,0 23	-0,071	-0,005	0,025	0,042	0,736**	1					
THC	-0,035	-0,133*	-0,052	0,060	-0,105	0,060	-0,051	0,064	0,108*	-0,158**	-0,125*	0,013	-0,238**	1				
PCC	-0,029	-0,041	-0,039	0,527**	0,050	-0,089	0,028	0,067	0,020	-0,017	0,032	0,806**	0,612**	0,233**	1			
GTC	-0,080	-0,009	-0,096	0,193**	0,025	-0,054	0,037	0,143*	0,095	-0,005	0,050	0,539**	0,255**	0,237**	0,751**	1		
BCC	0,072	-0,061	0,022	-0,139*	0,069	0,037	-0,065	-0,026	-0,063	0,073	0,024	-0,518**	-0,539**	-0,472**	-0,673**	-0,608**	1	
GDC	0,001	0,078	0,096	-0,424**	0,036	-0,006	-0,033	-0,036	-0,045	0,109	0,071	-0,610**	-0,607**	-0,447**	-0,717**	-0,420**	0,681**	1

**, * Significant at 0,01 and 0,05 respectively.

III. DISCUSSION

3.1. Usefulness of AFLP and SAMPL markers for genetic studies of *O. vulgare* L.

In recent decades, a variety of molecular techniques and genetic markers have been extensively developed to estimate genetic diversity, but no single technique is universally ideal; each available technique exhibits both strengths and weaknesses. Therefore, the choice of technique is often a compromise that depends on the research question pursued and the genetic resolution needed (Avise 1994). The precise assessment of genetic diversity through molecular markers depends on the type of DNA polymorphism (length vs. sequence variation) detected, and the proportion of the genome covered by the marker system used (Avise 1994). The nature of marker system (dominancy or co-dominancy) is also a very important factor for genetic studies. Many underrepresented plant species such as most of medicinal plants, or other crops with limited genomic information largely rely on dominant type of markers like RAPDs and AFLPs (Li et al. 2007).

One of the main aims in the study followed in this thesis was to investigate the efficiency of AFLP and SAMPL marker systems in surveying DNA polymorphisms and in detecting genetic relationships among 42 populations of *O. vulgare*. AFLP (amplified fragment length polymorphism) is a PCR-based technique that approaches an ideal tool as a relatively cheap, easy, fast and reliable method to generate hundreds of informative genetic markers (Vos et al. 1995). The main disadvantage of AFLP is the difficulty in identifying homologous markers (alleles), rendering this method less useful for studies that require precise assignment of allelic states, such as heterozygosity analyses (Mueller and Wolfenbarger 1999). In recent years, AFLP has been extensively used as a molecular marker system for detecting DNA polymorphisms and genetic mapping in several crops (Bensch and Åkesson 2005, Meudt and Clarke 2007).

SAMPL (Selectively Amplified Microsatellite Polymorphic Loci) is another molecular marker technique which utilizes the same template DNA as that of AFLP (Morgante and Vogel, 1994). In the SAMPL analysis, one of the AFLP primers in combination with an SAMPL primer is employed for the selective amplification step. The SAMPL primer essentially comprises of a compound microsatellite sequence, which is anchored. Such a SAMPL primer design ensures preferential amplification of microsatellite-like sequences (Rakoczy-Trojanowska and Bolibok 2004). Hence, SAMPL methodology was developed as an SSR-based modification of AFLP to combine the multiplex approach of AFLP with the hyper-variable nature of SSRs (Vogel and Scolnik 1998). Despite its many advantages, i.e., a high multiplex ratio, SAMPL has not been widely used for analysis of plant genomes.

Comparison between RAPD, AFLP and SSR markers in different plant species has revealed that codominant SSRs detect the highest level of polymorphism per locus as it surveys the hyper-variable microsatellite regions of the genome. Hence, they have higher information content (Powell et al. 1996, Russell et al. 1997). Nevertheless, molecular markers with higher multiplex ratio, such as RAPD,

AFLP and SAMPL may be more useful than SSRs despite their dominant nature (Powell et al. 1996, Russell et al. 1997, Raina et al. 2001). Their multilocus approach allows them to screen a wider region of the genome and estimate relationships with a high-resolution approach (Teulat et al. 2000).

As a part of the present thesis, a comparative analysis of AFLP and SAMPL in assessing the genetic diversity among the 42 oregano accessions was carried out and the results are (Azizi et al. 2009a, shown in table 2 of article 1). Five different aspects of the performance of both marker systems were considered. These included all informative bands amplified per assay (MR: multiplex ratio), the average number of polymorphic bands detected per assay (EMR: effective multiplex ratio), overall efficiency of detecting polymorphism in the germplasm pool and between any two accessions taken at random from that pool (%P: per cent polymorphism, $H_{av\text{-}p}$: average heterozygosity), overall utility of marker for detecting genetic variation (MI: marker index) and resolving power (RP) of the primer combinations (Azizi et al. 2009a : Table 2).

Seven primer combinations for both AFLP and SAMPL were utilized that generated a total of 596 and 558 fragments, respectively. Higher MR was detected among oregano accessions with the AFLP assay (MR = 85), compared to the SAMPL assay (MR = 80; Table 2). However, SAMPL assay detected higher polymorphism (%P = 92) compared to AFLP (%P = 71). Similar observations have been made in other medicinal plants such as *Azadirachta indica* (Singh et al. 2002) and *Withania somnifera* (Negi et al. 2006). Due to the high information content, the SAMPL analysis has previously been shown to be more suitable for studies where low genetic variation is expected (Witsenboer et al. 1998). This can be explained by the high levels of polymorphism related to the Microsatellite region, by which this variation is generated. The marker index (MI), which is calculated as the product of EMR and $H_{av\text{-}p}$ (Milbourne et al. 1997), marker utility was slightly higher for SAMPL than AFLP (14.25 vs. 13.53) in the 42 accessions evaluated in this study. This is because of the EMR component, which was shown to be higher for the SAMPL assay (Azizi et al. 2009a: Table 2). This result is in corroboration with the studies in other plant species such as *Vigna unguiculata* (Tosti and Negri 2002), *Withania somnifera* (Negi et al. 2006) and *Tribulus terrestris* (Sarwat et al. 2008). According to the values of resolving power (RP) estimated for all primer combinations, the AFLP primers discriminated the *O. vulgare* accessions better than the SAMPL primers (Azizi et al. 2009a: Table 2). This observation agrees with the results in a study of wheat by Altintas et al. (2008) where the higher average values of resolving power (RP) obtained for SAMPL primer combinations rather than those for AFLP.

In our study, an interesting feature of SAMPL fingerprint was the ladder-like banding pattern (stutter bands), a typical characteristic of SSR loci. These ladder-like sequences may have arisen due to replication slippage (Azizi et al 2009a: Figure 1). Replication slippage responsible for SSR diversity occurs more frequently than single-nucleotide mutations and deletions/insertion events that generate polymorphism detectable by AFLP analysis.

Considering association analysis, SAMPL markers seem to be more suitable than AFLP. We have identified 28 markers associated to economically and pharmaceutically important traits such drug fraction, essential oil yield, carvacrol content and thymol content (Azizi et al. Manuscript: Table 3).

Out of them 24 loci were SAMPL type markers. These results lead us to conclude that in comparison of these two multilocus marker systems, the SAMPL approach seems to be a powerful tool to strengthen the resolution of the AFLP technique. This finding is very important for taxonomic investigation of *O. vulgare* and also for germplasm management, genetic conservation and breeding programs in oregano. AFLP and SAMPL techniques can also be very useful tools for authentication of oregano populations and any other medicinally important herbs.

3.2. Genetic, chemical and agro-morphological variations in *O. vulgare* L.

Experimental studies in ecology, evolution and breeding often depend on accurate assessment of genetic diversity to address questions regarding genetic relatedness among individuals, population structure and phylogenetic relationships (Hillis et al. 1996). It can generally be achieved through the use of genetic polymorphism indicators such as morphology, cytogenetic, biochemical attributes including Isozyme patterns, chemical compounds like terpenes or PCR-based molecular markers (Brown et al. 1989). Terpenes are not much used unless the terpene chemicals themselves are of interest, for example in aromatic and spice plants. isozyme analysis is relatively easy to perform, but the other techniques for investigating genetic diversity indicators require a high level of technical skill, with good laboratory facilities (McKinnell 2002).

Identification of diverse germplasm that has high chances of detecting potentially useful genes for plant breeding is an essential prerequisite towards formulating conservation strategies for plant genetic resources. The present thesis offers a multidimensional approach to investigate diversities within a collection of *Origanum vulgare* based on molecular markers (AFLP and SAMPL), quantitative agronomic and morphological traits and chemical compounds of essential oils. Combined AFLP and SAMPL analysis offers the possibility to screen a large number of anonymous loci and to cover the whole genome, as specified by restriction sites and microsatelite loci, to detect polymorphisms.

In the present study, the UPGMA clustering, inferred population structure and principal component analysis based on genetic markers, quantitative agro-morphological traits and chemotypic characters revealed a high level of polymorphisms (Azizi et al. Manuscript). This finding is in good agreement with earlier reports by Chalchat and Pasquier (1998), D'antuono et al. (2000), Radušiene et al. (2005) and Katsiotis et al. (2009). However, the grouping patterns between *O. vulgare* accessions provided by three methods of diversity analysis were rather different (Azizi et al. Manuscript: Fig. 1). This difference may be related to phenotypic plasticity of the plants in response to changes in the habitat environment (West-Eberhard 1989). This relevant aspect of this study shows that genomic similarity does not necessarily reflect similarity or difference in output traits, such as oil composition, morphological characters or agronomic traits. For example, accessions ORI47 and ORI49 are quite different in their agro-morphological traits but genetically very similar (Azizi et al. Manuscript: Fig. 1). However in our study, a higher correlation was obtained between genetic and chemical polymorphism rather than between genetic and agro-morphological variations.

Based on the findings of the present thesis, AFLP and/or SAMPL dendrograms may be used for universal taxonomic studies, while dendrograms based on quantitative agro-morphological traits and chemical characteristics, may be of practical interest, but do not necessarily correlate with taxonomy. DNA genotyping offers the unique capacity to classify accessions regardless of environmental condition and plant growth stage. Morphological characters, which are the easiest to determine, may only provide a primary classification.

Population genetic structure is determined by joint effects of many factors including mating system, natural and artificial selection, mutation, migration and dispersal mechanism, drift, etc. (Brown et al. 1989). Natural populations of a large number of medicinal species, propagating through seeds, implicitly have a high level of genetic variation (Schippmann et al. 2002). The medicinal and spice herb *Origanum vulgare* investigated in the current study is a perennial with propagation through seeds. The reproductive system is also one of the important life-history characteristic that strongly influences genetic variability (Clegg et al. 1992). In *O. vulgare*, the mating system is mostly cross pollination (Kheyr-Pour 1981) which can cause a high level of genetic polymorphism and this variation may eventually led to differences in the genetic control of accumulation of monoterpenes such as carvacrol, thymol, *p*-cymene and *γ*-terpinene in the essential oils (Gershenzon and Croteau 1990). This wide variation in essential oil composition presumably has ecological advantages in protecting plants against different pests (Hough-Golstein and Hahn 1992).

Finally, the results obtained in the present thesis showed differences among the accessions of oregano in respect to morphological and agronomic traits and chemical constituents of essential oils, indicating the existence of intraspecific variation and chemical polymorphism. These natural biodiversity can be exploited for breeding programs of *O. vulgare* such as the identification and selection of accessions (chemotypes) with active compounds (carvacrol and thymol) and also with absence of undesirable compounds (germacrene D). The divers'natural populations of *O. vulgare* can also serve as starting material for intraspecific crossing programs including simple crosses (such as selfing, crossing, polycrossing, and backcrossing) and complex hybridization (Bernáth 2002).

In applied breeding for medicinal plants' improvement, chemotypic and genetic distances between genotypes are expected to provide predictors for high heterosis effects on pharmaceutical qualities and yield performance of their hybrids. In the present study, a high expression of germacrene-D (an undesirable compound) was observed in many wild accessions (Azizi et al. Manuscript: Fig. 1). Nonetheless, among these accessions, based on thymol content, essential oil yield and drug fraction, ORI8, ORI25, ORI27, ORI29, and ORI37 showed promising performance that can be exploited in breeding programmes. The knowledge of genetic, chemical and agro-morphological diversity of *O. vulgare*, germplasm, displayed through this study, will also let an improvement of homogeneous plant material of different types of essential oil depending on the demands of pharmaceutical and food industries for a specific use.

3.3. Enabling morphology- and marker-assisted selection of *O. vulgare* L.

The first step in the domestication process of medicinal and aromatic plants involves selection of plant material in nature, but the advent of modern plant breeding has accelerated their domestication considerably (Canter et al. 2005). The majority of medicinal plants under cultivation have been created using conventional selection methods. Even in the countries involved in the large scale production of medicinal and spice herbs, such as Hungary, 60-75 percent of cultivars are developed by simple selection methods from local, or introduced populations (Bernáth 2002).

Owing to the extremely large genetic, morphological and chemical variability encountered in *O. vulgare* (Radušiene et al. 2005, Azizi et al. 2009a, Azizi et al. Manuscript), selection programs represent an important part of the quality breeding activities. Breeding practices in oregano rely mostly on phenotypic selection on germplasm available in seed companies and botanical gardens so that the selection of new cultivars is underway. The oregano material which is already selected is characterized by 0.5-1.5% essential oil containing carvacrol and thymol as the main compounds (Bernáth 1997, Franz and Novak 2002). In the present thesis, we have studied trait-to-trait correlations (between all morphological, chemical and agronomic characters) and marker-trait associations (between AFLP and SAMPL markers, chemical and agronomic traits) to found an approach that could provide the indirect selection strategies using morphology and molecular markers.

In the selection process, it would be most useful to have morphological traits as indicators of chemotypes. For example, morphological traits with a high correlation to essential oil yield and content of phenolic monoterpenes (such as carvacrol and thymol) could provide a useful tool for indirect selection in breeding of pharmaceutical value of *O. vulgare*. These traits could be important because the essential oil-related traits like essential oil content and oil composition cannot reliably be measured during a breeding programme because of difficulty of oil distillation from the small amounts of plant material for single plants (Franz and Novak 1997). The trait-to-trait correlations between the major chemical components and morphological characters should also be considered during selecting the parental chemo- and phenotypes from oregano populations for breeding of pharmaceutical qualities.

In our study, for two pharmaceutically important traits for oregano, essential oil yield and carvacrol content, only one morphological trait, the stem diameter was found to be positively correlated to these traits (Azizi et al. Manuscript). Stem diameter could be considered as an indicator for indirect selection and breeding for these two economically important traits in oregano and may also be in other species of the genus *Origanum*. According to these results of our study, stem diameter is also negatively correlated to the undesirable compound germacrene-D in essential oil composition (Azizi et al. Manuscript). Our findings confirm the results of previous studies on other *Lamiaceae* plants such as mint, where stem diameter reported to be positively correlated to essential oil yield (Mirzaie-Nodoushan et al. 2001).

Marker-assisted selection (MAS) is another strategy for increasing the probability of selecting superior genotypes in indirect selection programs (Knapp 1998). Yousef and Juvik (2001) compared

phenotypic and marker-assisted selection (MAS) for quantitative traits in sweet corn and they concluded that incorporating DNA markers to traditional breeding programs could not only expedite selection progress but also be cost-effective. Knapp (1998) proposed that MAS would increase the efficiency of artificial selection and substantially decrease the resources required in breeding for a trait with low to moderate heritability.

The main purpose of marker-trait association analyses in the present thesis was to found a starting point for marker-assisted selection (MAS) in *O. vulgare* using AFLP, SAMPL, chemical and phenotypic polymorphisms. We have identified four AFLP and 24 SAMPL markers associated to the economically important traits including drug fraction, essential oil yield, carvacrol content and thymol content (Azizi et al. Manuscript: Table 3). SAMPL marker system seems to be more effective for association analyses and also for studies on intraspecific diversity and relationships among *O. vulgare* subspecies (Azizi et al. 2009a, Azizi et al. Manuscript). Because of the lack of mapping information for the AFLP and SAMPL markers, we were not able to examine the structure of disequilibrium among associated markers. However, considering that the gene-linked SSRs (simple sequence repeats) and locus-specific SNPs (single nucleotide polymorphisms) are still not developed for *O. vulgare*, the identified novel allelic polymorphisms for the pharmaceutically important traits should be of considerable interest for breeding purposes.

Marker-trait associations found in the present study were supported by 1,000-times permutated P-values of five different statistical models. However, it is still possible that some of the marker-trait associations identified in our study are false positives, therefore further validation is required. Further mapping studies in segregating populations will help to confirm whether the associated markers are linked to QTLs influencing the traits. Studies on QTLs linked to synthesis pathways of different monoterpenes in aromatic plants of the *Lamiaceae* family are very rare. However, with regard to broad variation in the essential oil profiles of *O. vulgare* populations, the biosynthetic pathway of carvacrol and thymol seems to be different and more complicated. This makes it difficult to detect QTLs for this pathway and also to identify individual genes because specific pathway branches control the synthesis of different monoterpenes (Vernet et al. 1986).

Among the identified markers showing strongest effects on four economically important traits (Azizi et al. Manuscript: Table 3), there are three markers that could be starting points for further studies for marker assisted selection (MAS):

I) AFLP-2_31 may be used in breeding for pharmaceutical quality because it was co-associated with two key traits, essential oil yield and carvacrol content; however the marker effects (R^2 values) were relatively low.
II) SAMPL-1_18 which is related to the drug fraction would be another considerable candidate to follow.
III) SAMPL-3_60 with a relatively strong effect (R^2 values) on thymol content could make it useful for marker-assisted selection (MAS) of this very important antimicrobial compound.

This study provides the first reported investigation of association analysis in the diverse populations of *O. vulgare*, and thus, it will provide a useful benchmark for comparison with future results and with results from other aromatic and medicinal species.

3.4. Effect of drought stress on oregano essential oil during the flowering phase

In order to assess the performance of oregano in different soil moisture regimes and nitrogen supplies, we have tested three cultivated types of accessions of *O. vulgare* under controlled greenhouse conditions (Azizi et al. 2009b). The results of our study showed that dry matter production and essential oil content of *O. vulgare* can be significantly affected by soil moisture regime, whereas percentage of main compounds of essential oil including monoterpenes such as carvacrol, thymol, γ-terpinene and *p*-cymene remained unaffected.

Several previous studies on aromatic and medicinal plants have proved that the water supply is one of the most determinative cultivation conditions which significantly affect the herbage yield and essential oil content of various spices and herb crops (Singh et al. 2000, Zehtabi-Salmasi et al. 2001, Delfine et al. 2005). The results of other studies indicated that drought stress during the vegetative period can reduce yield of medicinal and aromatic plants and optimal use of irrigation water improves the herbage yield of these plants (Ram et al. 1995, sing et al. 1997).

The effect of consistent drought stress on essential oil was studied in some medicinal and aromatic plants such as palmarosa (*Cymbopogon martinii*), citronella java (*Cymbopogon winterianus*), sweet basil (*Ocimum basilicum*) and American basil (*Ocimum americanum*). Essential oil content (percentage) was increased under drought stress but essential oil yield was reduced under these drought stress conditions (Fatima et al. 2006, Khalid 2006). This observation could be due to the interaction between essential oil content and herbage yield that are considered as two main components of the essential oil yield so that by exerting stress, increases the essential oil content but herbage yield (biomass yield) decreases by the drought stress, therefore essential oil yield reduces. However, most of the studies on drought stress in aromatic and medicinal plants were basically designed to find the response of the plants to consistent drought stress during the vegetative and reproductive period. Practically, the time at which the aromatic plants of *Lamiaceae* are irrigated is important for the efficiency of irrigation. For example, appropriate irrigation strategies showed a great potential for improvement of the yield of monoterpenes in field-grown of other *Lamiaceae* plants such as spearmint and rosemary (Delfine et al. 2005).

In our study, it could be observed that in comparison with optimal water supply, significantly lower dry matter production of oregano was caused by both water deficient treatments, consistent and later water deficiency (Azizi et al. 2009b: Fig.3a). Additionally, the consistent water deficiency caused higher degree of reduction of dry matter production than the water deficiency after flowering (Azizi et al. 2009b: Fig.3a). It is interesting to note that in comparison with control (optimal water supply), later

water deficiency (during the flowering phase) caused a significantly increase in essential oil content, whereas the consistent water deficiency failed to do it (Azizi et al. 2009b: Fig.3b). Our findings underline the conclusion that the amounts of essential oils produced under drought conditions were either maintained or enhanced, depending on the species and magnitude of the stress (Singh et al. 2000, Sangwan et al. 2001, Zehtabi-Salmasi et al. 2001, Delfine et al. 2005). In mints and sweet basil, it was found that higher essential oil content due to drought stress was related to higher density of essential oil glandular hairs (Sangwan et al., 2001). This may also be true for oregano. Later water deficiency significantly improved essential oil content and thus the quality of oregano herbage. In addition, this treatment reduces the water amount used for production and thus increases the efficiency of irrigation. However, this effect needs to be tested under field conditions.

It can be concluded therefore, the restriction of water supply after beginning of flowering may increase the content of essential oil and thus improves the quality of oregano herbage. This can significantly benefit the farm performance of oregano. This finding may give applicable advice to commercial farmers and researches for better management and proper allocation of water resources in medicinal and aromatic plants farming under drought conditions. Taking into account these results could improve the quantity and quality characteristics of these herb crops in arid and semi-arid areas.

IV. SUMMARY

Most of commercially used medicinal and aromatic plant species are collected from the wild flora. By bringing medicinal herbs into cultivation, conventional and biotechnological plant breeding techniques can be applied to improve herbage yield and plant uniformity as well as pharmaceutical properties. *Origanum vulgare* L. (oregano) is a perennial aromatic herb belonging to the family *Lamiaceae* used as medicinal plant because of the essential oil produced in the aerial parts.

The present study was basically designed to assess the genetic diversity of the oregano germplasm based on molecular markers, chemical compounds of essential oils and agro-morphological traits. The ultimate goal of our research was to use trait-to-trait correlation and marker-trait association analysis to identify morphologic characters and potential molecular markers for future use for indirect screening and marker-assisted selection, respectively. The other objective of this thesis was to identify the most appropriate soil moisture regime and nitrogen fertilization for the plant growth to obtain a high level of the essential oil content.

Twelve traits related to agronomic and morphological characteristics were measured. Components in the essential oils were identified by GC-MS and 18 major compounds of 60 identified constituents, were investigated. A total of 477 molecular polymorphisms including 214 AFLP (Amplified Fragment Length Polymorphisms) and 263 SAMPL (Selectively Amplified Microsatellite Polymorphic Loci) were used for genotyping. Associations between traits of interest and genetic markers were tested using five methods including three general linear models (GLM) and two unified mixed linear model (MLM). In this study, the relative efficiencies of two PCR-based marker approaches, AFLP and SAMPL, were also compared for surveying genetic diversity and subspecies discrimination among 42 oregano accessions.

The results of the greenhouse experiment with three populations of oregano cultivated in Germany (*O. vulgare* var. *creticum, O. vulgare* ssp. *hirtum* and *O. vulgare* var. *samothrake*) indicated that water deficiency after beginning of blooming can induce an increase in essential oil content and thus result in higher quality of oregano herbage and higher water use efficiency of oregano plants. This finding may give applicable advice to commercial farmers and researches for better management and proper allocate of water resources for cultivation of oregano in arid and semi-arid areas.

According to the results of diversity surveying in oregano germplasm, cluster analysis, population inference and principal component analysis (PCA) revealed a broad variation among accessions. A relatively high correlation between chemotypic patterns and genetic markers was identified while a lower correlation was found between the agro-morphological and genetic matrices. The results also showed that SAMPL marker system seems to be more effective than AFLP for studies on intraspecific diversity and relationships among *O. vulgare* subspecies and also for marker-trait association analyses. Considering the results of analyses of trait-to-trait correlations and marker-trait associations, we can conclude that stem diameter, an easily measurable morphological trait, could be considered for indirect selection and three identified molecular markers, AFLP-2_31, SAMPL-1_18 and SAMPL-3_60 may be included in marker-assisted programmes to improve breeding efficiency of

pharmaceutical properties. These findings can be very applicable for domestication and breeding strategies in *Origanum vulgare* and also for management of its genetic resources.

V. ZUSAMMENFASSUNG

Die Produktion von Arznei- und Gewürzpflanzen erfolgt heute zunehmend durch einen kontrollierten Anbau, mit dem hohe Drogenerträge und spezifische Qualitäten angestrebt werden. Voraussetzung dafür ist, dass mit Hilfe konventioneller und biotechnologischer Züchtungsmethoden leistungsfähige Zuchtsorten geschaffen werden, die sich durch verbesserte Erträge, eine gute Homogenität und spezielle pharmazeutische Eigenschaften auszeichnen. Das gilt auch für den Dost (*Origanum vulgare* L.), der wegen seines ätherischen Öles als mehrjährige Krautdrogenpflanze genutzt wird und als Gewürz- und Arzneipflanze eine große Bedeutung besitzt.

Das Ziel der durchgeführten Untersuchungen bestand darin, die Diversität von Oregano-Akzessionen aus einer Genbank-Kollektion bezüglich molekularer Marker, der chemischen Zusammensetzung der ätherischen Öle und agro-morphologischer Merkmale zu evaluieren. Dabei sollten mit Hilfe von Merkmal-zu-Merkmal-Korrelationen und Marker-Trait-Assoziationsanalysen potenzielle morphologische Merkmale sowie molekulare Marker identifiziert werden, die zukünftig in der Selektion von Oregano verwendet werden können. Ein weiteres Ziel der Arbeit bestand darin, den Einfluss unterschiedlicher Bodenfeuchteregime und Stickstoff-Düngung auf den Krautertrag sowie auf den Gehalt und die Zusammensetzung des ätherischen Öls zu klären.

Zur phänotypischen Charakterisierung der untersuchten Genbankkollektion wurden an den Oregano-Pflanzen insgesamt zwölf agronomische bzw. morphologische Merkmale erfasst. Daneben wurde das mit Hilfe der Wasserdampfdestillation gewonnene ätherische Öl mittels GC-MS analysiert, wonach insgesamt 18 Hauptkomponenten von 60 identifizierten Verbindungen detektiert wurden. Für die Genotypisierung von Oregano wurden insgesamt 477 molekulare Polymorphismen einschließlich 214 AFLP (Amplified Fragment Length Polymorphisms) und 263 SAMPL (Selectively Amplified Microsatellite Polymorphic Loci) verwendet. Assoziationen zwischen den interessierenden Merkmalen und den genetischen Markern wurden mit drei allgemeinen linearen Modellen (GLM) und zwei gemischten linearen Modellen (MLM) getestet. Daneben wurde auch die relative Effizienz von zwei PCR-basierten Markermethoden (AFLP und SAMPL) für die Bestimmung der genetischen Diversität sowie die Unterartendiskriminierung der insgesamt 42 Oregano-Akzessionen verglichen.

Die Untersuchungen zur Stickstoff- und Wasserversorgung von Oregano wurden als Gefäßversuche unter Freilandbedingungen durchgeführt. Einbezogen wurden drei in Deutschland kultivierte Populationen von Oregano: *O. vulgare* var. *creticum, O. vulgare* ssp. *hirtum* und *O. vulgare* var. *samothrake*. Die Ergebnisse dieser Versuche zeigten, dass der Wassermangel erst nach Beginn der Blühphase eine Erhöhung der Gehalte an ätherischem Öl und eine bessere Qualität der Oreganokräuter und somit eine höhere Effizienz der Wassernutzung induzieren kann.

Aus den Ergebnissen der Cluster-Analysen, der Bestimmung der Populations-Inferenz und der Komponentenanalysen (Principal Component Analysis) kann abgeleitet werden, dass zwischen den Oregano-Akzessionen eine große Variation besteht. Es wurde eine relativ enge Korrelation zwischen Chemotyp-Mustern und den genetischen Markern identifiziert. Eine geringere Korrelation wurde dagegen zwischen den genetischen und den agromorphologischen Merkmalen gefunden. Die

Ergebnisse zeigten auch, dass das SAMPL-Marker-System effektiver für Studien zur intraspezifischen Diversität und zur Erkennung der Beziehungen zwischen Oregano-Unterarten zu sein scheint.

Aus den Ergebnissen der Analysen der Merkmal-zu-Merkmal-Korrelationen und der Marker-Trait-Assoziationen kann geschlussfolgert werden, dass der Stängeldurchmesser als leicht messbares morphologisches Merkmal für die indirekte Auslese verwendet werden kann. Die drei identifiezierten molekularen Marker, AFLP-2_31, SAMPL-1_18 und SAMPL-3_60 können für die Marker-gestützte Selektion genutzt werden, um die Züchtungseffizienz von pharmazeutischen Eigenschaften zu verbessern. Die gewonnenen Erkenntnisse können sehr nützlich für die weitere Domestikation und Züchtung von *Origanum vulgare* und auch für die weitere Evaluierung der genetischen Ressourcen dieser Gewürzpflanzenart sein.

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