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DISSERTATION

Titel der Dissertation

Molecular and phytochemical analyses of the genus *Origanum* L. (Lamiaceae)

angestrebter akademischer Grad

Doktorin der Naturwissenschaften (Dr. rer.nat.)

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INTRODUCTION

Introduction

The genus *Origanum* L. comprises 43 species and 18 hybrids (Carlström, 1984; Danin, 1990; Danin and Künne, 1996; Duman et al., 1995; Duman et al., 1998; Ietswaart, 1980). *Origanum* species are usually subshrubs or woody perennial herbs that reach heights of up to 80 cm and have ovate leaves and white or purple flowers (Figure 1). The aerial organs of the plant, especially stems, leaves and bracts, are, often densely, covered by glandular and non-glandular hairs.

Figure 1: *O. cordifulium*, an endemic to Cyprus (Troodos Mountains), in its natural habitat.

Most of the *Origanum* species are locally distributed within the Mediterranean region where they grow in mountainous areas on dry, rocky, often calcareous soils. *Origanum vulgare* L. possesses by far the largest distribution and can be found throughout the Mediterranean region, in most parts of the Euro-Siberian region as well as in the Irano-Turanian region (Figure 2).

Figure 2: Distribution of the genus *Origanum* (dotted line: area of all sections; solid line: all sections except the section *Origanum*) (Ietwsaart, 1980).

About 60 percent of the *Origanum* taxa are recorded to grow in Turkey indicating this geographical area as the gene centre of the genus. The rate of endemism is high and about 70 percent of the *Origanum* species occur on an island or mountain (group) only.

The glandular hairs covering the aerial parts of *Origanum* plants secrete an essential oil with a very characteristic odour. Due to these essential oils *Origanum* species have locally been collected for centuries, for flavouring traditional dishes as well as for numerous purposes in traditional medicine. Today, two aromatic qualities of *Origanum* plant material, marjoram (*O. majorana* L.) and oregano (usually from *O. vulgare* and *O. onites* L.) are commercially traded and widely used all over the world as popular spices. Beside their sensorial qualities oregano and marjoram possess further valuable properties. Amongst others, antifungal, antibacterial, antioxidant, analgesic, and anticancer activities were observed (reviewed in Baričevič and Bartol, 2002). Apart from the traditional use as kitchen herb and folk remedy, preparations of *Origanum* plant material can be used in medicine as well as in feed-, food-, pharma- and cosmetic-industry in a wide variety of ways.

Despite its economic importance the genus *Origanum* is often referred to as an underutilized taxon with its potential for utilization not yet fully explored (Padulosi, 1997). This is partly due to a lack of knowledge on chemical and genetical diversity but also to the complex taxonomy of the genus that is complicated by the extent of morphological variation *Origanum* taxa exhibit in nature. With the intend to contribute to a refined and more complete picture of the genus my PhD studies addressed both, phylogenetic as well as phytochemical analyses of the genus *Origanum*. The following paragraphs of the introduction provide an overview about the systematical and phytochemical knowledge currently available and introduce the particular aims of my studies. In the subsequent parts of the thesis the results of my investigations as well as the major conclusions are summarized.

Taxonomy of the genus *Origanum*

The Lamiaceae family is a difficult plant group and its classification above the genus level has been heavily debated. According to the current status eight subfamilies are recognized (Cantino et al. 1992). The Nepetoiodeae, the largest and economically most important subfamily, is further divided into the four tribes Escholtzieae, Lavanduleae, Mentheae and Ocimeae (Cantino et al., 1992; Wagstaff et al., 1995). The genus *Origanum* is placed within the Mentheae tribe, with *Thymus* L., *Thymbra* L. and *Micromeria* Benth. as close relatives (Bräuchler et al., 2010; Ietswaart 1980).

The generic limits of *Origanum* were subject of intensive taxonomic discussions and up to recent times two different genus concepts were in use. Some authors followed Bentham's original genus concept (1834) and treated *Amaracus* Benth., *Majorana* Benth. and *Origanum* Benth. as separate genera. Others followed the Linnaean genus concept (1754) and considered *Origanum* in a broad sense. Today, the taxonomic revision of Ietswaart (1980) is widely accepted treating the genus *Origanum* as a whole. Within *Origanum* Ietswaart recognized three groups, ten sections and 38

species (one with six subspecies, one with three varieties). Since he published his monograph five more species were described (Carlström, 1984; Danin, 1990; Danin and Künne, 1996; Duman et al., 1995) raising the total number of species to 43 (Figure 3).

Group	Section	Number of species
A	*Amaracus* Bentham	7
	Anatolicon Bentham	8
	Brevifilamentum Ietswaart	7
	Longitubus Ietswaart	1
B	*Chilocalyx* Ietswaart	4
	Majorana Bentham	3
C	*Campanulaticalyx* Ietswaart	6
	Elongatispica Ietswaart	3
	Origanum	1
	Prolaticorolla Ietswaart	3

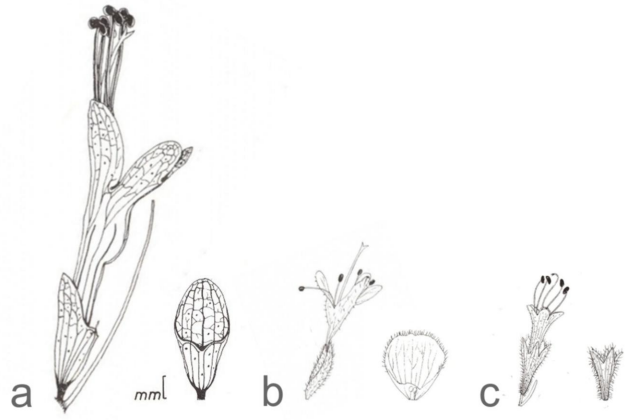

Figure 3: List of sections defined by Ietswaart (1980) and the number of species currently accepted. Shape and size of bracts and calyx are the basis for the differentiation of the three groups. Species of group A usually possess 2- or 1-lipped, large calyces, the bracts are also large, membranous and usually purple coloured (a = Flower and calyx of *O. dictamnus*, section *Amaracus*). Species of group B possess 2- or 1-lipped, rather small calyces as well as small, leaf-like bracts (b = Flower and calyx of *O. onites*, section *Majorana*). Species of group C usually have calyces with 5 (sub)equal teeth (c = Flower and calyx of *O. dayi*, section *Campanulaticalyx*) (Figures from Ietswaart (1980), modified).

Ietswaarts' delimitation of sections and species is based on the analysis of a number of morphological characters with shape and size of bracts, calyx teeth and corollas amongst the most important (Figure 3). However, the taxonomy of *Origanum* was found to be rather complex and nearly all of the sections are afflicted with some kind of taxonomic uncertainties. The genus exhibits a considerable extent of morphological variation, *Origanum* species, subspecies and varieties can be discerned in their typical form but nearly all of them gradually merge into at least one other form. Moreover, some *Origanum* species show morphological characteristics that are intermediate between two sections. Some others possess characters that can be found nowhere else in the genus but in closely related genera as e. g. *Thymus* or *Satureja* L. (Ietswaart, 1980). Hybrid speciation could be responsible for morphological tendencies as observed. On the basis of his comprehensive morphological analyses Ietswaart considered hybridisation, not only within the genus but also with related species from *Thymus* and *Satureja*, as the most important speciation mechanism in the genus *Origanum*. Hybridisation is still a frequent phenomenon in the genus and up to now eighteen *Origanum* hybrids were described (Duman et al., 1998; Ietswaart, 1980).

Molecular techniques provide valuable tools to gain additional evidence when the informational value of morphological characters is too low to resolve species boundaries and phylogenetic relationships satisfactorily. However, up to now only two investigations were published that applied DNA based methods to clarify taxonomic difficulties that concern the genus concept of *Origanum*. Both investigations focused on the widespread *O. vulgare*, and researched intraspecific genetic diversity as

well as the genetic integrity of the six *O. vulgare* subspecies and their relationships (Azizi et al. 2009; Katsiotis et al. 2009). A number of taxonomic uncertainties remain and demand for additional research with alternative methods. A part of my PhD studies was dedicated to this task and aimed to resolve phylogenetic relationships within *Origanum* by means of comparative DNA sequence analyses. As clarification of taxonomic ambiguities seemed to be especially urgent for commercially traded *Origanum* taxa a focus was given to section *Majorana* whose four member taxa are amongst the most widely used (Lukas et al., in preparation; part 1). When working on the four closely related taxa of section Majorana microsatellites proved to be a valuable tool to confirm assumptions regarding species boundaries and recent interspecific hybridisation. The characterization of the microsatellites used as well as the development of a novel microsatellite analyses strategy are documented in two manuscripts that have been published during my PhD research (Mader et al., 2008; Novak et al., 2007; annex to part 1). Phylogenetic analyses provide not only a new basis for the ongoing discussion about taxonomic uncertainties. Sequence analyses or sequence based approaches could serve as an additional tool for a botanical classification ("DNA-barcoding") of plant material (Ruzicka et al., 2009), with the major advantage that they would also allow an authentication of highly processed material such as plant powders or dried plant extracts (Müller et al., 2008; annex to part 1).

Origanum compounds of interest

The valuable sensorial and medicinal properties of *Origanum* species are mainly based on their essential oils, complex mixtures of around 30 to 50 mono- and sesquiterpenes that are produced in capitate and peltate glandular hairs covering the aerial parts of the plants (Figure 4).

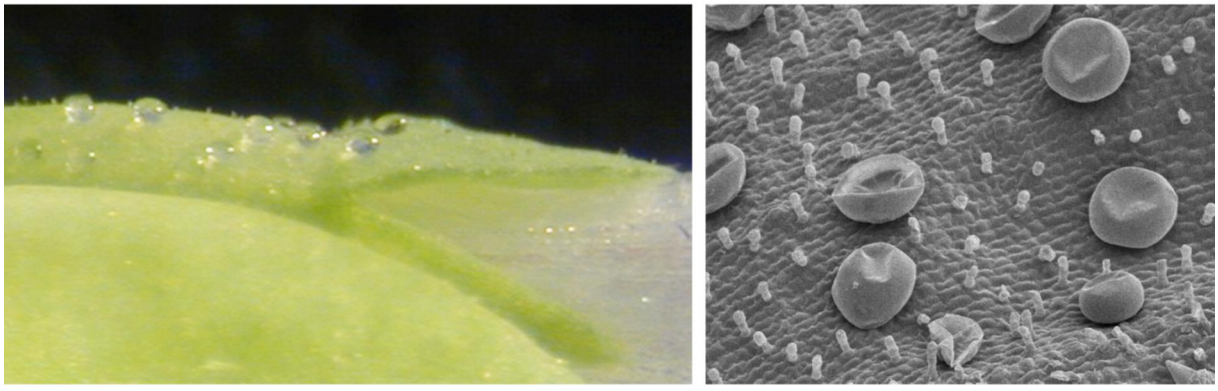

Figure 4: Peltate and capitate oil glands on the calyx surface of *O. vulgare* (left side) and *O. microphyllum* (right side; REM picture provided by Corinna Schmiderer).

Within the genus *Origanum* (but also within species and even populations) the qualitative and quantitative composition of the essential oils is rather variable and many different chemotypes were described (reviewed in Skoula and Harborne, 2002). In general, the essential oils of *Origanum* species circumscribed by the term oregano are rich in 'cymyl'-compounds (phenolic monoterpenoids, mainly

γ-terpinene, *p*-cymene, carvacrol and/or thymol deriving from the 'cymyl'-pathway). In the volatile oils of marjoram (*O. majorana*) 'cymyl'-compounds are almost completely absent and high percentages of 'sabinyl'-compounds (bicyclic monoterpenoids, mainly sabinene, *cis*- and *trans*-sabinene hydrate and *cis*-sabinene hydrate acetate deriving from the 'sabinyl'-pathway) are present. Compounds of three more biochemical groups, acyclic monoterpenoids (e. g. *β*-myrcene, geraniol, linalool), bornane type compounds (e. g. camphor, camphene, borneol) and sesquiterpenoids (e. g. *β*-caryophyllene, *β*-bisabolene, germacrene-D) can also be isolated but are quantitatively usually of less importance (Skoula and Harborne, 2002; Figure 5).

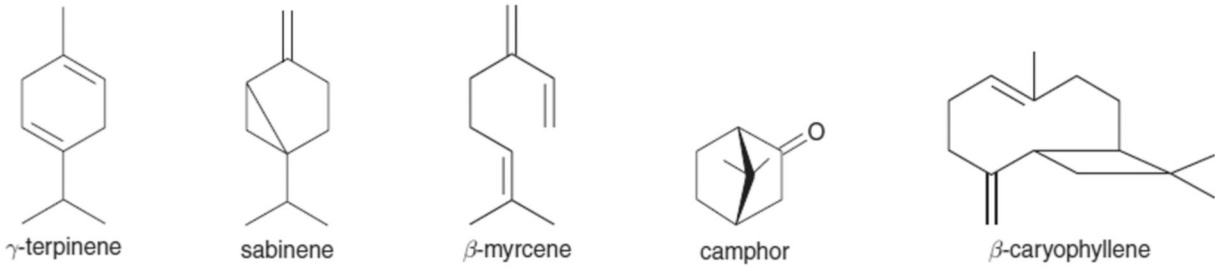

Figure 5: Examples for common monoterpenoids (*γ*-terpinene, sabinene, *β*-myrcene, camphor) and a sesquiterpenoid (*β*-caryophyllene) commonly found in *Origanum* spp.

Due to the heavy commercial interest scientific research was strongly concentrating on the volatile compounds. During the most recent years a number of further compounds, among them phenolic acids (e. g. rosmarinic acid, caffeic acid; Figure 6) and flavonoids (e. g. quercetin; galangin, Figure 6), became of increasing interest as they are thought to be involved in the high antioxidant activity that was observed for different preparations of *Origanum* plant material (e. g. Goze, 2009; Ozkan et al., 2007). As such investigations are often based on a small number of plant extracts or extracts deriving from pooled plant samples not too much is known about inter- and intraspecific variability of these compounds.

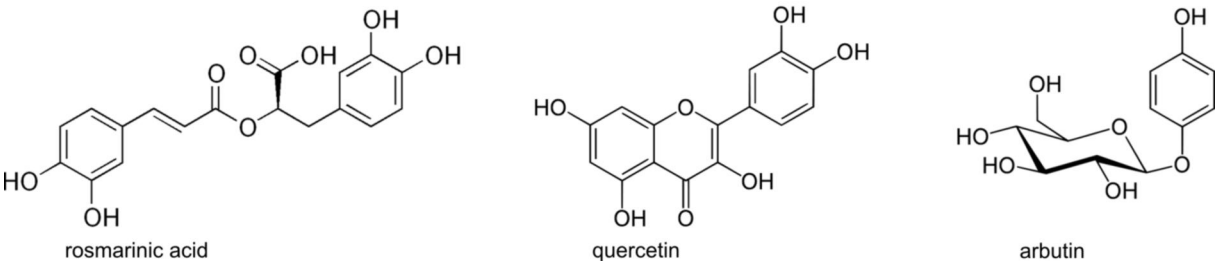

Figure 6: One example for phenolic acids (rosmarinic acid) and flavonoids (quercetin) present in *Origanum* spp. Structure of arbutin.

With arbutin (Figure 6) oregano and marjoram contain a compound non-desired in the human diet. Hydroquinone, a metabolite of arbutin, was found to be hepatotoxic, nephrotoxic, mutagenic and carcinogenic in animal studies (Nowak, et al., 1995; Peters et al., 1997). Furthermore, a hypothesis was published linking phenol and hydroquinone as causal factors for leukaemia (McDonald et al.,

2001). Up to now the knowledge about inter- and intraspecific arbutin variability in *Origanum* is scarce. High amounts of arbutin were isolated from an accession of *O. majorana* (marjoram) whereas plant material of *O. vulgare* (one of the commercial oregano species) was found to be free of this compound (Assaf et al., 1987; Kraus et al., 1996).

Today, oregano and marjoram are predominantly used as kitchen herbs but their richness in valuable compounds predestines them for further applications. Several in vivo studies proved the efficiency of oregano essential oils to substitute growth promoters in animal feed which will be banned in the EU in future (Docic and Bilkei, 2003). Essential oils or single essential oil components of oregano were also successfully tested for their food-preservative potential (e. g. Dagdemir et al., 2009; Olmedo et al., 2009). A number of in vitro and in vivo studies attested promising medicinal properties to *Origanum* essential oils or single *Origanum* compounds (e. g. Al-Howiriny et al., 2009; Cleff et al., 2010; Liang et al., 2010). These references represent just a small selection of recent investigations dealing with possible applications of *Origanum* plant material or its preparations.

The sensorial qualities of oregano and marjoram and the effects of *Origanum* preparations are of course closely associated with the chemical properties of the plant material processed. Whereas most of the traded marjoram is now standardized plant material from cultivation of *O. majorana*, the better part of oregano is still collected from the wild. This bears the danger of genetic erosion and results in heterogeneous crops, an undesirable situation that could be changed step by step by the expansion of cultivation. The natural variability of *Origanum* species would provide a wide range of interesting biotypes for selection and subsequent cultivation or breeding programs. However, to utilize the existing variability best comprehensive population studies are required that research the range of chemical variability present and reveal also rare chemotypes that are often overlooked. The studies included in part 2 of my thesis followed this objective and provide an overview about the composition of essential oil compounds in populations of Corsican *O. vulgare* (Lukas et al., 2008), Syrian *O. syriacum* (Lukas et al., 2009) and wild growing Cypriot *O. majorana* (Novak et al., 2008; annex to part 2). One more investigation researched the intrageneric and intraspecific variability of arbutin in *Origanum* and discussed approaches to breed cultivars low in arbutin or free of this compound (Lukas et al., 2010). Part 2 is completed by a methodological article that introduces a fast gas chromatographic method for the quantification of arbutin in extracts from marjoram and bearberry (Lamien-Meda et al., 2009; annex to part 2).

Essential oil polymorphisms in *Origanum*

Essential oils are concentrated, hydrophobic liquids composed of various terpenes. Terpenes are the largest and most widespread class of secondary metabolites in plants and more than 20.000 have been identified to date (Connolly and Hill, 1991). In plants terpenes have numerous biological functions. They are involved in growth and development, in climatic adaptation as well as in diverse plant-

environment interactions such as the attraction of pollinators, the defence of pathogens and herbivores or the signalling to neighbouring plants (Arimura et al., 2004; Baldwin et al., 2002; Pare and Tumlinson, 1999; Pichersky and Gershenzon, 2002).

Biosynthetically, all terpenes derive from the simple C_5-units isopentenyl pyrophosphate (IPP) and dimethylallyl diphosphate (DMAPP). Prenyltransferases fuse DMAPP with varying numbers of IPP to the terpenoid precursors geranyl diphosphate (GPP, C_{10}), farnesyl diphosphate (FPP, C_{15}) or geranylgeranyl diphosphate (GGPP, C_{20}). The unique terpene composition of a taxon is mainly controlled by a set of mono-, sesqui- and diterpene synthases which catalyze the transformation of the respective terpenoid precursor to the parent structures of each type (Tholl, 2006; Figure7 (a)).

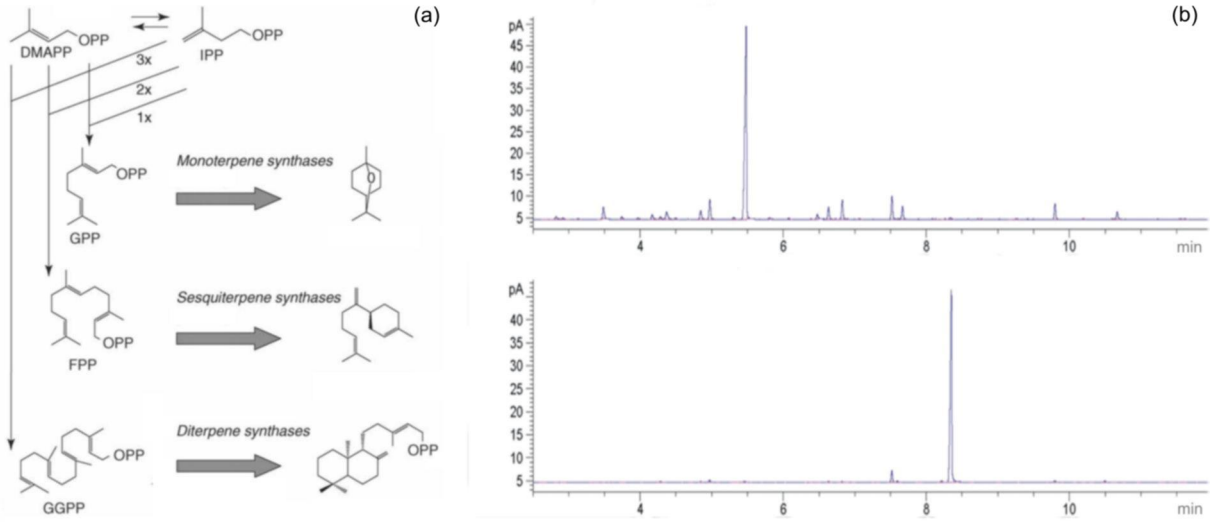

Figure 7: (a) Formation of plant terpenes (Tholl, 2006; modified). (b) GC-FID chromatograms of an almost pure 'sabinyl'-chemotype (*O. majorana*, above) and an almost pure 'cymyl'-chemotype (*O. dubium*, below). The dominant peaks represent cis-sabinene hydrate (above) and carvacrol (below).

The qualitative and quantitative essential oil composition is rather variable and different chemotypes were reported based on the dominant monoterpenes produced (reviewed in Skoula and Harborne, 2002). Polymorphic essential oil variation can be observed among and within *Origanum* species. Within the well investigated section *Majorana* three main essential oil chemotypes are present. The most abundant chemotype, the main chemotype of *O. dubium* (Arnold et al., 1993; Baser et al., 1993a), *O. onites* (e. g. Skoula et al., 1999; Vokou et al., 1988) and *O. syriacum* (e. g. Fleisher and Fleisher, 1991; Lukas et al., 2009) is the oregano flavoured 'cymyl'-chemotype (Figure 7 (b), below). In Turkish populations of both, *O. dubium* and *O. onites*, a second chemotype is additionally present and there 'cymyl'-chemotypes occur intermixed with almost pure linalool-chemotypes (Baser et al., 1993b; Lukas et al., data in preparation). *Origanum majorana* possesses a very different essential oil rich in 'sabinyl'-compounds (e. g. Fischer et al., 1987; Novak et al., 2008; Figure 7 (b), above). 'Mixed' chemotypes are rather rare but 'cymyl'/linalool chemotypes were observed in Turkish *O. onites* and *O. dubium* (Lukas et al., data in preparation).

Not much is known about the molecular mechanisms regulating such terpene variation within species or between taxa closely related. Differences in the qualitative essential oil composition can not only be due to the presence/absence of (functional) genes (Iijima et al., 2004; Köllner et al., 2004) but also to the mode of gene regulation (Crocoll et al., in print; Iijima et al., 2004). The aim of the investigation presented in part 3 of my thesis was to learn more about essential oil chemotype formation in *Origanum*. For this purpose sequence analyses of the γ-terpinene synthase gene, a key gene in the biosynthesis of 'cymyl'-compounds, were performed (Lukas et al., accepted). Part 3 of my thesis is completed by a study researching the influence of ecological conditions on the quantitative composition of *Origanum* essential oils (Novak et al., accepted; annex to part 3).

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PART 1

Tracing evolutionary relationships in section *Majorana* Benth.

Marjoram versus Oregano: Complex evolutionary relationships in the section *Majorana* Benth., genus *Origanum* L. (Lamiaceae)

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To be submitted

Abstract The genus *Origanum* (Lamiaceae) comprises a number of essential oil rich species that have traditionally been collected for centuries. Today, the four species of section *Majorana* (*O. onites*, *O. dubium*, *O. majorana* and *O. syriacum*) are amongst the most widely used. Despite the importance of this section, phylogenetic relationships and species boundaries among its four taxa are unclear. In the present investigation we used DNA sequence data from two nuclear regions (ITS and 1-deoxy-D-xylulose 5-phosphate synthase [DXS]) and one chloroplast region (*psbA-trnH* intergenic spacer), as well as five microsatellite loci to test the taxonomic status .of the four species of section *Majorana*. The combined DNA data revealed *O. onites* and *O. syriacum* as ancient species in the section. *Origanum dubium* was found to be of hybridogenous origin showing attributes of *O. onites*, *O. syriacum* and a third, unknown species. *Origanum majorana* directly derived from *O. syriacum*. Both, sequence and microsatellite analyses, provided evidence for recent hybridisation between *O. onites* and *O. dubium* in Turkey.

Key Words DXS*;* hybridisation*;* ITS*;* marjoram*; Origanum;* oregano

Introduction

The genus *Origanum* L. (Lamiaceae) comprises 43 species and 18 hybrids (Carlström 1984; Danin 1990; Danin and Künne 1996; Duman et al. 1995; Duman et al. 1998; Ietswaart 1980). Within the Lamiaceae, the genus is placed into the Mentheae tribe, with *Thymus* L., *Thymbra* L. and *Micromeria* Benth. as close relatives (Bräuchler et al. 2010). Most of the *Origanum* species are locally distributed in the Mediterranean where they have been traditionally collected for centuries (e. g. Della et al. 2006; Fleisher and Fleisher 1988). Today two 'aromatic qualities' of *Origanum* plant material, marjoram (from *Origanum majorana* L.) and oregano (from several *Origanum* species, but also from other genera of the Lamiaceae; Lawrence 1984), are commercially traded and widely used all over the world as popular spices. During the recent years the genus has also become of interest for cosmetical and pharmaceutical industry as preparations of oregano and marjoram were found to possess antifungal, antibacterial, antioxidant, analgesic, immunostimulant, anticancer, antiparasitic, insecticidial, nematicidial and molluscicidial properties (reviewed in Baričevič and Bartol 2002).

Despite its economic importance the genus *Origanum* is often referred to as an underutilized taxon with its potential for utilization not yet fully explored (Padulosi 1997). This is due to a lack of knowledge on chemical and genetic diversity but also to the complex taxonomy of the genus that is complicated by the considerable extent of morphological variation the genus exhibits in nature. *Origanum* species, subspecies and varieties can be discerned in their typical form but nearly all of them gradually merge into at least one other form (Ietswaart 1980). Mating barriers seem not to exist and hybridisation, even between distantly related species, is a frequent phenomenon in the genus *Origanum* (Ietswaart 1980). Among the different taxonomic concepts for *Origanum* (e. g. Bentham 1834; Bentham 1848; Boissier 1879; Briquet 1895) the taxonomic revision of Ietswaart (1980) is the most widely accepted. Based on morphological criteria Ietswaart (1980) arranged the *Origanum* species within three groups and ten sections (group A with sections *Amaracus* Benth., *Anatolicon* Benth, *Brevifilamentum* Ietsw. and *Longitubus* Ietsw.; group B with sections *Chilocalyx* Ietsw. and *Majorana* Benth.; group C with sections *Campanulaticalyx* Ietsw., *Elongataspica* Ietsw., *Origanum* and *Prolaticorolla* Ietsw.). Nearly all of the sections are afflicted with some kind of taxonomic uncertainties and thus details of Ietswaarts taxonomic concept are still an issue of debate. This is also true for the section *Majorana* (group B) where the taxonomic difficulties become especially obvious as its four essential oil rich taxa, *O. onites* L., *O. syriacum* L., *O. dubium* Boiss. and *O. majorana* L. are amongst the most widely used *Origanum* species and problems and confusion arise with the designation of traded and processed plant material (Baser 2002).

Within the genus *Origanum* the four species of section *Majorana* are united by a unique morphological feature: a one-lipped, bract-like calyx showing a nearly reduced lower lip. Within section *Majorana* morphological characters failed to explain satisfactorily evolutionary relationships, species boundaries and taxonomic status of its member taxa. *Origanum onites* appears to be the best

defined entity in section *Majorana* that can be differentiated by a characteristic corymbiform inflorescence and its often serrate leaves. Morphologically, *O. onites* has a somehow isolated position beside *O. syriacum*, *O. dubium* and *O. majorana,* latter three possess paniculate inflorescences and usually entire leaves. The species boundaries between these three species are not that clear and their differentiation partly relies on morphological characters that were found to be variable in natural populations. In *O. syriacum* stems and leaves are hirsute, hirsute-tomentose or tomentose and the leaves are usually acute and possess raised veins on the abaxial surface. In *O. dubium* and *O. majorana* the stems and leaves are tomentellous and the leaves are usually obtuse and have no raised veins on the abaxial surface. To differentiate the latter two taxa the inflorescence shape has been used (Ietswaart 1985). In *O. dubium* the inflorescence is usually thyrsoid or subcorymbose, more compact and does not extend so far down the stem whereas in *O. majorana* the inflorescence appears more elongated and narrow and extends far down the stem. However, the inflorescence shape was found to be quite variable in natural populations of *O. dubium* and *O. majorana* and thus their taxonomic status is still discussed. Both were treated as distinct species (e. g. Boissier 1879) before Ietswaart united them under *O. majorana* in his taxonomic revision of the genus (Ietswaart 1980) and in the *Origanum* chapter he wrote for the Flora of Turkey (Ietswaart 1982). After examination of additional specimens Ietswaart treated them again as separate species in the Flora of Cyprus (Ietswaart 1985).

Phytochemical characters are often helpful in phylogenetic considerations (Grayer et al. 1999; Larsson 2007; Wink 2003). *Origanum* species have been mainly used for their essential oils and because of the commercial interests especially their essential oil characteristics are well investigated. Three main essential oil chemotypes are present in section *Majorana* resulting in rather different 'aromatic qualities' of the respective plant material. The most abundant chemotype is the 'cymyl'-chemotype (Skoula and Harborne 2002) accumulating large amounts of γ-terpinene, *p*-cymene, carvacrol and/or thymol. Essential oils rich in 'cymyl'-compounds possess a pungent oregano flavour and are the characteristic of good quality oregano. The 'cymyl'-chemotype is the main chemotype of *O. dubium* (Arnold et al. 1993; Baser et al. 1993a), *O. onites* (e. g. Skoula et al. 1999; Vokou et al. 1988) and *O. syriacum* (e. g. Fleisher and Fleisher 1991; Lukas et al. 2009). In natural populations of Turkish *O. dubium* and *O. onites* a second chemotype is additionally present and here 'cymyl'-chemotypes occur intermixed with almost pure linalool-chemotypes (Baser et al. 1993b; Lukas et al. unpublished). *Origanum majorana* possesses a very different essential oil rich in 'sabinyl'-compounds (*cis*-/*trans*-sabinene hydrate and *cis*-sabinene hydrate acetate) (e. g. Fischer et al. 1987; Novak et al. 2008b) that are responsible for the specific flavour of marjoram. Regarding the essential oil chemistry with its 'sabinyl'-chemotype *O. majorana* has an isolated position within section *Majorana*. However, the chemosystematic relevance of essential oils for the taxa of this section is reduced by the trans-species occurrence of the 'cymyl'- and linalool-chemotype. The knowledge about other secondary compounds, among them flavonoids, phenols or phenolic acids (reviewed in Skoula and Harborne 2002; Skoula et al. 2008) is still too fragmentary to be helpful in systematic conclusions.

To evaluate the taxonomic status of *O. onites*, *O. syriacum*, *O. dubium* and *O. majorana* and to learn more about their evolutionary relationships we performed sequence analyses of the nuclear regions ITS (Internal transcribed spacers) and DXS (1-deoxy-D-xylulose 5-phosphate synthase) and the plastid psb*A*-trn*H* intergenic spacer region as well as microsatellite analyses of five polymorphic loci. The internal transcribed spacers (ITS1 and ITS2) are amongst the most commonly used molecular markers for evolutionary studies at the species level (e. g. Alvarez and Wendel 2003; Baldwin et al. 1995). Despite their high levels of variation ITS is a powerful tool to recognize ancient and recent hybridisation in flowering plants. During the last years in a number of taxa high intra-specific and intra-individual ITS variability was observed providing valuable insights into the history of complex taxa. (e. g. Denk et al. 2005; Koch et al. 2003; Mayol and Rosello 2001; Zheng et al. 2008). Single- or low copy genes of plant nuclear DNA, encompassing intron sequences with potentially high levels of polymorphism, are a promising tool to complement phylogenetic considerations based on ITS and plastid DNA loci (e. g. Schulte et al. 2009; Vaezi and Brouillet 2009). However, due to the lack of universal primers and the efforts often associated with the establishment of a new nuclear region for a taxon of interest they are still poorly used. With DXS a putative single-copy gene region in *Origanum* is introduced that may also be of interest for studying phylogenetic relationships in *Origanum* and closely related Lamiaceae genera. Psb*A*-trn*H* was shown to be among the most variable noncoding cpDNA regions (Shaw et al. 2007) and is frequently used for lower level taxonomic studies (e. g. Kim and Donoghue 2008; Peruzzi et al. 2008). In the discussion on the topic of DNA barcoding psbA-trn*H* was proposed as a useful marker for this purpose (Kress et al. 2005). Rapidly evolving microsatellites have developed into one of the most popular genetic markers to analyse inter-specific hybridisation (e. g. Edwards et al. 2008; Muir and Schlötterer 2005; Schwarzbach and Rieseberg 2002).

The aim of this investigation was to provide a new basis for the ongoing discussion about the taxonomic uncertainties concerning section *Majorana*, more specifically (1) to assess species limits and taxonomic status of *O. onites*, *O. syriacum*, *O. dubium* and *O. majorana* and to (2) discuss evolutionary relationships within the section *Majorana* by linking molecular with morphological and phytochemical evidence.

Material and Methods

Plant material

Individual plants of *O. dubium* Boiss., *O. majorana* L., *O. onites* L. and *O. syriacum* L. were collected during excursions to Italy, Greece, Turkey, Cyprus and Syria covering a wide range of the species natural distribution area (Figure 1). Additional plant material was taken from herbarium specimens and from plants grown in the greenhouse of the University of Veterinary Medicine, Vienna. Details about the geographic origin as well as accession number of the samples investigated for sequence and microsatellite analyses are given in Table 1. Species were identified by following the key of the

taxonomic revision of Ietswaart (1980). In case of *Origanum majorana* s. l. the Flora of Cyprus (Ietswaart 1985) was used as a second reference to distinguish between *O. dubium* and *O. majorana* s. str. Voucher specimens are kept at the herbarium of the Institute for Applied Botany and Pharmacognosy, University of Veterinary Medicine, Vienna.

DNA extraction, amplification, cloning and sequencing

Genomic DNA was extracted from young, silica gel dried leaves using a modified CTAB extraction procedure (Doyle and Doyle 1990). The nuclear ITS (Internal Transcribed Spacer) region was amplified using universal primers (ITS5 and ITS4; White et al. 1990, modified by Downie and Katz-Downie 1996). The amplification reactions were performed on a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, California, USA) in 20 µl volumes with the following reaction components: 0.5 µl template DNA (1-50 ng), 18 µl 1.1xReddyMixTM PCR Master Mix (2.5 mM $MgCl_2$) (ABGene, Epsom, UK), 0.5 µl DMSO, 0.2 µl ddH_20 and 0.4 µl (400 nM) of each primer (Invitrogen, Carlsbad, California, USA). 35 cycles of amplification with 1 min at 95°C, 1 min at 55°C and 1 min at 72°C were preceded by a 3 min denaturation step at 95°C and followed by an additional 7 min at 72°C. All PCR products were checked on 1.4% agarose gels before being purified with EXO1 and SAP (Fermentas, Burlington, Canada) according to the manufacturer's instructions. Sequencing of both strands was performed using BigDye Terminators (Applied Biosystems, Foster City, California, USA) and primers from the original amplifications. The sequences were generated with an ABI 3130x automated sequencer (Applied Biosystems, Foster City, USA) and edited using Chromas Vers. 2.24 (Technelyseum, Tewantin, Australia). Ambiguous sequences characterized by the appearance of few to many polymorphic sites and/or noise or divergence of the sequence after/to a specific point were cloned to isolate divergent ITS copies of individual plants. PCR products were cloned into the pGEM-T easy-cloning vector system (PROMEGA, Madison, Wisconsin, USA). Plasmid DNAs were reamplified from 10 to 16 clones for each individual using the original ITS primers and the same PCR conditions described above. After being checked on 1.4% agarose gels the PCR products were cleaned with EXO1 and SAP. Purified plasmid DNAs were sequenced in one direction according to the methods described for the direct sequencing of PCR products. A part of the nuclear DXS (1-deoxy-D-xylulose 5-phosphate synthase) gene was amplified using EST-derived primers (DXS24F1 and DXS390R1; Müller et al. 2008). The amplification protocol was the same as described for ITS. The thermal conditions were as follows: 35 cycles of amplification with 1 min at 95°C, 1 min at 60°C and 1 min at 72°C were preceded by a 3 min denaturation step at 95°C and followed by an additional 7 min at 72°C. The PCR products were directly sequenced in both directions according to the procedure described for the direct sequencing of ITS. Primers for the original amplification were used as sequencing primers. In heterozygote individuals (for a single or few substitutions) the alleles were determined through haplotype subtraction (Clark 1990). In most cases this method was successful to unambiguously separate different alleles from heterozygous plant individuals. PCR products

corresponding to strongly divergent alleles of heterozygote individuals were cloned with the same procedure already described for the cloning of ITS. The chloroplast psb*A*-trn*H* intergenic spacer region was amplified using universal primers (psb*A* and trnH^{GUG}; Sang et al. 1997; Tate and Simpson 2003). The amplification protocol was the same as described for ITS. The thermal conditions were: 35 cycles of amplification with 1 min at 95°C, 1 min at 60°C and 1 min at 72°C were preceded by a 3 min denaturation step at 95°C and followed by an additional 7 min at 72°C. The PCR products were directly sequenced according to the procedure described for the direct sequencing of ITS. Primers from the original amplification were used as sequencing primers.

Sequence analyses and phylogenetic reconstruction

ITS, DXS and psb*A*-trn*H* sequences were aligned using MEGA 4 (Tamura et al. 2007) with subsequent manual correction. Variable positions in the data matrices were checked against the original sequence chromatogram files.

The potential occurrence of pseudogenes among ITS repeats was assessed by checking the presence of conserved angiosperm motifs in ITS1 (Liu and Schardl 1994) and within the 5.8S rDNA (Jobes and Thien 1997). Secondary structure predictions and minimum free energy (deltaG at 37 °C) estimates were conducted using the web-based program mFold 3.1 (http://frontend.bioinfo.rpi.edu).

In order to gain information out of the intra-individual and intra-specific ITS diversity, the ITS clones of each accession and subsequently of each species were classified and grouped on the basis of alignment positions at which an overall variation of 5% or higher was observed (31 sites). For the ITS clones of each species the presence/absence of mutations at the variable nucleotide positions was assessed and recorded (Table 2). By this comparative view on the ITS clones, accessions exhibiting a deviating ITS pattern became obvious within the species *O. onites* and *O. syriacum* (putative recent hybrids between *O. onites and O. dubium* and between *O. majorana* and *O. syriacum*, respectively). To point out the particular character of these accessions their clones were united in sub-groups and treated separately in the subsequent ITS analyses. For each taxon the frequencies of the mutations were calculated. For *O. dubium*, *O. syriacum* and cultivated *O. majorana* ITS haplotypes (= ITS variants that were isolated from more than one accession of each species) were defined to distinguish frequent ITS haplotypes from clones with individual sequence composition. The occurrence and percentage of the different ITS haplotypes was calculated in each accession investigated (Figure 2).

For the phylogenetic analyses of the ITS clones the software programs G2CEF and EUKDIS (Göker and Grimm 2008) were used treating divergent ITS clones of one plant individual as ‘associates’ of a ‘host’. In a first step the sequence data of the associates was transformed into a character matrix of the hosts (program G2CEF, FRQ character transformation, gaps were treated as 5th character). In a second step host-host distances were computed from this character matrix (program EUKDIS, distance method: Euclidean distances). A split graph was computed on the resultant distance matrix by SplitsTree 4.8 (Huson and Bryant 2006) by using the Neighbor-Net algorithm.

For DXS a Neighbor-Net split graph was computed on the basis of uncorrected p distances using SplitsTree 4.8 (Huson and Bryant 2006).
Relationships among the chloroplast haplotypes were inferred using the software TCS 1.13 (Clement et al. 2000).
All sequences have been deposited in GenBank (Table 1).

Microsatellite analyses

The analysis of the 5 microsatellite loci (OR10, OR14, OR40, OR44, OR 64; Novak et al. 2008a) was done via High-Resolution-Melt-Analysis (HRM) according to the method described in Mader et al. (2008). The 10 µl PCR reaction volume contained 5.6 µl H_2O dest., 0.4 units Taq HOT FIREPol® polymerase (Solis BioDyne, Tartu, Estonia), 1µl Buffer B (Solis BioDyne, Tartu, Estonia), 1.4 µl $MgCl_2$ (25mM), 0.2 µl DMSO, 0.1 µl dNTP-mix (10mM), 0.1µl of each Primer (10pM) and 0.6µl BEBO 36µM (TATAA Biocenter, Bengtsson et al., 2003). 1 µl of DNA solution was added to each reaction, containing between 0.25 and 0.8ng/µl DNA. All reactions were done in duplicate. The PCR cycling started with an initial phase of 15min at 95°C, then 40 cycles of 10sec at 95°C, 20sec at 60°C and a 20sec elongation step at 72°C were performed. High resolution melting was carried out immediately following PCR from 70°C to 90°C at steps of 0.05°C, each step with a 1sec hold. PCR and subsequent HRM were performed on a RotorGene 6500 (Corbett Research Pty Ltd., Sydney, Australia) equipped with a HRM-module. The resulting melting curves were analysed using the RotorGene 6000 series software, Version 1.7.65. To obtain inter-run comparability standard samples were included in every run.

Microsatellite statistics

Alleles were scored according to allele identity and were named in order of appearance. Diversity indices (observed and expected heterozygosities, number of alleles) were calculated with GENALEX 6 (Peakall and Smouse 2006). To study population subdivision pairwise F_{ST} between all pairs of populations included in this study were calculated with FSTAT version 2.9.3 (Goudet 1995). The levels of significance were adjusted for multiple tests according to the Bonferroni criteria. PCA analysis was performed with GENALEX 6 and visualised with SPSS Version 16.0 (SPSS Inc. Chicago, Illinois, USA).

Results

ITS (Internal Transcribed Spacer)

A total of 277 ITS clones from 23 accessions of *O. dubium* (7), wild *O. majorana* (3), cultivated *O. majorana* (4), *O. onites* (5) and *O. syriacum* (4) were generated for the ITS analyses. The number of clones per individual plant ranged between 2 and 16. Four accessions of *O. onites* (SR810, SR1387,

SR1434, SR1440) and one of *O. syriacum* (SP3) were directly sequenced and included in the alignment. The length of the aligned sequences varied from 642 to 644 base pairs, the alignment of all ITS sequences resulted in a matrix of 647 characters of which 275 were variable.

Diversity of cloned ITS sequences and intra-specific ITS variability

High intra-individual ITS variability was observed and in nearly all accessions analysed, the number of ITS clones isolated corresponded to the number of divergent ITS clones. By a comparative view of the aligned sequences different degrees of intra-specific ITS variability became obvious (Table 2). In *O. onites* and wild *O. majorana* divergent ITS clones differed just by presence or absence of point mutations at some of the informative nucleotide positions defined. ITS of *O. onites* (oni) was characterised by mutations at ten of the informative nucleotide positions (Table 2). Five mutations were found in all of the clones, five nucleotide positions were polymorphic and mutations occurred in seven to 93% of the clones. These polymorphic sites corresponded to 'double-peaks' in the sequence chromatogram that became obvious when *O. onites* was directly sequenced. In ITS of *O. majorana* (maj) 14 informative nucleotide positions were present. Nine mutations were constantly found in all of the clones, five informative nucleotide positions were polymorphic and mutations occurred in two to 98% of the clones isolated from this species. In *O. dubium*, *O. syriacum* and cultivated *O. majorana* ITS appeared much more complex and was characterised by intra-specific polymorphisms that were shared with more than one other taxon, the presence of recombinant clones and a high number of clones (up to 78 % in accession Malta) that exhibited an individual sequence composition. To categorize this numerousness of rather different clones ITS haplotypes (ITS variants that were isolated from more than one accession of a species) were defined (Table 2). In ITS of *O. syriacum* (syr) point mutations at 13 informative nucleotide positions were present. Three different ITS haplotypes (syr1, syr2 and syr3) were isolated from more than one accession. Syr1 and syr2 differed just by the presence/absence of one mutation in ITS1, syr3 was clearly distinct. In ITS clones of cultivated *O. majorana* (cmaj) 18 informative nucleotide positions were present and four ITS haplotypes (cmaj1 to cmaj4) were observed. Haplotype cmaj1 was identical to haplotype syr3 that was described for *O. syriacum* before, haplotype cmaj2 was identical to ITS clones that were frequent in accessions of wild *O. majorana*. Cmaj3 and cmaj4 seemed to be recombinants, having ITS1 of cmaj1/cmaj2 and ITS2 of cmaj2/cmaj1, respectively. For ITS of *O. dubium* (dub) 24 informative nucleotide positions were diagnostic. More than half of them were shared with either *O. syriacum*/O. *majorana* (ITS1) or with *O. onites* (mostly ITS2). Five ITS haplotypes (variants dub1 to dub5) were isolated from more than one accession of *O. dubium*. Haplotype dub1 was characterised by seven mutations of which five were species specific for *O. dubium.* Haplotype dub2 exhibited mutations that were shared with syr1/syr2/maj (five mutations in ITS1) and oni (three mutations in ITS2). Two further mutations of this haplotype were specific for *O. dubium.* Dub3 was characterised by the presence of four of the five mutations that were found to be constant in *O. onites* and two *O. dubium* specific mutations. Dub4 and

dub5 appeared to be recombinants, dub4 had combined features of dub1 (in ITS1) and dub2 (in ITS2), dub5 exhibited dub2 (in ITS1) and dub1 (in ITS2).

An ITS pattern at the first look rather similar to that described for *O. dubium* was found in three Turkish accessions of *O. onites* (SR530, SR777, SR786). On closer examination of ITS of these accessions complete oni clones were revealed and it became obvious that all point mutations present in Turkish *O. onites* today were involved in their intra-individual ITS polymorphisms whereas three of them (sites 15, 425 and 582) were never found in the clones of *O. dubium*. These three Turkish accessions were treated as recent hybrids between *O. onites* and *O. dubium*. In H104, the accession of *O. syriacum* from Egypt that was assigned to O. *syriacum* var. *sinaicum*, beside syr2, ITS clones identical to that of wild *O. majorana* and also cmaj4 of cultivated *O. majorana* were found. As ITS haplotypes identical to variants of both, *O. syriacum* and *O. majorana*, were isolated, this accession was thought to be a recent hybrid between *O. syriacum* and (cultivated ?) *O. majorana*.

All ITS sequences were checked for the presence of conserved angiosperm motifs in ITS1 (Liu and Schardl 1994) and 5.8S (Jobes and Thien 1997). The two motifs were present in all of the clones, with the exception of one of cultivated *O. majorana* that lacked the conserved angiosperm motif in ITS1 (deletion). In two clones of *O. dubium* (similar to the putative recombinants dub2 and dub4, respectively) a deletion (bps 493-510) in ITS2 was present. From these two clones no valid secondary structures of ITS2 were obtained indicating recombinant ITS haplotypes of *O. dubium* to represent pseudogenes. Some of the clones of *O. dubium* with individual sequences resulted also in chaotic secondary structures. In *O. dubium* the overall highest free energies of the ITS2 region were observed for sequences exhibiting haplotype dub1. As this haplotype was also frequently isolated from all accessions analysed we assume clones exhibiting haplotype dub1 to represent functional ITS arrays of *O. dubium*.

Occurrence and frequencies of ITS haplotypes

Accessions of *O. dubium*, *O. syriacum* and cultivated *O. majorana* exhibited individual combinations of ITS haplotypes (summarised in Figure 2). In *Origanum dubium*, dub1 was abundant in all of the accessions analysed. The four other haplotypes defined were in each case present in two to three of the six accessions. About 45% of the ITS clones of *O. dubium* exhibited an individual sequence composition. In the four accessions of *O. syriacum* the closely related haplotypes syr1 and syr2 were most abundant. From SR438 exclusively these two ITS haplotypes were isolated and syr1 seems to be the only one haplotype present in the directly sequenced accession SP3. Accession LC7 possessed all three haplotypes defined for *O. syriacum*. From accession H50 solely the rare haplotype syr3 was isolated, together with a number of ITS clones (about 70 %) that exhibited individual sequences and could not clearly be assigned to one of the three haplotypes described. These clones were either rather similar to syr2 or united sequence characteristics of syr2 and syr3 in various combinations. Cultivated *O. majorana* exhibited haplotype cmaj1 (the haplotype similar to syr3 of *O. syriacum*) in accessions

OBI, Malta and Mira2/3 and haplotype cmaj2 (= the haplotype similar to ITS of wild *O. majorana*, Table 2) in accessions OBI, Mira2/3 and LC10. In addition haplotype cmaj3, a combination of cmaj1 (ITS1) and cmaj2 (ITS2), was present in accession OBI. Haplotype cmaj4, a combination of cmaj2 (ITS1) and cmaj1 (ITS2), was isolated from the accessions Mira2/3 and LC10. About 50% of the ITS clones from cultivated *O. majorana* were not isolated from more than one of the four accessions or exhibited specific sequences. They differed from the haplotypes described by additional presence or absence of point mutations or by showing cmaj typical mutations in various combinations.

Phylogenetic analyses of ITS

High intra-individual/intra-specific ITS variability and random combinations of shared and specific mutations, as found in many clones of *O. syriacum*, cultivated *O. majorana* and *O. dubium* accounted for weak resolution in phylogenetic trees. In order to use the high ITS variability as a tool an alternative, distance-based approach was chosen that groups the individuals according to their patterns of ITS variability. This approach was already shown to have a high potential to reflect evolutionary relationships within angiosperm genera (Göker and Grimm 2008). In the resulting ITS network three major groups can be distinguished (Figure 3). The first group is composed of plant individuals of *O. onites* from Italy (SR1434, SR1440), Greece (SR1372, SR1387) and Turkey (H106, SR810). From these accessions solely ITS clones of the oni type were isolated and these plant individuals seem to represent the 'pure' *O. onites*. The second group comprises the accessions of *O. syriacum*, *O. majorana* and cultivated *O. majorana*. These three taxa are united by the presence of either the ITS haplotypes syr1/syr2/syr3 or of ITS clones of the maj type that is closely related to syr1/syr2. In *O. syriacum* from Israel (SP3, LC7) and Syria (SR438) syr1 and syr2 were the predominant ITS haplotypes. These accessions appear as sister group to *O. majorana* from Cyprus (SR849, SR998, SR1087) that possessed solely ITS of the closely related maj type. Clones of H104 (originally designated to *O. syriacum* var. *sinaicum* but probably of hybrid origin) exhibited mostly syr2 and maj characteristics and therefore this accession is placed in-between. In Lebanese *O. syriacum* (H50) and cultivated *O. majorana* (Malta, OBI, Mira2/3, LC10), beside syr2 and maj characteristics, haplotype syr3 was frequent. Additionally a large percentage of different recombinants were isolated, the high intra- and inter-individual variability in these accessions is reflected by the formation of a conspicuous sub-network. The third group comprises the accessions of *O dubium* (H67, H137, SR558, SR725, SR1028, SR1054, SR1200) and putative hybrids between *O. onites and O. dubium* (SR530, SR777, SR786). The relationship of *O. dubium* to both, *O. onites* and *O. syriacum* becomes clearly obvious. The high intra- and inter-individual variability is reflected by the conspicuous sub-network. In clones of SR1054 a large proportion of oni specific mutations was present resulting in an ITS pattern that was related to the ITS patterns found in the accessions of Turkish *O. onites* that were of putative hybrid origin (SR777, SR786, SR530).

DXS (1-deoxy-D-xylulose 5-phosphate synthase)

With DXS a new nuclear gene region was established to trace species relationships in the genus *Origanum*. DXS is involved in the formation of IPP (Isopentenylphosphat), a basic structure for molecules of the primary- and secondary metabolism (Cordoba et al. 2009). The DXS gene seems to be part of a small gene family with two distinct gene subfamilies, classified as DXS1 and DXS2. In *Medicago trunculata* both genes were identified sharing about 70% identity in their amino acid sequences (Walter et al. 2002). In *Origanum* sequencing and cloning of DXS did not reveal more than two different, not strongly diverging alleles from a single plant individual. Therefore we are confident that all DXS fragments amplified correspond to a single locus. DXS was found to be useful for phylogenetic considerations in *Origanum* and could probably also serve as nuclear marker region in other Lamiaceae genera closely related to *Origanum*. Cross-amplification of primers was already successfully tested in species of the genera *Thymus* and *Satureja*, respectively.
A total of 86 DXS sequences from 66 accessions of *O. dubium* (20), wild *O. majorana* (6), cultivated *O. majorana* (4), *O. onites* (16) and *O. syriacum* (20) were included in the DXS analyses. Twenty of the 66 individuals examined showed heterozygosity at the DXS locus. The length of the aligned sequences varied from 413 to 421 base pairs. The total length of the alignment was 422 bp including three gaps, accommodating relatively small indels (5/6, 1, and 2 bp long). An intron of 108 bp was localized accounting for bp 216 to 324 of the alignment. A total of 40 variable characters were found.

Phylogenetic analyses of DXS

Seventeen different DXS alleles (alleles 1 to 17, Figure 4) were isolated from the four species investigated. In the DXS-network (Figure 4, accessions that were included in the ITS analyses are indicated in the network, DXS alleles of all accessions that were included in the analyses are listed in Table 1) three distinct major groups can clearly be distinguished. The first group comprises DXS alleles 1, 2 and 3 that were exclusively isolated from *O. onites*. DXS allele 3 was the most frequent in *O. onites* and was found throughout its distribution area, in accessions of Turkey, Greece and Italy. The second group of DXS alleles is the most diverse comprising 12 closely related alleles that were isolated from Turkish and Cypriot *O. dubium* (allele 4), wild *O. majorana* (alleles 7, 8 and 9), cultivated *O. majorana* (alleles 5, 6 and 10) and *O. syriacum* (alleles 5, 11, 12, 13, 14 and 15). With the exception of LC10 (allele 10) that seemed to be more closely related to DXS of *O. syriacum*, the different DXS alleles isolated from wild and cultivated *O. majorana* clustered closely together. In accessions of *O. syriacum* allele 12 was the most frequent. The two accessions of *O. syriacum* that did not derive from Syria (H50, SP3) exhibited two of the more rare DXS alleles (alleles 11 and 14). H104, classified as *O. syriacum* var. *sinaicum* but, according to ITS, probably a recent hybrid between *O. majorana* and *O. syriacum* possessed DXS similar or closely related to that isolated from cultivated *O. majorana* (allele 5) supporting the hypothesis of a hybridogenous origin of this accession. The two DXS alleles that compose the third group (alleles 16 and 17) were predominantly present in *O.*

dubium. Concerning DXS, *O. dubium* was exceptional as it exhibited rather distinct DXS alleles (4 and 16/17) indicating that this gene is not monophyletic in the accessions assigned to this species. DXS allele 4, isolated from Turkish and Cypriot accessions, seems to share a common ancestor with the DXS alleles isolated from *O. majorana*. DXS allele 17, its most frequent allele, as well as the rarer allele 16 appeared clearly separated in the network. These two DXS alleles were also found in some Turkish accessions of *O. onites* that exhibited one DXS allele 3 together with one allele 16 or 17, respectively. Accessions SR777 and 786 were also included in the ITS analyses and were, according to their ITS pattern, thought to be recent hybrids between *O. onites* and *O. dubium*. The co-occurrence of an *O. onites* specific and a putative *O. dubium* specific DXS allele in these accessions directly indicates hybridisation. Another putative Turkish hybrid accession (SR530) exhibited both of the *O. dubium* specific DXS alleles indicating introgression of genetic material from *O. dubium* into the genetic background of *O. onites.*

psbA-trnH intergenic spacer

Considerable efforts were made to find chloroplast regions suitable for this investigation. Of the most variable non-coding cpDNA regions (Shaw et al. 2007) 22 were amplified and sequenced but only little or no sequence variation was found between the species under study (data not shown). The psb*A*-trn*H* region was the only one chloroplast region where at least some informative sequence variation (about 4 % variability) was present. The psb*A*-trn*H* alignment included 94 accessions (45 of *O. onites*, 8 of *O. syriacum*, 28 of *O. dubium*, 11 of wild *O. majorana*, 2 of cultivated *O. majorana*). The length of the aligned sequences varied from 418 to 433 base pairs, the alignment resulted in a matrix of 445 characters. Within the alignment three indels (6/15/6 bps), one inversion (bp 308 to 325) and 2 variable characters were present.

Haplotype analyses of psbA-trnH

Six different psb*A*-trn*H* haplotypes were identified. Software analyses required five steps to connect four of them (haplotypes 1, 2, 3, 5) to a most parsimonious network, two (haplotypes 4 and 6) could not be attached. After visual inspection of the sequences haplotype 4 and 6 were related to haplotype 3 and 5, respectively (Figure 5, haplotypes of all accessions included in the analysis are indicated in Table 1). Within section *Majorana* five of the six psb*A*-trn*H* haplotypes were not species-specific. Each with two different haplotypes *O. dubium* and *O. majorana* were the most homogenous species. In *O. dubium*, in Turkish as well as Cypriot accessions haplotype 1 was predominant. Haplotype 6 was present in one plant individual only that was sampled from a Turkish population where *O. dubium* and *O. onites* grew intermixed (population dubium1). This accession probably represents a recent example of cytoplasmic introgression. In wild and cultivated *O. majorana* haplotypes 1 and 5 were common. Haplotype 1 was shared with Turkish *O. onites* and *O. dubium* and was found in natural populations as well as in cultivated plants. Haplotype 5 was shared with *O. onites* and was present in wild *O.*

majorana only. Three different haplotypes (3, 4 and 6) were isolated from *O. syriacum*. No clear grouping according to geographical position or variety could be observed. *O. onites* possessed all of the six haplotypes that were found. Four of them were isolated from Greek as well as from Turkish accessions. Haplotype 1 was found only in Turkish *O. onites*, mostly in individuals sampled from the population where *O. onites* and *O. dubium* were intermixed (population onites4). Probably haplotype 1 was recovered recently from *O. dubium* by cytoplasmic introgression what indicating hybridisation between the two species. When more individuals of a population were analysed always divergent psb*A*-trn*H* haplotypes were present. From the mixed population of *O. onites* and *O. dubium* (populations dubium1 and onites4) five different haplotypes were isolated (haplotypes 1 and 6 in 19 individual plants of *O. dubium*, haplotypes 1, 2 and 6 in eleven individual plants of *O. onites*). Two Greek populations of *O. onites* were also analysed in more detail. Three haplotypes (3, 4 and 5) were present in nine individual plants of population onites2, two haplotypes (4 and 5) in eleven individual plants of population onites3.

SSRs

Five polymorphic microsatellites were screened for three populations of 'pure' *O. onites* (onites1, onites2 and onites3), sympatrically occurring *O. onites* and *O. dubium* (onitesmix = onites4, dubiummix = dubium1), two populations of 'pure' *O. dubium* (dubium2 and dubium3), two populations of *O. syriacum* (syriacum1 and syriacum2), two populations of *O. majorana* (majorana1 and majorana2) and one population of an inbreeding line of cultivated *O. majorana* (cmajorana) (Figure 1). The expected heterozygosity at each locus in each species ranged from 0 to 0.71, the observed heterozygositiy ranged from 0 to 0.58 and the number of alleles in each locus ranged from 1 to 5 (Table 3). Cultivated *O. majorana* was monomorphic at each of the loci, the population onitesmix possessed the highest number of alleles per locus. Pairwise F_{ST} values varied widely between population pairs (Table 4). Comparisons between populations of different species resulted in highly significant *P* values ($P < 0.001$). Non-significant pairwise F_{ST} values were found primarily between populations of one species but also intra-specific comparisons resulted sometimes in highly significant pairwise F_{ST} values. However, significant F_{ST} values yielding from intra-specific comparisons were commonly smaller (0.169-0.439) than those resulting from inter-specific comparisons (0.23-0.74). In *O. onites* high intra-specific pairwise F_{ST} values (0.30-0.49) were found between the Sicilian and Greek populations (onites1, onites2, onites3) on the one hand, and the Turkish 'mixed' population (onites4) on the other hand. These high pairwise F_{ST} values between geographically separated populations are indicative for a high population differentiation in *O. onites*. Although there seem to be a geographical gradient (higher pairwise F_{ST} values between Sicilian and Turkish *O. onites* than between Greek and Turkish *O. onites*) the high diversity in *O. onites* could be also partly due to gene flow between *O. dubium* and *O. onites* in Turkey. The pairwise F_{ST} values between Turkish *O. onites* and populations of *O. dubium* (0.28-0.33) were conspicuously lower than pairwise F_{ST} values between

Sicilian and Greek populations of *O. onites* and *O. dubium* (0.47 and 0.56). In *O. dubium* no significant pairwise F_{ST} values were found between the two Cypriot populations whereas significant genetic divergence exists between the Turkish 'mixed' population and the Cypriot populations (0.17-0.18). As only one (not 'pure') population of Turkish *O. dubium* was included in the microsatellite study it is not clear if the genetic divergence is due to gene flow from *O. onites* to *O. dubium* or to the geographical isolation of continental and island populations. Within the species *O. syriacum* and *O. majorana* no significant pairwise F_{ST} values were found between populations. The pairwise F_{ST} values between populations of *O. syriacum* and *O. majorana* are amongst the lowest inter-specific pairwise F_{ST} values (0.23-0.35). Interestingly the high pairwise F_{ST} values were calculated between cultivated *O. majorana* and wild *O. majorana* (0.74 and 0.82). In the PCA analysis (Figure 6) four different clusters became obvious. *O. onites* is clearly separating into two groups. Individuals of onites1, onites2 and onites3 cluster together without any geographical pattern whereas plant individuals of onites4 (onitesmix) accumulate to a second group that tends clearly towards *O. dubium*. No such apparent differentiation was visible between dubium1 (dubiummix) and individuals of dubium2/dubium3 although the group centroids of dubiummix and dubium1/2 are slightly separated. The fourth cluster is composed of intermixing individuals of *O. syriacum* and *O. majorana* indicating again a close relationship of these two species. The fourth cluster includes also the uniform individuals of cultivated *O. majorana*.

Discussion

Evaluation of species and species boundaries

Taxonomically the section *Majorana* has been regarded as a difficult group and species boundaries as well as the taxonomic status of *O. dubium* and *O. majorana* remained unresolved (Boissier 1879; Ietswaart 1980; Ietswaart 1982, Ietswaart 1985). Morphologically, *O. onites* appeared to be the best defined and most homogenous species of section *Majorana* that can be distinguished by its corymbiform inflorescence and its often serrate leaves. When considering *O. onites* from Greece and Sicily to represent a 'pure' *O. onites* (i. e. geographically isolated from the other members of section *Majorana*) the results from the ITS, DXS and SSR analyses confirmed the stable and independent character of this species. In this 'pure' *O. onites* intra-individual ITS polymorphisms were comparatively low. Three closely related DXS alleles (alleles 1 to 3) were present, separating clearly from DXS of *O. syriacum, O. majorana* and *O dubium* in the network (Figure 4). In the SSR analyses no significant differentiation could be observed between the 'pure' populations from Sicily and Greece but *O. onites* sampled in Turkey was genetically distinct (Figure 6). Sequence patterns that were found in several Turkish accessions of *O. onites* strongly suggest that gene flow from *O. dubium* to *O. onites* is significant and contributes to the genetic differentiation. DXS directly indicated recent hybridisations between the two species as several Turkish hybrid accessions (morphologically

classified as *O. onites* before) were found to possess an allele of *O. onites* specific DXS (alleles 1 to 3) together with one of an *O. dubium* specific DXS allele (alleles 16 or 17). Two of these putative hybrids were included in the ITS analyses and exhibited a complex mixture of the *O. onites* and the *O. dubium* specific ITS pattern. Hybridisation had probably also an effect on the essential oil chemistry of *O. onites* and O. *dubium*. It was shown that hybrids may express all or only some of the secondary compounds of the parental taxa but probably also novel compounds that are absent in each parent. Quantitatively, concentrations of parental chemicals may vary (Orians 2000; Pichersky and Gang 2000; Schwab 2003). The essential oils of both, *O. onites* and *O. dubium*, usually contain large amounts of cyclic 'cymyl'-compounds (carvacrol and biosynthetically related monoterpenes) but in some Turkish populations of both species, besides the common 'cymyl'-chemotype, an almost pure linalool chemotype is present (Baser et al. 1993a; Baser et al. 1993b, Lukas et al., unpublished). This linalool type is exceptional as the synthesis of cyclic 'cymyl'-compounds seem to be almost completely suppressed whereas considerable amounts of the non-cyclic linalool, usually a minor compound, are accumulated. The exclusive, trans-species occurrence of the linalool chemotype in a geographical area where *O. onites* and *O. dubium* are supposed to hybridise could indicate that it has its origin in the genetic consequences following hybridisation of the two species.

Unlike *O. onites*, the other three species*, O syriacum*, *O. majorana* and *O. dubium* possess paniculate inflorescences and usually entire leaves. The differentiation of the latter three species relies on indumentum as well as on leaves and inflorescence characteristics but high intra-specific variability and conflicting morphological signals complicate the determination of species boundaries. In *O. syriacum* the stems and leaves are usually hirsute, hirsute-tomentose or tomentose and the leaves are acute and have raised veins on the abaxial leaf surface. Morphologically *O. syriacum* appears quite heterogeneous and mainly due to the high variability of indumentum and leave characteristics three varieties, var. *syriacum*, var *bevanii* and var. *sinaicum* were recognized (Ietswaart 1980). Heterogeneity was also observed in the sequence and SSR data of *O. syriacum*. Three different ITS haplotypes were present of which two, syr1 and syr2, were closely related whereas syr3 was clearly distinct (Table 2). The occurrence of divergent ITS types indicate ancient hybridisation with an unknown, closely related *Origanum* species. The haplotypes syr1 and syr2 were most abundant in *O. syriacum* and one or even both of them were present in three of the four accessions analysed. The rarer haplotype syr3 was isolated from a Lebanese and an Israeli accession. From the present results it can not clearly be concluded if more northern populations of *O. syriacum* (represented here by accession SR438) lost an ancient ITS polymorphism or if hybridisation only occurred in more southern populations (represented by H50 and LC7). Five different DXS alleles (alleles 11 to 15) were observed in *O. syriacum*. Three of them (alleles 12, 13 and 15) were restricted to Syrian accessions, the Israeli and the Lebanese accession exhibited two of the rare DXS alleles (alleles 11 and 14). Two geographically distant Syrian populations of *O. syriacum* were included in the microsatellite analyses. Similar to the 'pure' *O. onites*, no grouping of plant individuals according to the geographical location

was evident. The accession from Egypt originally assigned to *O. syriacum* var. *sinaicum* is probably of hybridogenous origin as it appeared intermediate between *O. syriacum* and *O. majorana* in the ITS analysis and exhibited a DXS allele (allele 5) similar to DXS of cultivated *O. majorana*. This specimen was also found to be intermediate in its essential oil composition as beside γ-terpinene and thymol also *cis*-sabinene hydrate was detected (Baser et al. 2003), a component that is never present in *O. syriacum* but is one of the main compounds of *O. majorana*. Accession H104 was originally taken from the area of an agricultural experimental station where a conjunction of *O. syriacum* and *O. majorana* might have occurred.

Origanum majorana and *O. dubium* both differ from *O. syriacum* by their tomentellous leaves and stems and by obtuse leaves that have no raised veins on the abaxial surface. The taxonomic status of *O. dubium* has remained unresolved which is not only a scientific problem as both species are extensively used. In Ietswaart's taxonomic revision (1980) and in the Flora of Turkey (Ietswaart 1982) both are united under *O. majorana*. In the Flora of Cyprus (Ietswaart 1985) the inflorescence shape is used to distinguish and they are treated separately. The DNA data now provides evidence that *O. majorana* and *O. dubium* possess different species histories having *O. syriacum* as a common ancestor.

Origanum dubium was revealed as the most complex species of section *Majorana*. In ITS high intra individual polymorphism was observed attributing three different ancestors. The ITS variability could be traced back to ITS isolated from *O. onites* and *O. syriacum* (dub2 and dub 3) and suggested the participation of a third ITS type (dub1) from an *Origanum* species that could not be identified. Surprisingly, ITS closely related to dub1 was isolated from *Origanum* species of group A (from section *Brevifilamentum* (Lukas et al. unpublished)) relating *O. dubium* to a group of taxa that is morphologically distinct. An interesting phenomenon is the co-occurrence of distinct DXS alleles. Allele 4 of *O. dubium* is closely related to DXS of *O. syriacum*, *O. majorana* and cultivated *O. majorana* whereas the other two alleles (alleles 16 and 17) were, similar to ITS haplotype dub1, found to be closely related to DXS of *Origanum* species from group A (from the sections Anatolicon and Amaracus (Lukas et al. unpublished)). This supports the conclusion that, beside *O. onites* and *O. syriacum* a third, currently unknown *Origanum* species was involved in the speciation of *O. dubium*. According to our sequence and microsatellite data, gene-flow from *O. dubium* to *O. onites* seems to be more prominent than vice versa. In ITS and DXS of *O. dubium* no evidence for recent gene flow from *O. onites* into *O. dubium* was found. In the SSR-PCA analyses no clear differentiation between *O. dubium* from the 'mixed' population in Turkey (dubium1) and the two 'pure' Cypriot populations was visible. Both species are flowering during the same season and possess similar flower morphology. Eventually postzygotic mechanisms impede introgression of genetic material from *O. onites* into the genetic background of *O. dubium*. However, the rare psb*A*-trn*H* haplotype (haplotype 6) that is shared with *O. onites* and *O. syriacum* was isolated only once from a Turkish accession deriving from the population where *O. dubium* and *O. onites* grew intermixed (dubium1). This haplotype could be

indicative for cytoplasmic introgression from *O. onites* into *O. dubium*. The linalool chemotype, supposed to originate from the genetic consequences following hybridisation, is distributed in both, *O. onites* and *O. dubium*. This would indicate that *O. dubium* may also be affected by hybridisation. However more investigation is needed to assess the influence of hybridisation on the genetic integrity of *O. onites* and *O. dubium*.

For *Origanum majorana* a close relationship to *O. syriacum* was observed. This was especially evident from the SSR results where populations of *O. majorana* and Syrian *O. syriacum* were intermixed (Figure 6). In ITS and DXS the species character of *O. majorana* was more pronounced although its genetic distance to *O. syriacum* remains quite close. One of the main ITS haplotypes of *O. syriacum* (syr2) appeared to be ancestral to ITS of *O. majorana* (Table 2). Some of the polymorphic informative nucleotide positions of ITS of *O. majorana* are not shared by syr2 of *O. syriacum*. Probably these mutations have been gathered recently during the speciation process and have not yet been established completely in the maj ITS arrays. The rare ITS haplotype of *O. syriacum*, syr3, was not detected in wild *O. majorana*. *Origanum syriacum* usually accumulates high amounts of 'cymyl'-compounds (e. g. Fleisher and Fleisher 1991; Lukas et al. 2009). *Origanum majorana* possesses an essential oil chemotype unique in section *Majorana* completely lacking 'cymly'-compounds but accumulating high amounts of 'sabinyl'-compounds (*cis*- and *trans*-sabinene hydrate, *cis*-sabinene hydrate acetate; e. g. Fischer et al. 1987; Novak et al. 2008b). Based on the results from the DNA analyses wild *O. majorana* could be considered as a specialised chemo-variant of *O. syriacum*.

Cultivated marjoram is thought to have its origin in Cypriot wild *O. majorana*. However, morphologically the modern cultivars differ to some extent from the natural populations in Cyprus, probably due to strict selection of the cultivated types by man. The cultivars are less compact, possess longer branches and a less dense indumentum. The leaves are larger, having a longer petiolate and appear greener than the greyish leaves of the wild form. In the microsatellite analyses cultivated *O. majorana* clustered closely together with wild *O. majorana* and *O. syriacum*. Not surprisingly, the population of the cultivar appeared completely homogenous. Cultivated and wild marjoram share a psb*A*-trn*H* haplotype (haplotype 1) and similar DXS alleles (alleles 5 to 10) but in the case of ITS different patterns were observed. From Cypriot, wild-growing *O. majorana* solely maj ITS was isolated whereas in all four marjoram cultivars beside maj ITS (= haplotype cmaj2) also ITS haplotype syr3 (= haplotype cmaj1) was present which is difficult to interpret. There is only limited knowledge about the early days of the cultivated plant and the origin of the modern cultivars commercially grown today. Probably crossings with *O. syriacum* took place in their early history what could, to some degree, also explain morphological tendencies of cultivated *O. majorana*. H104, the putative hybrid of *O. majorana* and *O. syriacum* could be a connecting link between wild form and cultivar.

Evolutionary relationships in the section *Majorana*

Ietswaart (1982) considered section *Majorana* to contain some of the most ancient species of the genus *Origanum*. Based on our results, *O. onites* and *O. syriacum* were considered to be ancient species in section *Majorana*. Probably both derived from the same ancestor, *O. onites* spread over Turkey and Greece whereas *O. syriacum* developed in the eastern Mediterranean. Complex species histories are indicated by the presence of six cpDNA haplotypes in *O. onites* and three in O. *syriacum*, respectively (Figure 5). In more southern accessions of *O. syriacum* in ITS evidence was found for ancient gene flow between *O. syriacum* and an unidentified, possibly extinct species. Ancient hybridisation between *O. onites* and *O. syriacum* presumably laid the basis for the development of *O. dubium*, leading to the formation of a 'proto-*O. dubium*'. Recurrent, independent hybridisation and backcrossing with the two parental species may have occurred. *Origanum syriacum* var. *bevanii*, the variety distributed in Turkey and the Northern parts of Syria, is described as somewhat intermediate between *O. onites* and *O. syriacum* var. *syriacum* (Ietswaart 1982). The morphological tendencies characteristic for *O. syriacum* var. *bevanii* could be due to introgression of genetic material during this period. More than one third of the variable ITS nucleotide positions of modern *O. onites* and *O. syriacum* are not shared by *O. dubium* and for some reason genetic exchange with the parental species must have stopped after some time. Maybe the 'proto-*O. dubium*' was able to undergo some time of undisturbed speciation before a second hybridisation event, involving a third, unknown species, led to the final formation of *O. dubium*. *Origanum onites* is not present in Cyprus and the closest relatives of the predominant ITS haplotype and the most frequent DXS alleles of *O. dubium* were found in *Origanum* species endemic to the Taurus Mountains. Therefore *O. dubium* is assumed to have its origin in this geographical area (Figure 1). The coastal mountains of Turkey are the diversity hot spot for the genus *Origanum* and 14 species are exclusively distributed along the Turkish coast. Most of them are narrow endemics and are thought to be of recent (hybrid?) origin (Ietswaart 1980). Their formation is likely to be associated with range alternations and secondary contact of previously isolated species induced by Quaternary climatic oscillations. Probably hybridisation between the 'proto-*O. dubium'* and the third species took place during this geohistorical period. *Origanum majorana* seems to have its origin in Syrian *O. syriacum* and has probably developed straight-forward by isolation and differentiation on the island of Cyprus. Cyprus is an island of oceanic origin which has never been connected to the mainland (Moores et al. 1984) and long distance dispersal (± 200 km open sea) must have been responsible for its colonisation with *O. dubium* and *O. majorana*. The small population differentiation observed indicates that the colonisation of Cyprus by *O. dubium* was a more recent event.

Reconstructing past evolutionary events is difficult and DNA-based methods can only reveal corner-stones in species histories. Evolutionary interlocking of the species of section *Majorana,* especially of *O. syriacum*, *O. dubium* and *O. majorana,* is most probably more complex as can be seen from the current data. The partly overlapping distribution of *O. onites*, *O. dubium and O. syriacum* in Turkey as

well as the parapatric occurrence of *O. majorana*, *O. dubium* and *O. syriacum* in Cyprus would offer the possibility for (repeated) genetic exchange. Repeated long distance dispersal between Cyprus and the coastal region of the Eastern Mediterranean seems to be within the realms of possibility. More extended microsatellite analysis including continuous fine-scale population-level sampling would be needed to get a refined picture of the evolutionary processes relating *O. onites*, *O. syriacum*, *O. dubium* and *O. majorana*.

Species distribution and recent hybridisation

Hybridisation between co-occurring species, even between species of different sections and groups, is a frequent phenomenon in the genus *Origanum* and eight hybrids of *O. onites*, *O. majorana* and *O. syriacum* with species of section Anatolicon, Brevifilamentum, Origanum and Prolaticorolla have been described (Ietswaart 1980). Six of these hybrids can be found in natural environments, often with both parental species growing sympatrically. Although species of the section *Majorana* are reported to co-occur in parts of Turkey and Cyprus, so far no morphological hybrid within the section was recorded. Nevertheless, in our DNA data we found clear evidence for recent hybridisation between *O. onites* and *O. dubium*. All of the putative hybrid accessions of *O. onites* that were identified by DNA analyses originated from the district of Antalya (Köprülü National Park and ancient city of Termessos). From the present results it can not be predicted whether there is a continuous hybridisation zone or whether the common distribution area of *O. onites* and *O. dubium* is interspersed with small hybrid populations. Further study is needed to assess the geographical dimensions of inter-specific gene flow between *O. onites* and *O. dubium*.

Recent hybridisation has often been correlated with migration and range expansions following the last cold period of the Pleistocene. In case of *Origanum* species also the human factor has to be considered as species of the genus have been used as economic plants for centuries. The popularity of oregano and marjoram in ancient times is well documented. Pedanios Dioscurides mentioned different *Origanum* qualities (later identified as *O. vulgare*, *O. onites*, *O. dictamnus* and *O. majorana*) and their medicinal application in his Materia Medica (1st century AD) (Berendes 1902). Pliny the Elder alluded in his Historia Naturalis (ca. 77 AD) to sampsuchus (later identified as *O. majorana*) as valuable ingredient of fragrances and unguent oils (Bostock and Riley 1855) that were commonly used for cosmetic and medicinal purposes in ancient cultures. Marjoram was also part of Greek burial ceremonies. In graeco-roman burials (dated to the 1st century BC - the 4th century AD), located in the necropolis of Hawara, funeral wreaths made of *O. majorana* were discovered (Edmondson and Bierkowski 1993). Recently *Origanum* DNA was amplified from clay material of a 2400 year old Greek amphora (Hansson and Foley 2008). As oregano and marjoram have been played a role in the daily life for centuries it can be assumed that the current distribution of *Origanum* species is not only a result of natural migration but also of distribution by man along trade routes.

Origanum majorana is endemic to Cyprus but due to its use as a kitchen herb it occurs as a cultivated and naturalized plant in many parts of the world today. From ancient literature there is evidence that already in ancient times the distribution of *O. majorana* was not restricted to the island of Cyprus. In the Materia Medica beside the medicinal applications of sampsuchus also the origin of the best quality herb is mentioned: the ancient city of Cyzicus (today located in Turkey), Cyprus and Egypt (Berendes 1902). In his Historia Naturalis Pliny the Elder also refers to Cyzicus as a supplier for marjoram plant material and mentioned an unguent of marjoram from Cos (Bostock and Riley 1855), a Greek island that was, according to Athenaeus, the best source for perfume from marjoram (Yonge 1854). Probably *O. majorana* has been already actively distributed in ancient times to satisfy the local requirements for sampsuchus. However, it would also be possible that in the ancient world *O. majorana* was naturally more widespread (e. g. along the coast of the Eastern Mediterranean and Egypt) and that its today narrow distribution in Cyprus is a consequence of recent climatic and ecological changes that were reported for the Eastern Mediterranean (Orland et al. 2009).

The geographically isolated population of *O. onites* present on the island of Sicily (onites1) is located in and around the ancient city of Syracuse that was founded by Greek settlers 733 BC. The isolated occurrence of this population, together with its narrow distribution on historical ground and its close genetic relationship to two Greek populations provokes the conclusion that *O. onites* was actively brought to Sicily by the Greeks. In Greece and Turkey *O. onites* can often be found in and around archaeological excavations of ancient Greek cities. As *O. onites* is widely distributed its frequent occurrence on historical ground could of course be a result of its common distribution but it is also possible that in parts of Greece and Turkey this species was recently (re)introduced by man giving rise to gene flow between distantly related plant individuals, a broader distribution and probably to the conjunction with *O. dubium*.

Concluding remarks

The four species of section *Majorana* are widely used and therewith traded and processed in big quantities (e. g. Baser 2002; Fleisher and Fleisher 1988). When *Origanum* plant material is commercially used, taxonomic ambiguities constitute not only a scientific challenge. They concern also industry and consumers as the unambiguous identification and classification of the original plant material is one of the prerequisites for good quality products with the properties expected. As the taxonomic status of *O. dubium* is not clear, confusion arise when carvacrol-rich, oregano-flavoured plant material of *O. dubium* is designated to *O. majorana*, the species that has traditionally been linked to sweet marjoram. To provide a basis for the ongoing discussion about species boundaries, taxonomic status and the correct designation of plant material the primary goal of this investigation was the evaluation of the taxonomic entities of section *Majorana*. The present results stressed the independent character of *O. dubium* and *O. majorana* but simultaneously revealed low genetic differentiation between *O. syriacum* and *O. majorana*. However, regarding the distinguishing essential oil

composition of *O. majorana* a four-species concept, treating *O. syriacum*, *O. majorana* and *O. dubium* as separate species, seems to be reasonable. Nevertheless, further analyses that address the genetic diversity of *O. syriacum* as well as the genetic integrity of *O. onites* and *O. dubium* are desirable.

There seem to be no stable macroscopic character that reliably separates *O. dubium* from *O. majorana*. In Cyprus their different ecological preferences and the limited geographical distribution of *O. dubium* will ease the differentiation in the field. Sequence analyses or sequence based approaches could serve as an additional tool for a botanical classification with the advantage that they would also allow the authentification of processed and highly processed plant material. In the genus *Origanum* the use of cpDNA sequences, as proposed in literature addressing methods for DNA-barcoding (e. g. Ford et al. 2009), seem not to be useful. The variability in a number of frequently used chloroplast regions (Shaw et al. 2007) was neglectable whereas in psb*A*-trn*H*, the cpDNA region that was analysed further in this investigation, intra-specific variability and the sharing of cpDNA haplotypes between species was observed. A combination of nuclear ITS and DXS seem to be more useful for a reliable identification and differentiation (Müller et al. 2008). As the extent of intra-specific and intra-individual ITS variability is known 'double peaks' in direct sequences can be interpreted or used as a tool. Difficulties may arise in the differentiation of cultivated *O. majorana* and certain accessions of *O. syriacum* as well as in the correct designation of hybrids between *O. onites* and *O. dubium* but could be overcome by the use of DXS that can be sequenced directly in most cases.

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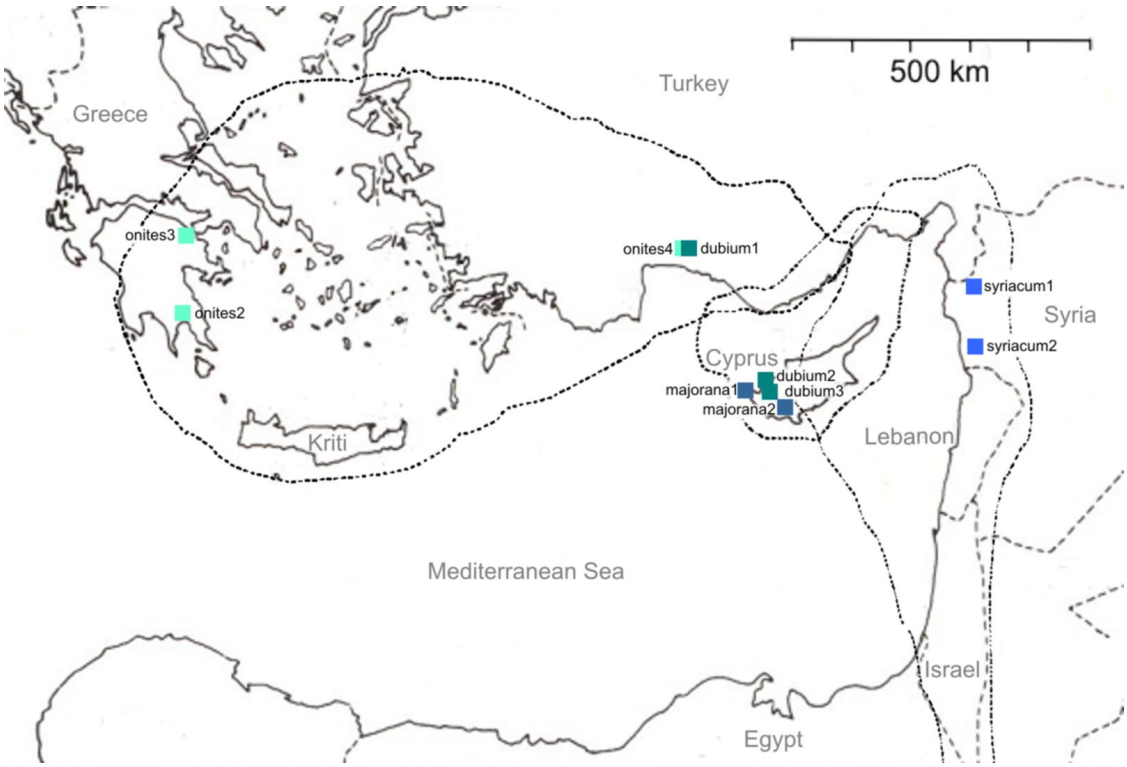

Fig. 1 Natural distribution of *Origanum onites*, *O. majorana* s. l. and *O. syriacum* as indicated in Ietswaart (1980). The geographical location of the populations sampled is indicated (with the exception of the Sicilian population onites1). In Cyprus the distribution of *O. majorana*, *O. dubium* seem not to overlap. *O. majorana* is distributed in the South, *O. dubium* seem to be restricted to the North (Troodos massif). *O. syriacum* occurs solely in the Turkish North-East of Cyprus. In Turkey *O. dubium* is more wide distributed to the West than indicated in the Figure

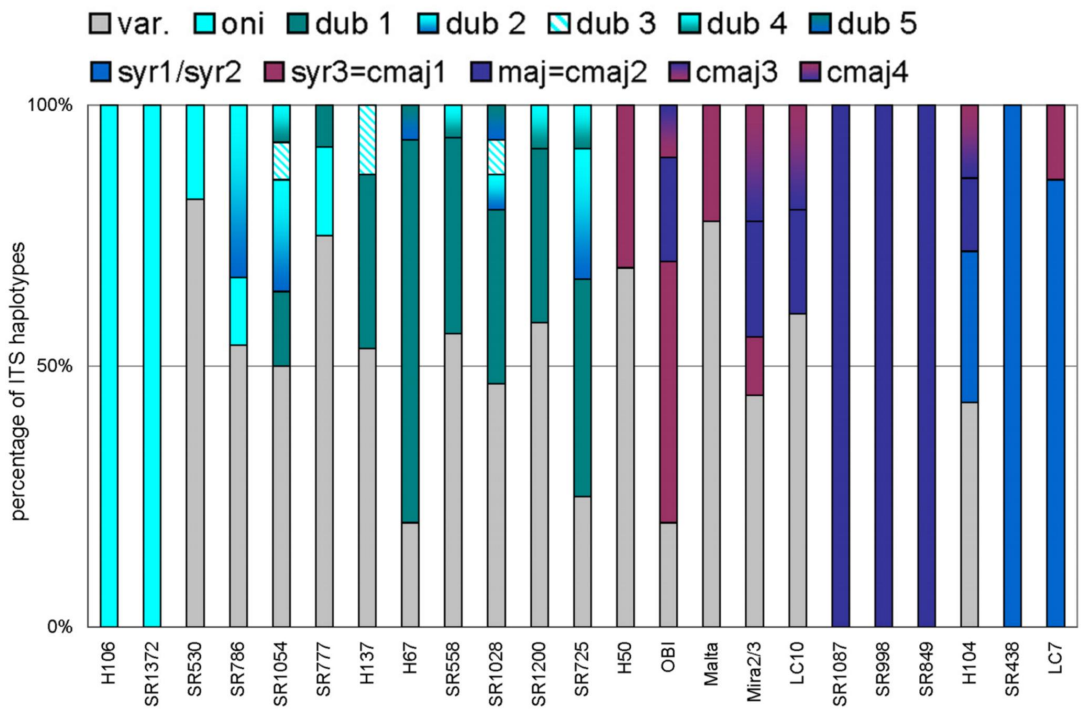

Fig. 2 Occurrence and percentages of ITS haplotypes in the cloned accessions of *Origanum onites* (H106, SR1372), putative recent hybrids between *O. onites* and *O. dubium* (SR530, SR777 SR786), *O. dubium* (H67, H137, SR558, SR725, SR1028, SR1054, SR1200), *O. syriacum* (H50, LC7, SR438), a putative recent hybrid between *O. syriacum* and *O. majorana* (H104), O. *majorana* (SR849, SR998, SR1087) and cultivated *O. majorana* (Malta, Mira 2/3, OBI)

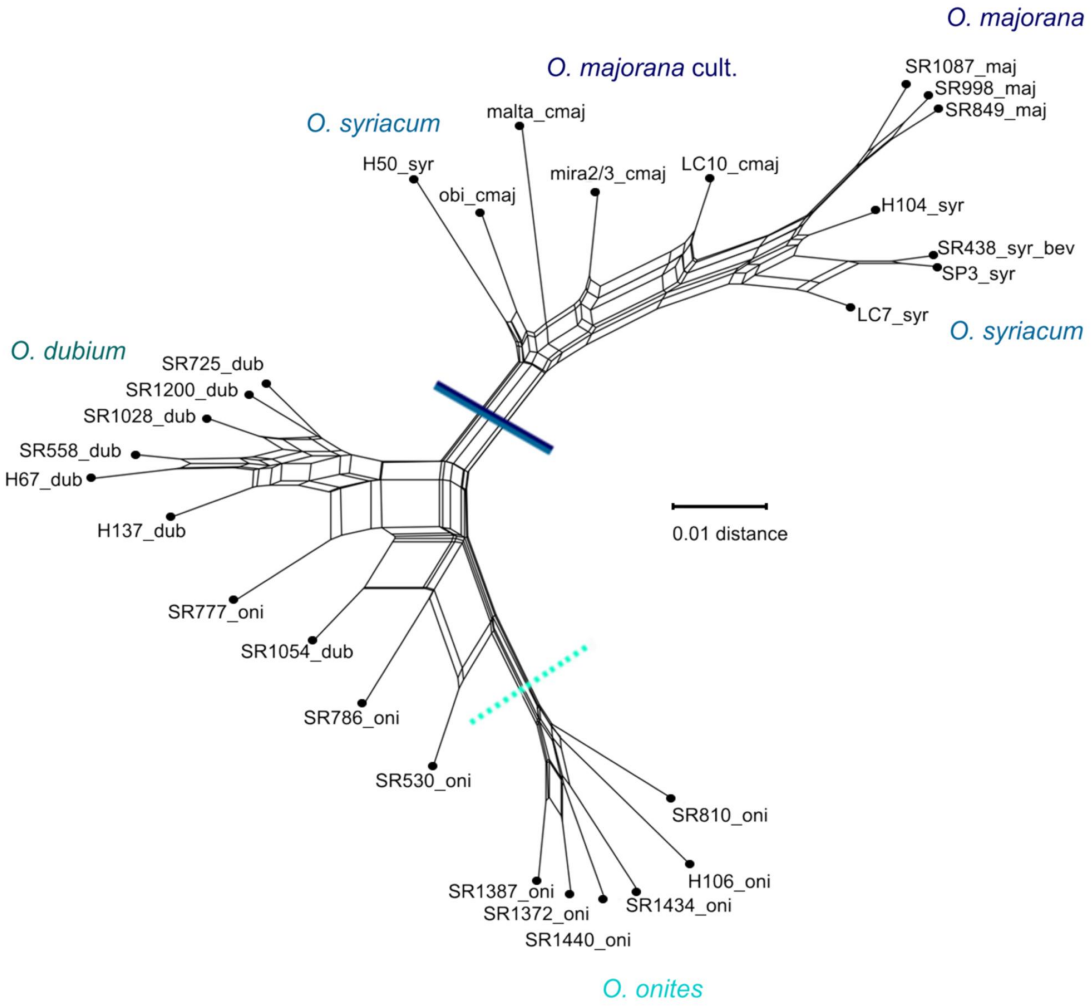

Fig. 3 Neighbor-Net splits graph based on FRQ-inferred distances: ITS data (cmaj = cultivated marjoram; dub, *Origanum dubium*; maj, *O. majorana*; oni, *O. onites*; syr, *O. syriacum;* syr_bev, *O. syriacum* var. *bevanii*)

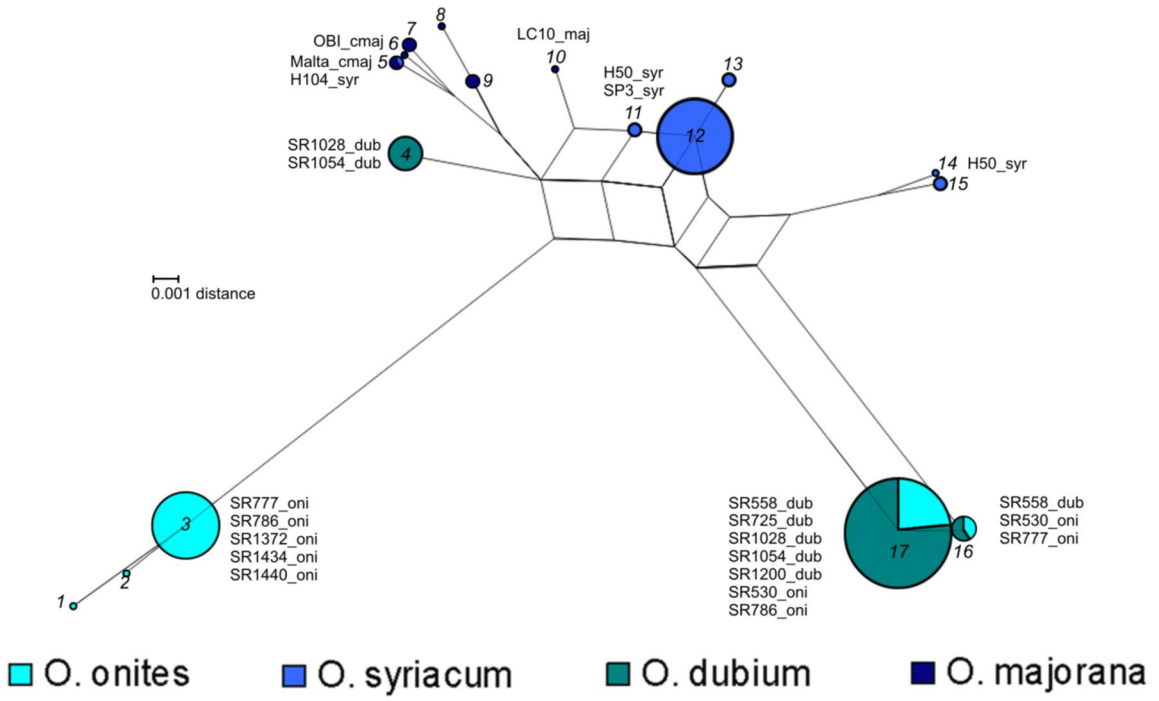

Fig. 4 Neighbor-Net splits graph based on uncorrected p distances: DXS data. Figures refer to the different DXS alleles (alleles 1-17) isolated. The sizes of circles roughly correspond to the frequencies of the respective DXS allele across the four species analysed. The colours of circles refer to *Origanum onites* (turquoise), *O. syriacum* (mid blue), *O. dubium* (green) and *O. majorana* (dark blue). In *O. majorana* alleles 7, 8 and 9 were isolated from wild accessions, alleles 5, 6 and 10 derived from different cultivars. Plant accessions indicated in the network were also included in the ITS analyses (cmaj = cultivated marjoram; dub, *Origanum dubium*; maj, *O. majorana*; oni, *O. onites*; syr, *O. syriacum*)

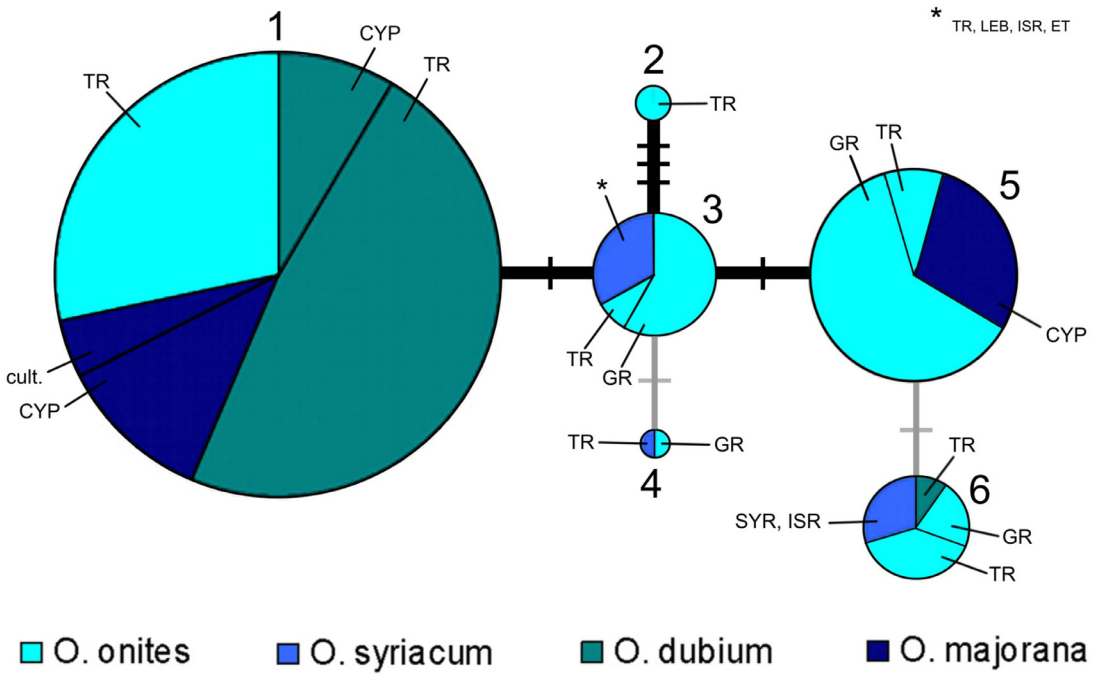

Fig. 5 Psb*A*-trn*H* haplotypes of the species under study (the geographical origin of *O. onites*, *O. syriacum*, *O. dubium* and *O. majorana* is indicated (cult, cultivated *O. majorana;* CYP, Cyprus; ET, EGYPT; GR, Greece; ISR, Israel; LEB, Lebanon; SYR, Syria; TR, Turkey). The sizes of the circles roughly correspond to the frequencies of the haplotypes across the four species of section Majorana. Bars between haplotypes indicate mutational steps

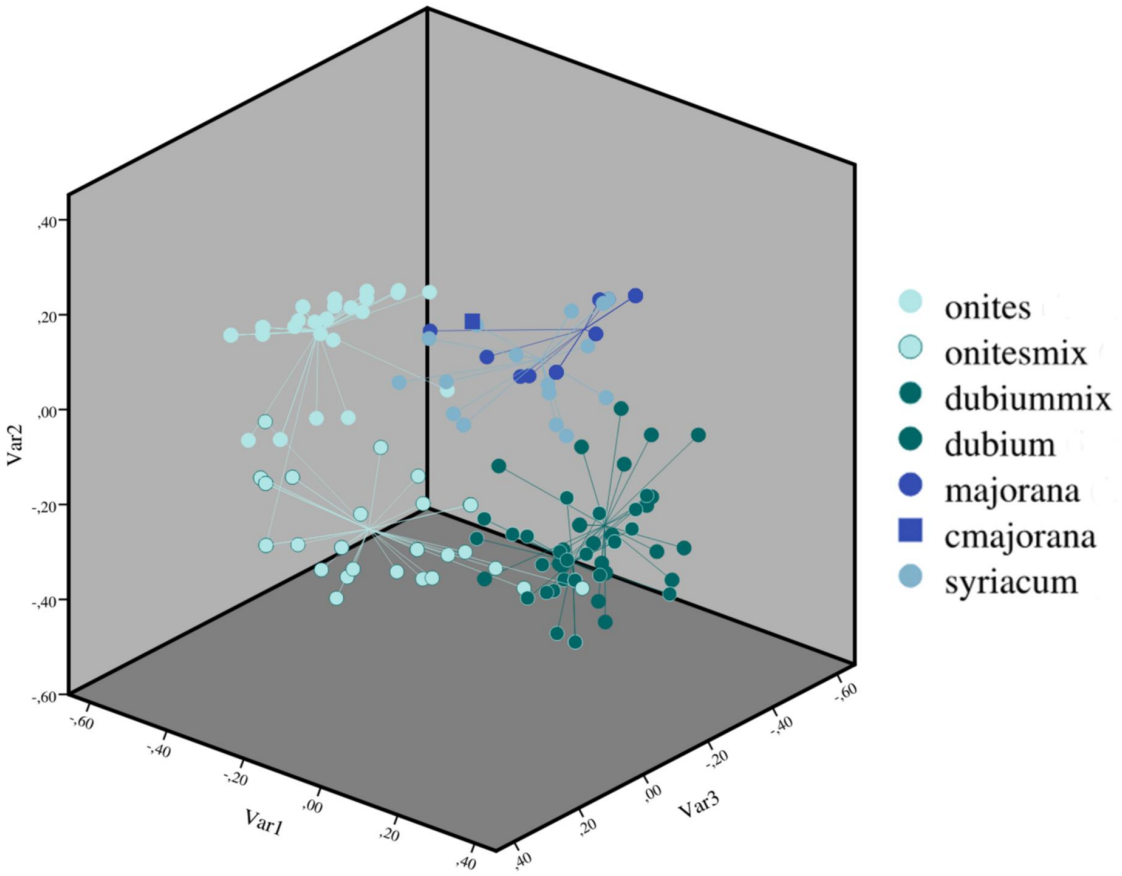

Fig. 6 PCA analysis of SSR data. Centroids of groups ('pure' *Origanum onites* (including populations onites1, onites2 and onites3), 'pure' *O. dubium* (dubium2 and dubium3), sympatric occurring *O. onites* and *O. dubium* (onitesmix = onites4, dubiummix = dubium1), *O. majorana* (majorana1 and majorana2), cultivated *O. majorana* and *O. syriacum* (syriacum1 and syriacum2)) are indicated

Table 1 List of accessions examined in this study. Species, identification number (H-, accessions sampled in Herbaria; LC-, Living collection University of Veterinary Medicine Vienna; SR-, accessions collected from wild populations; affiliation of wild accessions to SSR populations is indicated), geographic origin (cult., cultivated plant; CYP, Cyprus; ET, Egypt; GR, Greece; ISR, Israel; I, Italy;; LEB, Lebanon;; SYR, Syria; TR, Turkey) and GenBank accession numbers. DXS allele types and psb*A*-trn*H* haplotypes are indicated

species	ID	voucher/ pop.	origin	Geographical position N	E	ITS GenBank accession Nrs.	DXS allele/s	DXS GenBank accession Nrs.	*psbA-trnH* haplotype	*psbA-trnH* GenBank accession Nrs.
O. dubium	H45^{RNG}	-	TR	-	-		-		1	
O. dubium	H65^{ESSE}	10974	TR	-	-		-		1	
O. dubium	H67^{ESSE}	10659	TR	-	-		-		1	
O. dubium	H70^{ESSE}	10796	TR	-	-		-		5	
O. dubium	H137^{GAZI}	-	TR	-	-		-		1	
O. dubium	SR531	dubium1	TR	36°56’9.21”	31°28’34.06”		-		1	
O. dubium	SR535	dubium1	TR	36°56’9.21”	31°28’34.06”		16/17		-	
O. dubium	SR536	dubium1	TR	36°56’9.21”	31°28’34.06”		-		1	
O. dubium	SR540	dubium1	TR	36°56’9.21”	31°28’34.06”		16/17		-	
O. dubium	SR541	dubium1	TR	36°56’9.21”	31°28’34.06”		-		1	
O. dubium	SR546	dubium1	TR	36°56’9.21”	31°28’34.06”		-		1	
O. dubium	SR551	dubium1	TR	36°56’9.21”	31°28’34.06”		-		1	
O. dubium	SR553	dubium1	TR	36°56’9.21”	31°28’34.06”		-		1	
O. dubium	SR555	dubium1	TR	36°56’9.21”	31°28’34.06”		-		1	
O. dubium	SR556	dubium1	TR	36°56’9.21”	31°28’34.06”		17/17		1	
O. dubium	SR557	dubium1	TR	36°56’9.21”	31°28’34.06”		-		1	
O. dubium	SR558	dubium1	TR	36°56’9.21”	31°28’34.06”		16/17		6	
O. dubium	SR603		TR	36°29'47.2"	32°07'13.5"		4/4		1	
O. dubium	SR684		TR	-	-		-		1	
O. dubium	SR725		TR	36°06'14"	32°34'55.5"		17/17		1	
O. dubium	SR745		TR	36°01'57,6"	32°46'12,4"		-		-	
O. dubium	SR750		TR	36°05'30.5"	32°55'14.6"		4/4		1	
O. dubium	SR772		TR	36°20'40,4"	32°12'42,9"		-		1	
O. dubium	SR778		TR	36°59'18,3"	31°12'25,4"		-		1	
O. dubium	SR820		TR	37°10'09,1"	31°11'32,2"		-		1	
O. dubium	SR831		TR	36°58'08,7"	31°12'24,5"		-		1	

species	ID	voucher/ pop.	origin	Geographical position N	E	ITS GenBank accession Nrs.	DXS allele/s	DXS GenBank accession Nrs.	*psbA-trnH* haplotype	*psbA-trnH* GenBank accession Nrs.
O. dubium	SR1028		CYP	35°05'31.2"	32°32'31.8"		4/17		1	
O. dubium	SR1054		CYP	35°05'31,2"	32°32'31,8"		4/17		-	
O. dubium	SR1056	dubium2	CYP	35°08'45.6"	32°31'58.5"		17/17		-	
O. dubium	SR1060	dubium2	CYP	35°08'45.6"	32°31'58.5"		4/17		-	
O. dubium	SR1072	dubium2	CYP	35°08'45.6"	32°31'58.5"		17/17		-	
O. dubium	SR1076	dubium2	CYP	35°08'45.6"	32°31'58.5"		17/17		-	
O. dubium	SR1162	dubium3	CYP	35°01'19.1"	32°34'49.8"		17/17		-	
O. dubium	SR1165	dubium3	CYP	35°01'19.1"	32°34'49.8"		17/17		-	
O. dubium	SR1184	dubium3	CYP	35°01'19.1"	32°34'49.8"		17/17		-	
O. dubium	SR1187	dubium3	CYP	35°01'19.1"	32°34'49.8"		4/17		-	
O. dubium	SR1189	dubium3	CYP	35°01'19.1"	32°34'49.8"		17/17		-	
O. dubium	SR1200		CYP	35°01'42.9"	32°36'50.3"		17/17		1	
O. dubium	SR1204		CYP	35°01'42.9"	32°36'50.3"		4/17		1	
O. dubium	SR1208		CYP	35°01'42.9"	32°36'50.3"		-		1	
O. majorana	H44^{RNG}	-	CYP	-	-		9/9		1	
O. majorana	SR837	majorana1	CYP	35°04’54.1“	32°17’36.87“		9/9		-	
O. majorana	SR839	majorana1	CYP	35°04’54.1“	32°17’36.87“		8/9		-	
O. majorana	SR849	majorana1	CYP	35°04’54.1“	32°17’36.87“		-		5	
O. majorana	SR850	majorana1	CYP	35°04’54.1“	32°17’36.87“		-		5	
O. majorana	SR941	majorana2	CAP	34°49'39.3"	32°49'43.6"		-		1	
O. majorana	SR942	majorana2	CYP	34°49'39.3"	32°49'43.6"		7/7		-	
O. majorana	SR951	majorana2	CYP	34°49'39.3"	32°49'43.6"		7/7		-	
O. majorana	SR961	majorana2	CYP	34°49'39.3"	32°49'43.6"		7/7		-	
O. majorana	SR976	majorana2	CYP	34°49'39,3"	32°49'43.6"		-		1	
O. majorana	SR998		CYP	34°58'36,4"	32°28'23,0"		-		5	
O. majorana	SR999		CYP	34°58'36,4"	32°28'23,0"		-		5	
O. majorana	SR1077		CYP	-	-		-		5	
O. majorana	SR1087		CYP	34°55'43,6"	32°26'32,5"		-		1	
O. majorana	SR1139		CYP	34°50'15,5"	32°35'48,9"		-		5	
O. majorana	SR1161		CYP	34°48'56,9"	32°39'47,5"		-		1	
O. majorana	LC10		cult.	-	-		10/10		1	

species	ID	voucher/ pop.	origin	Geographical position N	E	ITS GenBank accession Nrs.	DXS allele/s	DXS GenBank accession Nrs.	*psbA-trnH* haplotype	*psbA-trnH* GenBank accession Nrs.
O. majorana	Malta		cult.	-	-		5/5		-	
O. majorana	OBI		cult.	-	-		6/6		-	
O. majorana	Petronell		cult.	-	-		5/5		-	
O. majorana	Mira 2/3		cult.	-	-		-		1	
O. onites	H38^{RNG}	-	GR	-	-		3/3		6	
O. onites	H106^{ESSE}	8972	TR	-	-		-		2	
O. onites	H107^{ESSE}	13856	TR	-	-		-		3	
O. onites	H108^{ESSE}	9824	TR	-	-		-		6	
O. onites	H109^{ESSE}	13024	TR	-	-		-		4	
O. onites	H136^{GAZ}I	-	TR	-	-		-		6	
O. onites	LC3		cult.	-	-		1/3		-	
O. onites	SR498	onites4	TR	36°56’9.21”	31°28’34.06”		3/3		1	
O. onites	SR502	onites4	TR	36°56’9.21”	31°28’34.06”		-		1	
O. onites	SR507	onites4	TR	36°56’9.21”	31°28’34.06”		-		1	
O. onites	SR512	onites4	TR	36°56’9.21”	31°28’34.06”		-		1	
O. onites	SR517	onites4	TR	36°56’9.21”	31°28’34.06”		-		1	
O. onites	SR522	onites4	TR	36°56’9.21”	31°28’34.06”		-		1	
O. onites	SR527	onites4	TR	36°56’9.21”	31°28’34.06”		-		6	
O. onites	SR528	onites4	TR	36°56’9.21”	31°28’34.06”		3/17		1	
O. onites	SR529	onites4	TR	36°56’9.21”	31°28’34.06”		-		1	
O. onites	SR530	onites4	TR	36°56’9.21”	31°28’34.06”		16/17		1	
O. onites	SR563		TR	36°50'01,7"	30°35'01,0"		-		6	
O. onites	SR580		TR	36°35'38.5"	30°28'23.3"		3/3		2	
O. onites	SR656		TR	36°59'18.5"	30°28'05.1"		3/3		-	
O. onites	SR658		TR	36°59'18,5"	30°28'05,1"		17/17		1	
O. onites	SR691		TR	36°05'56,8"	32°32'02,8"		-		1	
O. onites	SR724		TR	36°05'56,8"	32°32'02,8"		-		1	
O. onites	SR777		TR	36°59'18.3"	31°12'25.4"		3/16		1	
O. onites	SR779		TR	37°05'06.2"	31°13'51"		3/3		5	
O. onites	SR786		TR	37°15'55.8"	31°12'48.8"		3/17		-	
O. onites	SR1299	onites2	GR	36°51'05.5"	22°39'16.2"		2/3		3	

species	ID	voucher/ pop.	origin	Geographical position N	E	ITS GenBank accession Nrs.	DXS allele/s	DXS GenBank accession Nrs.	*psbA-trnH* haplotype	*psbA-trnH* GenBank accession Nrs.
O. onites	SR1300	onites2	GR	36°51'05.5"	22°39'16.2"		-		3	
O. onites	SR1301	onites2	GR	36°51'05.5"	22°39'16.2"		-		3	
O. onites	SR1302	onites2	GR	36°51'05.5"	22°39'16.2"		-		3	
O. onites	SR1302	onites2	GR	36°51'05.5"	22°39'16.2"		-		3	
O. onites	SR1303	onites2	GR	36°51'05.5"	22°39'16.2"		-		3	
O. onites	SR1309	onites2	GR	36°51'05.5"	22°39'16.2"		-		5	
O. onites	SR1318	onites2	GR	36°51'05.5"	22°39'16.2"		-		3	
O. onites	SR1320	onites2	GR	36°51'05.5"	22°39'16.2"		-		3	
O. onites	SR1323	onites2	GR	36°51'05.5"	22°39'16.2"		-		6	
O. onites	SR1339		GR	36°40'13,3"	23°01'47,5"		-		5	
O. onites	SR1372	onites3	GR	37°43’49.89”	22°45’22.77”		3/3		5	
O. onites	SR1373	onites3	GR	37°43’49.89”	22°45’22.77”		-		5	
O. onites	SR1374	onites3	GR	37°43’49.89”	22°45’22.77”		-		5	
O. onites	SR1375	onites3	GR	37°43’49.89”	22°45’22.77”		-		5	
O. onites	SR1376	onites3	GR	37°43’49.89”	22°45’22.77”		-		5	
O. onites	SR1377	onites3	GR	37°43’49.89”	22°45’22.77”		-		5	
O. onites	SR1378	onites3	GR	37°43’49.89”	22°45’22.77”		-		5	
O. onites	SR1379	onites3	GR	37°43’49.89”	22°45’22.77”		-		5	
O. onites	SR1380	onites3	GR	37°43’49.89”	22°45’22.77”		-		5	
O. onites	SR1381	onites3	GR	37°43’49.89”	22°45’22.77”		-		5	
O. onites	SR1382	onites3	GR	37°43’49.89”	22°45’22.77”		-		4	
O. onites	SR1387	onites3	GR	37°43’49.89”	22°45’22.77”		-		5	
O. onites	SR1434	onites1	I	37°04’33.72“	15°16’30.43“		3/3		-	
O. onites	SR1440	onites1	I	37°04’33.72“	15°16’30.43“		3/3		-	
O. onites	SR1460	onites1	I	37°04’33.72“	15°16’30.43“		3/3		-	
O. syriacum	H50^{RNG}	-	LEB	34°11'	35°38'		11/14		3	
O. syriacum var. *sinaicum*	H104^{ESSE}	13338	ET	-	-		5/5		3	
O. syriacum	H148^{ANK}	-	TR	-	-		-		4	
O. syriacum	LC7		cult.	-	-		-		3	
O. syriacum	SP3		ISR	-	-		11/11		6	

species	ID	voucher/ pop.	origin	Geographical position N	E	ITS GenBank accession Nrs.	DXS allele/s	DXS GenBank accession Nrs.	*psbA-trnH* haplotype	*psbA-trnH* GenBank accession Nrs.
O. syriacum	SR326		SYR	-	-		-		6	
O. syriacum	SR342	syriacum1	SYR	35°49'53.7"	36°15'51"		12/15		-	
O. syriacum	SR346	syriacum1	SYR	35°49'53.7"	36°15'51"		12/12		-	
O. syriacum	SR349	syriacum1	SYR	35°49'53.7"	36°15'51"		15/15		-	
O. syriacum	SR369	syriacum1	SYR	35°42'40"	36°05'58.3"		12/12		-	
O. syriacum	SR371	syriacum1	SYR	35°47'16.6"	36°02'12"		12/12		-	
O. syriacum	SR374	syriacum1	SYR	35°47'16.6"	36°02'12"		12/12		-	
O. syriacum	SR378	syriacum1	SYR	35°47'16.6"	36°02'12"		12/12		-	
O. syriacum	SR400	syriacum1	SYR	35°20'57.2"	36°08'55.3"		12/13		-	
O. syriacum	SR401	syriacum1	SYR	35°20'57.2"	36°08'55.3"		12/12		-	
O. syriacum	SR402	syriacum1	SYR	35°20'57.2"	36°08'55.3"		12/12		-	
O. syriacum	SR416	syriacum2	SYR	35°05'49.7"	36°12'16"		12/12		-	
O. syriacum	SR418	syriacum2	SYR	35°05'49.7"	36°12'16"		12/12		-	
O. syriacum	SR420	syriacum2	SYR	35°05'49.7"	36°12'16"		12/13		-	
O. syriacum	SR434		SYR	34°59'46.7"	36°11'45.9"		12/12		-	
O. syriacum	SR437		SYR	34°59'46.7"	36°11'45.9"		12/15		-	
O. syriacum	SR438		SYR	34°47'09,7"	36°09'28,9"		-		6	
O. syriacum	SR442		SYR	34°47'09,7"	36°09'28,9"		12		-	
O. syriacum	SR449		SYR	34°47'09,7"	36°09'28,9"		12		-	

-, information not available; [RNG] Centre for Plant Diversity and Systematics, University of Reading, Plant Science Laboratories, Reading, England, U.K.; [ESSE] Faculty of Pharmacy, Anadolu University, Eskisehir, Turkey; [GAZI] Biyoloji Bölümü, Fen Edebiyat Fakültesi, Gazi Üniversitesi, Ankara, Turkey; [ANK] Biyoloji Bölümü, Ankara Üniversitesi, Ankara, Turkey

Table 2 Informative rDNA (ITS) nucleotide positions of the *Origanum* species under study[1] and the frequencies (%) of mutations at these sites in the clones isolated from a species. For dub, syr and cmaj ITS variants (= ITS haplotypes) that were detected in more than one accession were listed. The order of haplotpyes corresponds to their frequencies in the respective taxon. Shading refers to mutations that were frequently shared between *O. onites*/*O. dubium*, *O. syriacum*/*O. dubium* and *O. syriacum*/*O. majorana*

	ITS1																						ITS2											
	013	013	014	015	015	026	028	039	045	054	072	087	096	107	118	161	174	179	180	223	311		410	420	425	426	445	479	506	509	545	546	573	582
Consensus	T	T	T	G	G	C	C	T	T	A	T	C	G	C	T	T	T	C	C	T	G		C	C	A	A	C	C	T	C	C	T	A	A
O. onites	.	.	.	**A**	.	.	.	**A**	.	.	.	.	.	.	**C**	.	.	.	.	.	.		**T**	**G**	**G**	**T**	.	**A**	.	.	.	**G**	**G**
				100				100							36								100	93	64	100		100				7	57
oni x dub	C	A	.	A	C	T	.	A	.	G	C	T	A	.	.	A	C	T	.	A	.		T	G	G	T	T	A	T	.	C	G	G
O. dubium	**C**	**A**	**C**	.	**C**	.	**T**	**A**	**C**	**G**	**C**	**T**	**A**	.	.	**A**	**C**	**T**	.	**A**	**A**		**T**	.	**G**	**T**	**T**	**A**	**T**	.	**C**	**G**	.
	61	12	1		26		6	14	1	24	66	22	23			25	26	66		37	1		8		28	36	62	35	62		62	64	
dub1	C	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	T	.	.	.		.	.	.	.	T	.	T	.	C	G	.
dub2	.	.	.	.	C	.	.	.	.	G	.	T	A	.	.	A	C	.	.	A	.		.	.	G	T	.	A	.	.	.	.	.
dub3	A	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	A	.		T	.	.	T	.	A	.	.	.	.	.
dub4	C	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	T	.	.	.		.	.	G	T	.	A	.	.	.	.	.
dub5	.	.	.	.	C	.	T	.	.	G	.	T	A	.	.	.	.	T	.	.	.		.	.	.	.	T	.	T	.	C	G	.
O. syriacum	**C**	.	**C**	.	.	**T**	.	.	**C**	**G**	.	**T**	**A**	**T**	**C**	**A**	**C**	.	**T**	.	.		.	.	.	.	.	.	.	.	.	**G**	.	.	.
	76		24			59			38	59		59	59	62	38	71	71		71													68			
syr1	C	.	.	.	.	T	.	.	.	G	.	T	A	T	.	A	C	.	T	.	.		.	.	.	.	.	.	.	.	.	G	.	.	.
syr2	.	.	C	.	.	T	.	.	.	G	.	T	A	T	.	A	C	.	T	.	.		.	.	.	.	.	.	.	.	.	G	.	.	.
syr3	C	.	.	.	.	.	.	.	C	.	.	.	.	.	C	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	.	.	.	.
syr x maj	C	.	C	.	.	T	T	.	C	G	.	T	A	T	C	A	C	.	T	.	.		T	.	.	.	.	.	.	.	.	G	.	.	G
O. majorana	.	.	**C**	.	.	**T**	**T**	.	.	**G**	.	**T**	**A**	**T**	.	**A**	**C**	.	**T**	.	.		**T**	**T**	.	.	.	.	.	.	.	**G**	.	.	**G**
			100			100	96			100		100	98	100		100	100		100				56	2								100			98
cult. *O. majorana*	**C**	.	**C**	.	.	**T**	**T**	.	**C**	**G**	.	**T**	**A**	**T**	**C**	**A**	**C**	.	**T**	.	**A**		**T**	.	.	.	.	.	.	.	.	**G**	.	**G**	**G**
	50		53			37	45		53	39		39	39	39	55	42	42		45		13		16									53		3	50
cmaj1	C	.	.	.	.	.	.	.	C	.	.	.	.	.	C	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	.	.	.	.
cmaj2	.	.	C	.	.	T	T	.	.	G	.	T	A	T	.	A	C	.	T	.	.		T	.	.	.	.	.	.	.	.	G	.	.	G
cmaj3	C	.	.	.	.	.	.	.	C	.	.	.	.	.	C	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	G	.	.	G
cmaj4	.	.	C	.	.	T	T	.	.	G	.	T	A	T	.	A	C	.	T	.	.		.	.	.	.	.	.	.	.	.	.	.	.	.

Consensus = consensus sequence, oni = *O. onites*, dub = *O. dubium*; syr = *O. syriacum*, maj = *O. majorana*, cmaj = cultivated *O. majorana*

[1]The following accessions/number of clones were included: oni (H106/2, SR1372/12); oni x dub (SR530/11, SR777/12, SR786/15); dub (H67/15, H137/15, SR558/16, SR725/12, SR1028/15, SR1054/14, SR1200/13); syr (H50/16, LC7/7, SR438/11), syr x maj (H104/7); maj (SR849/16, SR998/16, SR1087/13), cmaj (LC10/10, OBI/10, Malta/9, Mira2-3/9)

Table 3 Diversity indices for the five investigated microsatellite loci in 'pure' *Origanum onites* (including populations onites1, onites2 and onites3), 'pure' *O. dubium* (dubium2 and dubium3), sympatric occurring *O. onites* and *O. dubium* (onitesmix = onites4, dubiummix = dubium1), *O. majorana* (majorana1 and majorana2), cultivated *O. majorana* and *O. syriacum* (syriacum1 and syriacum2). He, expected heterozygosity; Ho, observed heterozygosity; a, number of alleles

	O. onites (n=49)			'onitesmix' (n=33)			'dubiummix' (n=27)			*O. dubium* (n=24)			*O. majorana* (n=24)			cultivated *O. majorana* (n=10)			*O. syriacum* (n=23)		
Locus	He	Ho	a	He	Ho	a	He	Ho	a	He	Ho	a	He	Ho	a	He	Ho	a	He	Ho	a
SSR10	0.52	0.14	4	0.17	0.12	3	0.48	0.56	2	0.63	0.25	3	0	0	1	0	0	1	0	0	1
SSR14	0	0	1	0.09	0.09	2	0.43	0.48	2	0.29	0.33	3	0	0	1	0	0	1	0	0	1
SSR40	0.35	0.41	2	0.62	0.39	4	0.20	0.22	2	0.47	0.58	3	0.32	0.29	3	0	0	1	0.04	0.04	2
SSR44	0.55	0.51	3	0.40	0.36	5	0.44	0.56	3	0.29	0.33	3	0	0	1	0	0	1	0.71	0.48	5
SSR64	0.10	0.10	3	0.46	0.52	4	0.56	0.52	5	0.51	0.54	5	0.55	0.17	5	0	0	1	0.60	0.48	4

Table 4 Pairwise F_{ST} values between all populations of *Origanum onites* (oni), *O. dubium* (dub), *O. syriacum* (syr), *O. majorana* (maj) and cultivated *O. majorana* (cmaj). Shading refers to F_{ST} values higher than 0.5. Numerals in bold indicate non-significant F_{ST} values

	*oni*1	*oni*2	*oni*3	*oni*4	*dub*1	*dub*2	*dub*3	*maj*1	*maj*2	c*maj*	*syr*1	*syr*2
*oni*1												
*oni*2	**0.091**											
*oni*3	**0.146**	**-0.023**										
*oni*4	0.439	0.306	0.300									
*dub*1	0.563	0.522	0.513	0.295								
*dub*2	0.513	0.479	0.473	0.283	0.169							
*dub*3	0.549	0.509	0.499	0.329	0.181	**-0.008**						
*maj*1	0.683	0.553	0.537	0.451	0.357	0.419	0.366					
*maj*2	0.740	0.604	0.602	0.450	0.389	0.454	0.383	**-0.002**				
c*maj*	0.806	0.658	0.700	0.582	0.521	0.613	0.616	0.740	0.819			
*syr*1	0.595	0.510	0.510	0.383	0.289	0.350	0.305	0.336	0.347	0.503		
*syr*2	0.605	0.505	0.497	0.376	0.259	0.309	0.248	0.233	0.246	0.615	**-0.016**	

PART 2

Phytochemical aspects of the genus *Origanum*

Essential Oil Compounds of *Origanum vulgare* L. (Lamiaceae) from Corsica

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Essential Oil Compounds of *Origanum vulgare* L. (Lamiaceae) from Corsica

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The essential oil compounds of 274 individual plants belonging to 13 populations of *Origanum vulgare* L. (Lamiaceae) from Corsica were studied by GC/FID and GC/MS. On the basis of their monoterpene chemistry three different chemotypes were obvious in the *Origanum* populations: The first and most common type was a 'cymyl' type, with either carvacrol (0.6–65.5%) or thymol (0.0–49.5%) as the main compound. The second, which was very rare, was a 'sabinyl' type with sabinene (7.8–20.2%) and *cis*-sabinene hydrate (0.7–24.8%) as main compounds. The third type was a 'mixed' type combining compounds of the 'cymyl' and the 'sabinyl' pathway. The 'mixed' type was rich in *cis*-sabinene hydrate (0.0–52.4%) and γ-terpinene (0.6–35.4%).

Keywords: *Origanum vulgare* L. ssp. *viride*, *O. vulgare* L. ssp. *vulgare*, essential oil compounds, chemotypes, Corsica.

The genus *Origanum* comprises several species with a long tradition as medicinal and aromatic plants. Due to their content of essential oils two different economic plants (two different 'qualities'), marjoram (*O. majorana* L.) and oregano (several other species of the genus) have been used by man for many centuries. While the volatile oils of marjoram are rich in bicyclic monoterpenoids (*cis*- and *trans*-sabinene hydrate) those of oregano are containing primarily phenolic monoterpenes (mainly carvacrol, occasionally thymol).

In most European countries the commonly used 'oregano' derives from plant material of the species *O. vulgare* L. This species possesses the largest area of all the species in the genus and is widely distributed in Europe as well as in parts of Asia and North Africa. The species is extremely variable in its morphological characters, leading to the differentiation of six subspecies in the presently accepted taxonomic reference for the genus [1]. Despite morphology, a wide diversity can also be found in the total amount and the qualitative composition of the essential oils produced. In principle, the subspecies on the southernmost range of the distribution area, ssp. *glandulosum* (Desfontaines) Ietswaart, ssp. *hirtum* (Link) Ietswaart and ssp. *gracile* (Koch) Ietswaart are the subspecies rich in essential oil, whereas those in the northern part, ssp. *virens* (Hoffmannsegg et Link) Ietswaart, ssp. *vulgare* and ssp. *viride* (Boissier) Hayek are poor sources of volatiles [2]. Those individuals rich in essential oil are usually rich in 'cymyl' compounds (mainly carvacrol and/or thymol), while those with poor or intermediate oil content often contain acyclic compounds and sesquiterpenes [3]. Although there seem to be subtle chemical differences, no clear correlation between the presently recognized subspecies and the qualitative composition of their essential oil can be observed. The current concept of *O. vulgare* is still an issue of debate and these taxonomic difficulties seem to have their counterpart in the volatiles.

As they are of immediate commercial interest, published analyses dealing with the chemistry of essential oils of *O. vulgare* mostly concentrate on the oil-rich subspecies of the southern Mediterranean region. There is only fragmentary knowledge about the essential oil characteristics of *O. vulgare* from the northern Mediterranean area and of central and northern Europe. On Corsica two of the oil-poor

subspecies of *O. vulgare* are reported to co-occur, ssp. *viride* and ssp. *vulgare* [1,4], common in the northern part of the island over limestone and schist. It has been found before that ssp. *vulgare* is rich in acyclic compounds and sesquiterpenes whereas ssp. *viride* is either rich in acyclic compounds and sesquiterpenes or in carvacrol/thymol [3].

This study has the aim to contribute to the fragmentary knowledge of the essential oil chemistry of *O. vulgare* from the northern Mediterranean area and we present here analyses of the essential oil compounds of 13 populations of *O. vulgare* collected on Corsica.

Individual plants (274) were analyzed for their essential oil compounds. Morphological characters suggested that the samples belonged to the relationship of *O. vulgare* ssp. *vulgare* and *O. vulgare* ssp. *viride*. Nevertheless, an attribution of the sampled plants to one of the two subspecies was often doubtful due to the subtle morphological differences that are used for the taxonomical discrimination. Green bracts and white to slightly pink flowers are the characteristics of subspecies *viride*, whereas subspecies *vulgare* usually has violet bracts and pink corollas. On Corsica, none of the examined populations showed homogeneity in these characters and individuals with intermediate flower characters were found. This observation is in accordance with the reported gradual change of subspecies *vulgare* into subspecies *viride* within their shared distribution area [1].

On the basis of their monoterpene chemistry three different types were obvious in the *Origanum* populations of Corsica (Table 1).

The first and most common type was a 'cymyl' type, with carvacrol (0.6–65.5%) and/or thymol (0.0–49.5%) as main compounds. These two monoterpenes, their precursors *p*-cymene (0.0–26.6%) and γ-terpinene (0.0–42.8%), *trans*-β-ocimene (0.0–19.3%), as well as the sesquiterpenes β-caryophyllene (0.0–15.6%) and germacrene D (0.0.–19.3%) represented the bulk of the essential oil compounds. The sometimes high amounts of *p*-cymene and γ-terpinene are probably due to the early collection date. The often high content (in accordance with the dominating monoterpene phenol) of carvacrol methyl ether (0.1–20.5%) or thymol methyl ether (0.0–5.8%) is remarkable as these compounds are usually not detected in *O. vulgare* in high amounts. In the 'cymyl' type, compounds of the 'sabinyl' pathway were rare and were detected in either minor amounts or traces only.

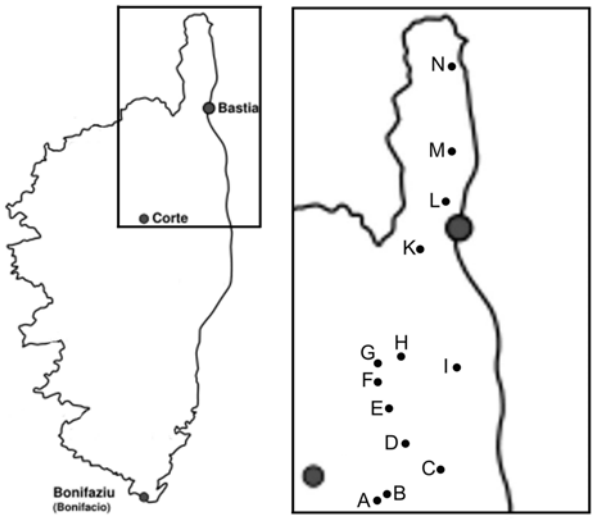

Figure 1: Location of the 13 populations sampled for this study.

The second, very rare type was a 'sabinyl' type with sabinene (7.8–20.2%) and *cis*-sabinene hydrate (0.7–24.8%) as main compounds, accompanied by *trans*-sabinene hydrate (0.8–6.8%), *trans*-β-ocimene (1.2–8.2%) and the sesquiterpenes β-caryophyllene (2.3–12.5%), germacrene D (2.3–18.3%), bicyclogermacrene (1.5–13.6%) and germacrene D-4-ol (2.5–17.0%). In this type 'cymyl' compounds were rare and were mostly found in traces only.

The third type seemed to be 'mixed' combining both, compounds of the 'cymyl' and the 'sabinyl' pathway. In the 'mixed' type, sabinene (1.5–18.4%) and *cis*-sabinene hydrate (0.0–52.4%), as well as γ-terpinene (0.6–35.4%), *p*-cymene (0.8–18.0%), carvacrol (0.0–37.4%) or thymol (0.0–23.9%), carvacrol methyl ether (0.5–11.6%), *trans*-β-ocimene (2.2–13.0%) and the sesquiterpenes β-caryophyllene (0.3–18.0%) and germacrene D (1.6–15.3%) were detected in the extracts in higher amounts.

As already mentioned, the 'cymyl' type was the most common one and in populations B to N most of the individuals represented this type. In populations A, C, D, F, H, M, N and K also the 'mixed' type was found, usually in the minority, with the exception of populations A and D, where the majority of the individuals were of the 'mixed' type. Population A was the only one where individuals of the rare 'sabinyl' type were found and was also special in that the 'cymyl' type was very rare (Table 2).

Table 1: Mean values, standard deviations and range of essential oil compounds (peak area percentages) of the three Corsican chemotypes (mean values above 5 % are in bold). Total yield in mg/g dry mass.

RI		cymyl type n = 222			sabinyl type n = 5			mixed type n = 47		
931	α-Thujene	0.8 ± 0.3	(0.0-1.9)		0.2 ± 0.3	(0.0-0.5)		0.7 ± 0.3	(0.0-1.2)	
939	α-Pinene	0.4 ± 0.1	(0.0-1.2)		0.9 ± 0.7	(0.0-1.7)		0.6 ± 0.3	(0.2-1.5)	
977	Sabinene	0.1 ± 0.3	(0.0-1.9)		**11.9** ± 4.9	(7.8-20.2)		**6.9** ± 3.9	(1.5-18.4)	
981	β-Pinene	2.6 ± 1.0	(0.0-5.7)		3.8 ± 1.4	(2.4-5.7)		2.4 ± 0.9	(0.4-4.0)	
986	3-Octanone	0.4 ± 0.4	(0.0-1.8)		0.1 ± 0.2	(0.0-0.4)		0.3 ± 0.3	(0.0-1.2)	
992	Myrcene	1.7 ± 0.6	(0.0-7.8)		3.9 ± 3.0	(0.0-7.7)		1.9 ± 0.8	(1.1-5.2)	
996	3-Octanol	0.3 ± 0.4	(0.0-2.2)		0.4 ± 0.7	(0.0-1.6)		0.2 ± 0.2	(0.0-1.0)	
1029	*p*-Cymene	**5.3** ± 4.3	(0.0-26.6)		0.8 ± 1.1	(0.0-2.6)		**5.3** ± 4.4	(0.8-18.0)	
1034	Limonene/1,8-cineole	0.3 ± 0.2	(0.0-1.7)		**8.1** ± 6.3	(0.0-15.4)		1.6 ± 1.4	(0.0-5.2)	
1043	*trans*-β-Ocimene	**6.9** ± 3.7	(0.0-19.3)		**5.9** ± 2.8	(1.2-8.2)		**6.9** ± 2.9	(2.2-13.0)	
1054	*cis*-β-Ocimene	1.1 ± 0.8	(0.0-6.3)		2.5 ± 1.7	(0.0-4.1)		2.3 ± 1.3	(0.0-4.7)	
1065	γ-Terpinene	**20.7** ± 9.7	(0.0-42.8)		0.1 ± 0.2	(0.0-0.4)		**20.1** ± 8.2	(0.6-35.4)	
1072	*trans*-Sabinene hydrate	0.4 ± 0.2	(0.0-1.2)		3.6 ± 2.8	(0.8-6.8)		0.7 ± 0.6	(0.0-2.4)	
1076	*trans*-Linalool oxide furanoid	0.0 ± 0.1	(0.0-0.5)		1.4 ± 2.9	(0.0-6.6)		0.0 ± 0.1	(0.0-0.2)	
1100	*cis*-Sabinene hydrate	0.4 ± 0.2	(0.0-1.6)		**10.3** ± 12.6	(0.7-24.8)		**9.2** ± 14.1	(0.0-52.4)	
1171	Borneol	0.0 ± 0.2	(0.0-2.3)		0.0 ± 0.0	(0.0-0.0)		0.1 ± 0.3	(0.0-1.7)	
1181	Terpinene-4-ol	0.1 ± 0.1	(0.0-0.4)		0.1 ± 0.2	(0.0-0.4)		0.1 ± 0.1	(0.0-0.2)	
1200	α-Terpineol	0.1 ± 0.2	(0.0-2.4)		3.0 ± 2.9	(0.0-7.5)		0.4 ± 0.5	(0.0-1.8)	
1242	Thymol methyl ether	0.1 ± 0.5	(0.0-5.8)		0.0 ± 0.0	(0.0-0.0)		0.0 ± 0.2	(0.0-1.3)	
1252	Carvacrol methyl ether	**7.5** ± 3.5	(0.1-20.5)		0.1 ± 0.2	(0.0-0.4)		**6.2** ± 3.0	(0.5-11.6)	
1254	Thymoquinone	0.4 ± 0.4	(0.0-3.2)		0.2 ± 0.5	(0.0-1.0)		0.3 ± 0.5	(0.0-2.4)	
1297	Thymol	1.1 ± 5.5	(0.0-49.5)		0.0 ± 0.0	(0.0-0.0)		0.7 ± 3.5	(0.0-23.9)	
1309	Carvacrol	**26.7** ± 20.5	(0.6-65.5)		0.0 ± 0.0	(0.0-0.0)		**7.9** ± 8.5	(0.0-37.4)	
1383	α-Copaene	0.1 ± 0.1	(0.0-0.8)		0.4 ± 0.6	(0.0-1.4)		0.1 ± 0.2	(0.0-0.6)	
1393	β-Bourbonene	0.6 ± 0.5	(0.0-2.7)		1.8 ± 1.0	(0.2-2.6)		0.6 ± 0.4	(0.0-1.6)	
1431	β-Caryophyllene	**5.6** ± 3.2	(0.0-15.6)		**7.3** ± 3.8	(2.3-12.5)		**5.8** ± 3.1	(0.3-18.0)	
1439	β-Copaene	0.2 ± 0.2	(0.0-0.9)		0.1 ± 0.2	(0.0-0.5)		0.1 ± 0.1	(0.0-0.5)	
1450	Aromadendrene	0.3 ± 0.3	(0.0-1.4)		0.2 ± 0.5	(0.0-1.0)		0.1 ± 0.1	(0.0-0.4)	
1465	α-Humulene	0.6 ± 0.5	(0.0-2.3)		0.8 ± 0.5	(0.0-1.2)		0.6 ± 0.5	(0.0-1.7)	
1472	*allo*-Aromadendrene	0.3 ± 0.3	(0.0-2.1)		1.1 ± 0.7	(0.0-1.7)		0.5 ± 0.4	(0.0-1.6)	
1485	γ-Muurolene	0.4 ± 0.3	(0.0-1.2)		0.0 ± 0.0	(0.0-0.0)		0.1 ± 0.1	(0.0-0.7)	
1491	Germacrene D	4.9 ± 4.2	(0.0-19.3)		**8.5** ± 6.4	(2.3-18.3)		**7.3** ± 2.8	(1.6-15.3)	
1506	Bicyclogermacrene	1.9 ± 1.8	(0.0-11.7)		**5.0** ± 5.1	(1.5-13.6)		3.3 ± 1.7	(0.7-7.5)	
1510	(*E*,*E*)-α-Farnesene	1.2 ± 0.9	(0.0-5.5)		0.5 ± 0.5	(0.0-1.1)		0.8 ± 0.5	(0.0-1.9)	
1514	β-Bisabolene	0.8 ± 1.1	(0.0-7.4)		0.0 ± 0.0	(0.0-0.0)		0.3 ± 0.6	(0.0-2.2)	
1524	γ-Cadinene	0.5 ± 0.3	(0.0-1.6)		0.0 ± 0.0	(0.0-0.0)		0.1 ± 0.1	(0.0-0.7)	
1531	δ-Cadinene	0.8 ± 0.5	(0.0-2.3)		0.2 ± 0.4	(0.0-0.7)		0.3 ± 0.3	(0.0-1.1)	
1588	Germacrene D-4-ol	2.0 ± 2.1	(0.0-18.0)		**9.5** ± 5.2	(2.5-17.0)		2.9 ± 1.8	(0.2-8.5)	
	Total yield [mg/g]	7.9 ± 3.4	(1.8-18.4)		3.6 ± 1.5	(1.3-5.1)		7.2 ± 2.9	(2.6-13.8)	

O. vulgare from Corsica was generally low in essential oil compounds (0.14 to 1.85 mg per g dry mass) and this poor amount does not qualify the Corsican oregano for commercial use.

The 'cymyl' type seems to be similar to types already described for populations of *O. vulgare* ssp. *viride* from Liguria, north Italy [5] and Greece [6]. A characteristic of the Corsican type was the presence of *trans*-β-ocimene and carvacrol methyl ether. Carvacrol methyl ether is a compound commonly not detected in *O. vulgare*. In *O. vulgare* ssp. *glandulosum* from Algeria, carvacrol methyl ether was found in higher amounts [7]. Carvacrol was the dominating monoterpene in the 'cymyl' individuals from Corsica. Only in populations K and L were there samples with thymol present sporadically; three individuals (population C, F, K) showed a balanced oil composition.

Compounds of the 'sabinyl' pathway are rare in *O. vulgare* and seem to be restricted to subspecies (or individuals) with poor or intermediate oil content. In *O. vulgare* ssp. *viride* from Iran [8], in ssp. *vulgare* from Italy [5], Turkey [9] and Lithuania [10], as well ssp. *virens* from Turkey [9], 'sabinyl' compounds were reported in combination with high amounts of acyclic compounds and/or sesquiterpenes. In relation to its qualitative composition, the Corsican 'sabinyl' type is similar, but resembles best the geographically near *O. vulgare* ssp. *vulgare* from Italy [5]. Like in the Corsican *Origanum*, beside sabinene, *cis*- and *trans*-sabinene hydrate where also detected, while the other authors found sabinene only.

Table 2: Occurrence of the three chemotypes in the 13 Corsican populations of *Origanum vulgare* (n = individuals sampled; C = carvacrol, T = thymol), total yield (mg/g dry mass) and geographical position.

	n	cymyl type			sabinyl type	mixed type	total yield	position		
		C	T	C/T			mg/g	N	E	s. l.
A	26	3	-	-	5	18	5.8 ± 2.3	42°18'13,6"	9°18'10,7"	763 m
B	9	9	-	-	-	-	5.3 ± 1.3	42°18'35,2"	9°28'31,9"	387 m
C	29	25	-	1	-	3	6.9 ± 2.6	42°19'07"	9°26'49,2"	344 m
D	10	3	-	-	-	7	6.6 ± 2.3	42°21'55,8"	9°21'28"	676 m
E	12	12	-	-	-	-	5.8 ± 1.8	42°25'34,2"	9°20'04,3"	875 m
F	34	28	-	1	-	5	8.1 ± 3.3	42°28'26,3"	9°17'34,5"	353 m
G	9	9	-	-	-	-	9.6 ± 2.1	42°29'05,4"	9°18'32,5"	280 m
H	20	19	-	-	-	1	8.3 ± 3.7	42°30'13,3"	9°22'20,7"	155 m
I	42	42	-	-	-	-	11.1 ± 3.6	42°29'24,5"	9°26'48,2"	207 m
K	26	16	5	1	-	4	5.6 ± 2.4	42°40'55,8"	9°23'12,1"	498 m
L	2	1	1	-	-	-	8.6 ± 1.7	42°44'54,7"	9°25'16,6"	312 m
M	49	43	-	-	-	6	7.8 ± 2.7	42°48'49,6"	9°26'32,8"	184 m
N	6	6	-	-	-	-	5.5 ± 2.0	42°56'47,4"	9°26'13"	183 m

The Corsican 'mixed' type combining high amounts of 'sabinyl', 'cymyl' and acyclic compounds is neither common in ssp. *vulgare* nor in ssp. *viride*. The qualitative composition of the 'mixed' type resembles closer the essential oil composition of a sample of *O. vulgare* ssp. *virens* originating from France [11] suggesting again a similarity to material sampled in an area geographically near.

This study contributes to the primary knowledge of *Origanum* populations from an area previously not explored. The results reveal once more the taxonomic ambiguities in the current concept of *O. vulgare*. In the Corsican populations, the morphological characters varied gradually and a clear assignment of the sampled plants to one of the two subspecies in question was difficult. The chemotype dominating in Corsica, with its high proportion of components belonging to the carvacrol/thymol pathway, resembles the chemistry of *O. vulgare* ssp. *viride* from Greece and Italy, but also shows some characteristics, like the high amounts of carvacrol methyl ether. 'Sabinyl' compounds, together with acyclic compounds and/or sesquiterpenes, were previously found in all the three subspecies generally poor in essential oil. The Corsican 'sabinyl' type most resembles the qualitative composition of the essential oil of *O. vulgare* ssp. *vulgare* sampled in Italy. The combination of 'sabinyl' and 'cymyl' compounds, as found in the Corsican 'mixed' type, is neither common in ssp. *vulgare* nor in ssp. *viride* but a similar type was found geographically near in *O. vulgare* ssp. *virens* from France.

Experimental

Plant material: *Origanum vulgare* [274 samples (13 populations)] was collected from the wild in various areas in the north-east of Corsica (Figure 1), before or when starting to bloom in June 2006. The plants were cut above ground and air-dried at room temperature. The leaves and inflorescences were analyzed by GC and GC-MS. Voucher specimens of the populations are kept at the herbarium of the Institute for Applied Botany.

Extraction: Dried plant material (55 mg) was extracted in 1 mL dichloromethane for 30 min in an ultrasonic water bath. The extracts were filtered through cotton pads on a Pasteur pipette and were directly used for GC-analysis.

GC and GC/MS analysis: The quantitative GC analyses were performed on an HP6890 machine equipped with a FID and fitted with an Agilent DB-5 narrow bore capillary column (10 m x 0.1 mm, 0.17 µm film thickness). Helium was used as carrier gas (average velocity 45 cm/s). The inlet was kept at 260°C in split mode (split ratio: 100:1). Temperature program: 60°C for 1 min.; from 60°C to 85°C with 8°C/min increases; 85°C to 280°C with 12°C/min increments. Fenchone was used as internal standard. The quantitative composition was obtained by peak area normalisation, and the response factor for each component was considered to equal 1.

The analyses for the identification of the compounds were performed on an HP 6890 machine coupled with an HP 5972 MSD (mass-range 50-550 *m/z*) Agilent DB-5MS narrow bore capillary column (30 m x 0.25 mm, 0.25 µm film thickness). Helium was used as carrier gas (average velocity 42 cm/s). The inlet was kept at 260°C in split mode (split ratio: 100:1). Temperature program: 60°C for 4 min.; from 60°C to 100°C with 5°C/min increases; 100°C to 280°C with 9°C/min rises. Retention Indices (RI) of

the sample components were determined on the basis of homologous *n*-alkane hydrocarbons under the same conditions. The compounds were identified by comparing their retention indices and mass spectra with published data [12,13].

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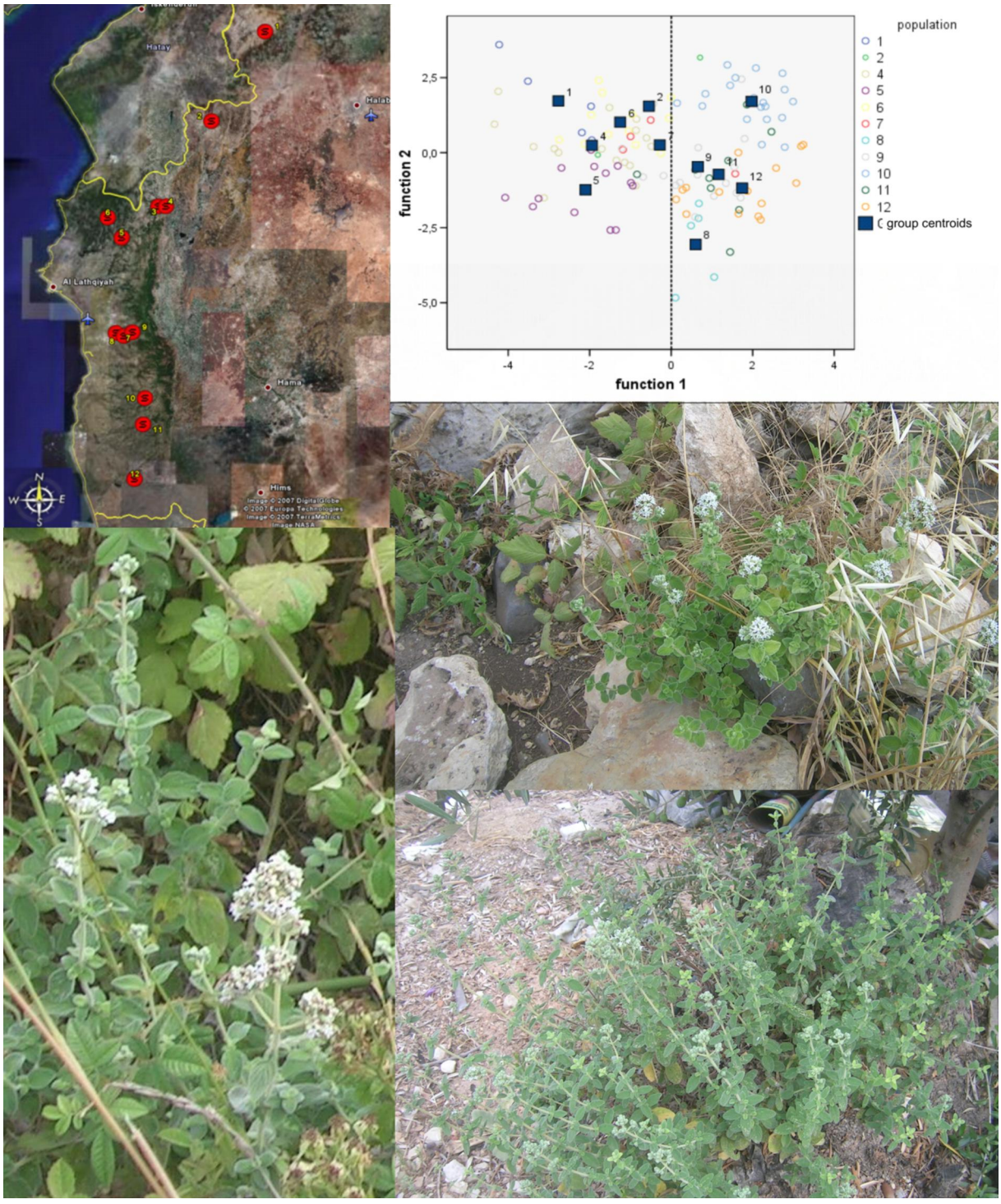

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Composition of Essential Oil Compounds from Different Syrian Populations of *Origanum syriacum* L. (Lamiaceae)

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The chemical compositions of the essential oil compounds of 117 individual plants belonging to 11 Syrian populations of *Origanum syriacum* L. (Lamiaceae) were studied by GC-FID and GC-MS. The composition was dominated by carvacrol and/or thymol with a high degree of polymorphism in the occurrence of these two compounds between the different populations. In three populations carvacrol was dominating, with thymol being present only in minor amounts, whereas in only one population thymol was the main compound, with carvacrol only in traces. In all other populations both carvacrol and thymol were present as major compounds. No geographical pattern could be detected for the occurrence of the chemotypes. Thymoquinone, a promising anticancer candidate, was present in the extracts in a wide range between 0.04 and 23.7%.

KEYWORDS: ***Origanum syriacum*; essential oil compounds; carvacrol; thymol; thymoquinone; Syria**

INTRODUCTION

The genus *Origanum* (Lamiaceae) consists of 43 species and 18 hybrids arranged in 3 groups and 10 sections (*1*). *Origanum syriacum* L. is placed in the section Majorana, together with *Origanum majorana* L. and *Origanum onites* L (*2*). Within *O. syriacum* three varieties, var. *syriacum*, var. *bevanii* (Holmes) Ietswaart, and var. *sinaicum* (Boissier) Ietswaart, are described (*2*). *O. syriacum* inhabits a large area in the eastern Mediterranean. It can be found in southern Turkey, on Cyprus, in Syria, Lebanon, Israel, Jordan, and on the Sinai Peninsula and grows from nearly sea level up to at least 2000 m in rocky soils, often on limestone (*2*). In Syria the plant is called "soba'a" (whirlwind) in the north of the western mountain range and "za'atar khalil" (meaning not known) on the coast and in the southern part of the coastal mountain range. In Syrian folk medicine *O. syriacum* is used for treating gastrointestinal problems and respiratory diseases (Gadoua, personal communication). Treatment against respiratory diseases is also reported as the main medicinal use of this species from neighboring countries such as Jordan (*3, 4*), Israel (*5*), and Palestine (*6*). In Jordan the plant is reported to be additionally used as a carminative, pectoral, antitussive, aperitif, and antistomachache and against arthritis (*3, 4*). In different historical records from the area of Bilad al-Sham (a historical geographical term by former Arab rulers that included significant parts of present-day Syria, Lebanon, Israel, Palestine, and Jordan) *Origanum* sp. was used against internal diseases, hemorrhoids, sexual diseases, pains, animals bites, and poison (*7*). Because *O. syriacum* is the dominating species of the genus in this region, it is very likely that this species was meant by the historical records. Today the quantitatively predominating use, however, is for human consumption as a basic ingredient in za'atar. Many other species from the Labiatae family are also used as za'atar such as *Satureja thymbra* L., called "za'atar rumi" or "za'atar franji" (Roman or European hyssop), *Thymbra spicata* L., called "za'atar hommar" or "za'atar sahrawi" (donkey or desert hyssop), and *Coridothymus capitatus* (L.) Reichenb., called "za'atar farsi" (Persian hyssop). But za'atar par excellence prevailing in terms of sensorial quality and in market quantities is *O. syriacum* (*8*). The basic mixture of za'atar consists of dried and ground leaves of *O. syriacum* (za'atar), powdered seed coats of sumac (*Rhus coriaria*, Anacardiaceae) as acidulant and responsible for the typical red color, salt, roasted sesame seeds, and olive oil. This mixture is often refined by other spices, walnuts, etc. The large quantities needed for preparing za'atar are exclusively collected from the wild.

The essential oil of *O. syriacum* is dominated by carvacrol and thymol (*8*−*18*). Only in one case the bicyclic *cis*-sabinene hydrate was described as a major compound in this species (*19*). Essential oil chemotypes are frequently occurring in species of the genus *Origanum*, whereas rare chemotypes are often overlooked when pooled plant samples are analyzed. In *O. majorana* L. from Cyprus, for example, two new chemotypes could be found by analyzing individual plants (*20*), whereas previous studies about wild-growing *O. majorana* analyzed only the essential oil distilled from a mixture of plants. Also in *O.*

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Table 1. Geographical Localization of the 11 Populations of *Origanum syriacum* Collected in Syria

population	variety	no. of individuals sampled	elevation (m)	GPS N	GPS E
1	var. *bevanii*	5	600	36° 30′ 04.2″	36° 44′ 46.0″
2	var. *bevanii*	2	235	36° 09′ 27.4″	36° 30′ 10.2″
3	var. *bevanii*	16	525	35° 49′ 53.7″	36° 15′ 51.0″
4	var. *bevanii*	13	347	35° 42′ 40.0″	36° 05′ 58.3″
5	var. *bevanii*	12	523	35° 47′ 16.6″	36° 02′ 12.0″
6	var. *syriacum*	4	283	35° 20′ 48.6″	36° 04′ 27.8″
7	var. *syriacum*	5	538	35° 20′ 06.5″	36° 06′ 38.3″
8	var. *syriacum*	16	860	35° 20′ 57.2″	36° 08′ 55.3″
9	var. *bevanii*	20	937	35° 05′ 49.7″	36° 12′ 16.0″
10	var. *bevanii*	9	575	34° 59′ 46.7″	36° 11′ 45.9″
11	var. *bevanii*	15	270	34° 47′ 09.7″	36° 09′ 28.9″

Table 2. Essential Oil Compounds of 11 Different Populations of *Origanum syriacum* from Syria[a]

		population										
compound	RI	1	2	3	4	5	6	7	8	9	10	11
α-thujene	932	0.9 ± 0.4	0.9 ± 0.0	1.0 ± 0.3	1.0 ± 0.2	1.0 ± 0.1	0.8 ± 0.1	0.8 ± 0.3	0.9 ± 0.2	0.8 ± 0.2	0.9 ± 0.1	0.9 ± 0.6
α-pinene	941	0.7 ± 0.1	0.7 ± 0.0	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.6 ± 0.2	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.0	0.6 ± 0.1
sabinene + β-pinene	980	0.9 ± 0.5	0.8 ± 0.1	0.9 ± 0.4	1.6 ± 2.1	0.8 ± 0.5	0.4 ± 0.3	1.0 ± 0.4	1.0 ± 0.4	0.9 ± 0.4	1.2 ± 0.4	1.0 ± 0.4
myrcene	992	1.3 ± 0.8	2.0 ± 0.0	1.7 ± 0.6	1.8 ± 0.3	1.7 ± 0.6	1.8 ± 0.4	1.4 ± 0.6	1.6 ± 0.5	1.8 ± 0.3	1.7 ± 0.4	1.5 ± 0.4
α-phellandrene	1008	0.2 ± 0.1	0.3 ± 0.0	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.0	0.1 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.1
α-terpinene	1021	0.9 ± 0.8	1.7 ± 0.7	1.7 ± 0.6	1.5 ± 0.5	1.6 ± 0.9	1.2 ± 0.3	1.1 ± 0.5	1.3 ± 0.4	1.8 ± 0.5	1.5 ± 0.6	1.0 ± 0.4
p-cymene	1029	6.1 ± 2.4	5.3 ± 3.3	9.8 ± 7.5	6.9 ± 7.7	8.0 ± 5.1	6.7 ± 2.5	17.7 ± 20.1	10.0 ± 7.7	8.4 ± 5.4	7.9 ± 5.6	8.0 ± 6.4
limonene	1034	0.4 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.3 ± 0.2	0.4 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
γ-terpinene	1064	5.4 ± 5.6	11.2 ± 8.7	11.3 ± 5.1	9.8 ± 3.9	8.9 ± 5.5	6.8 ± 1.8	8.0 ± 3.4	9.0 ± 4.0	14.3 ± 6.4	11.8 ± 6.7	6.7 ± 4.0
trans-sabinene hydrate	1072	1.0 ± 0.1	0.9 ± 0.0	1.0 ± 0.2	0.9 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	0.9 ± 0.2	0.9 ± 0.3	0.9 ± 0.1	1.0 ± 0.2	1.0 ± 0.1
cis-sabinene hydrate	1101	0.4 ± 0.0	0.6 ± 0.2	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.0	0.4 ± 0.0	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
terpinen-4-ol	1183	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.1	0.2 ± 0.0	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.1
thymoquinone	1253	4.3 ± 4.1	1.6 ± 1.4	4.7 ± 5.8	2.5 ± 1.9	6.0 ± 6.1	5.1 ± 3.7	4.5 ± 3.1	5.5 ± 5.6	3.1 ± 3.3	5.6 ± 6.2	5.7 ± 5.7
thymol	1296	0.4 ± 0.1	27.8 ± 38.7	15.4 ± 23.7	0.3 ± 0.1	22.2 ± 28.7	10.6 ± 11.3	0.4 ± 0.2	17.6 ± 25.0	57.6 ± 10.3	14.2 ± 22.2	13.3 ± 21.9
carvacrol	1306	69.8 ± 3.6	40.7 ± 53.6	42.1 ± 23.3	63.8 ± 12.0	41.7 ± 29.8	58.2 ± 5.8	53.9 ± 21.6	44.1 ± 31.8	2.7 ± 0.6	45.1 ± 26.6	52.0 ± 31.7
β-caryophyllene	1431	1.7 ± 0.4	2.4 ± 0.3	2.8 ± 0.8	3.2 ± 0.7	2.1 ± 0.3	2.3 ± 0.4	3.5 ± 1.3	3.2 ± 1.0	3.0 ± 0.9	3.9 ± 0.7	4.1 ± 0.9
α-humulene	1466	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.1	0.4 ± 0.2	0.2 ± 0.1	0.2 ± 0.1	0.4 ± 0.3	0.3 ± 0.2	0.2 ± 0.1	0.4 ± 0.2	0.3 ± 0.2
β-bisabolene	1516	0.3 ± 0.3	0.4 ± 0.3	1.2 ± 0.8	1.7 ± 1.1	0.2 ± 0.2	0.9 ± 0.6	1.9 ± 1.8	0.5 ± 0.4	0.6 ± 0.5	0.9 ± 0.4	0.8 ± 0.4
germacrene B	1561	2.8 ± 2.2	0.3 ± 0.2	1.6 ± 2.0	0.5 ± 0.4	1.1 ± 1.1	0.8 ± 0.4	0.4 ± 0.2	0.4 ± 0.4	0.3 ± 0.4	0.3 ± 0.4	0.3 ± 0.3
caryophyllene oxide	1596	0.7 ± 0.5	0.2 ± 0.0	0.6 ± 0.5	0.4 ± 0.2	0.3 ± 0.4	0.3 ± 0.2	0.5 ± 0.4	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.2	0.2 ± 0.1

[a] Values are arithmetic mean peak area percentages ± standard deviations.

syriacum so far only the essential oil composition of mixed plant samples is reported. In this work we present an analysis of the essential oil compounds from individual plants of 11 populations of *O. syriacum* from Syria, collected along a north−south transect in its distribution area.

MATERIALS AND METHODS

Plant Material. One hundred and seventeen samples of 11 populations of *O. syriacum* L. (Lamiaceae) were collected in Syria in full bloom following a north−south transect in July 2006 (**Table 1**). The identification of plant material was done according to the method of Ietswaart (*2*). The attribution of sampled plants to one of the two varieties in question (var. *syriacum* or var. *bevanii*) was not always unambiguous due to the subtle morphological differences that are used for their discrimination. Voucher specimens of the populations are kept at the herbarium of the Institute for Applied Botany.

Extraction. Seventy milligrams of dried plant material was extracted in 1 mL of dichloromethane for 30 min in an ultrasonic water bath. The extracts were filtered with cotton pads on a Pasteur pipet. Solvent extraction instead of distillation was chosen for the following reason: some essential oil compounds are labile to temperature and/or water and are rearranged during distillation as it is the case for, for example, *cis*-sabinene hydrate, a compound often present in the genus *Origanum* (*21*). Solvent extraction often avoids this problem and enables the detection of the native plant composition (*22*).

GC and GC-MS Analyses. *Fast-GC-FID.* The quantitative GC analyses were performed on an HP 6890 equipped with a FID and fitted with an Agilent DB-5 narrow-bore capillary column (10 m × 0.1 mm, 0.17 μm film thickness). Helium was used as carrier gas. The front inlet was kept at 260 °C in split mode (split ratio = 100:1); injection volume, 0.2 μL; temperature program, 60 °C for 1 min, from 60 to 85 °C at 8 °C/min, from 85 to 280 °C at 12 °C/min.

GC/MS. Analyses to identify the compounds were performed on an HP 6890 coupled with an HP 5972 MSD (mass range *m*/*z* 50−550) and an Agilent DB-5MS narrow-bore capillary column (30 m × 0.25 mm, 0.25 μm film thickness). Helium was used as carrier gas. The inlet was kept at 260 °C in split mode (split ratio = 100:1); injection volume, 1 μL; temperature program, 60 °C for 4 min, from 60 to 100 °C at 5 °C/min, from 100 to 280 °C at 9 °C/min.

Retention indices (RI) of the sample components were determined on the basis of homologous *n*-alkane hydrocarbons under the same conditions. Fenchone was used as internal standard at a concentration of 0.1 mg/mL dichloromethane. The quantitative composition was obtained by peak area normalization, and the response factor for each component was considered to equal 1. The compounds were identified by comparing their retention indices and mass spectra with published data (*23, 24*).

RESULTS AND DISCUSSION

O. syriacum is an important "cash crop" in western Syria, collected exclusively from the wild. In this study the composition of essential oil compounds is dominated by carvacrol and thymol followed by their precursors γ-terpinene and *p*-cymene as well as thymoquinone (**Table 2**).

With regard to its main compounds carvacrol and thymol the species is highly polymorphic in Syria. In our samples the population average values for carvacrol are in a range of

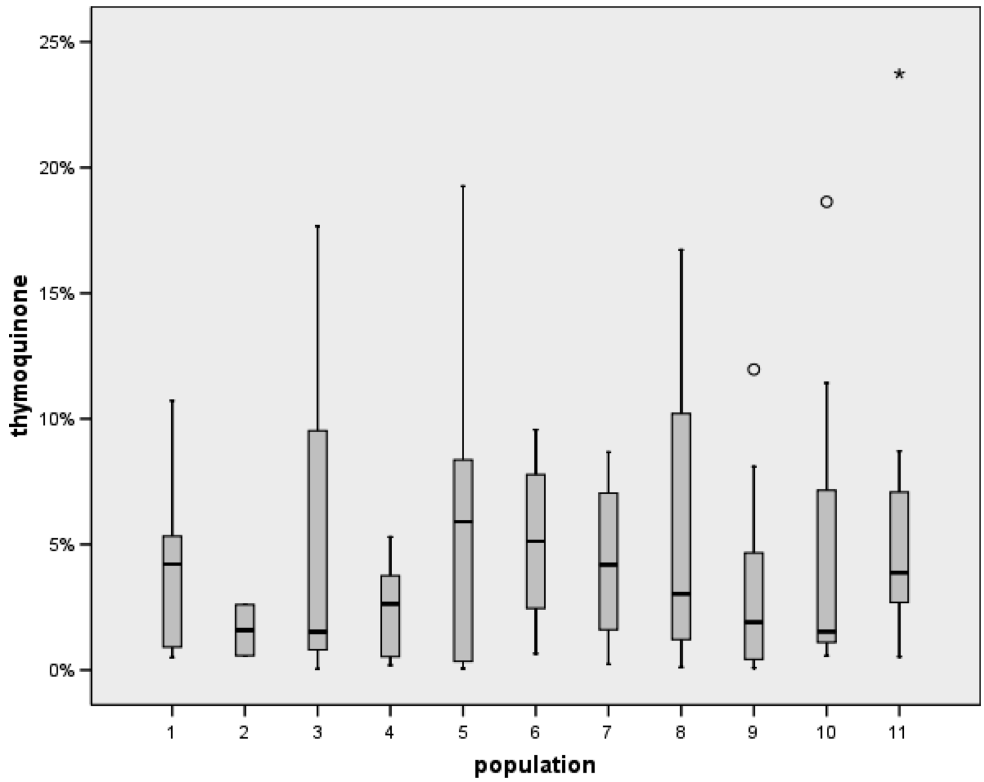

Figure 1. Box plot of thymoquinone contents in the different populations of *Origanum syriacum*.

2.7−69.8%, whereas thymol values were between 0.3 and 57.6%. In Syria 4 of 11 populations were dominated by carvacrol (populations 1, 4, and 7) or thymol (population 9); in the remaining 7 populations both compounds occurred at higher levels. Comparable results were found in previous studies about *O. syriacum* sampled in Turkey, Lebanon, Israel, and Egypt where sometimes one of the two main compounds was over dominating (*9, 14, 15*) or both were present in higher amounts (*8, 10, 11*). The ratio between the main compounds, however, may change during the vegetation period (*16*). The only deviating essential oil composition was reported by Baser et al. (*19*), who found besides thymol as main compound also *cis*-sabinene hydrate at approximately the same quantity as thymol. In our samples no geographical pattern could be observed regarding the distribution of the two compositional types. Carvacrol and thymol are compounds with remarkable antioxidant activity. In *O. syriacum*, however, the essential oil as well as the pure compounds carvacrol and thymol exhibit lower antioxidant activities than the dichloromethane and methanol/water extracts. Therefore, the major effect of the high antioxidant activity of *O. syriacum* is due to other polar and nonpolar phenolics (*25*).

The average population values of γ-terpinene and *p*-cymene varied between 5.4 and 14.3% and between 5.3 and 17.7%, respectively. The value of 17.7% for *p*-cymene in population 7 was an extreme value based on one sample with an extraordinarily high value of 52%; all other samples showed a continuous variation for *p*-cymene of up to 30%.

Thymoquinone was present in the samples in a wide range between 0.04 and 24%. The average mean of 4.5% together with a median of 3% already indicates a high positive skewness (1.57) of the distribution of thymoquinone. The populations themselves showed mean values for thymoquinone between 1.6 and 6% with very high variability within the populations (**Figure 1**). Especially populations 3, 5, and 8 showed a wide range with values of up to almost 20%. The highest value of 24% was found as an extreme outlier in population 11, where the rest of the population has thymoquinone contents of below 10%.

Thymoquinone is a bioactive compound with interesting properties such as antioxidant effects and anti-inflammatory and analgesic actions (*26, 27*). Furthermore, the molecule has become a promising anticancer candidate (*28, 29*). Thymoquinone is often the major aglycone in glycosidically bound volatiles within the genus *Origanum* (*30*−*32*). However, it has been reported as a free (not glycosidically bound) volatile compound only by Economakis et al. (*33, 34*) for *Origanum dictamnus* and by Johnson et al. (*35*) for *Origanum vulgare* ssp. *hirtum*. First indications for polymorphisms between genotypes of this compound were demonstrated by Economakis et al. (*34*), who found for the two genotypes examined very distinct levels of thymoquinone. Hirobe et al. (*36*) demonstrated the high cytotoxic activity of this compound and published the only report of the presence of low amounts of unbound thymoquinone in the species *O. syriacum*.

Nigella sativa was always propagated as a natural source of thymoquinone (*26*−*29*) and has a 2-fold higher content of thymoquinone in the essential oil (*26*) compared to the best individuals of *O. syriacum*. The >12-fold higher essential oil content in *O. syriacum* together with an 8−9-fold higher yield (*18*), however, demonstrates impressively the higher production potential of *O. syriacum* as a natural source for thymoquinone.

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Arbutin in marjoram and oregano

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ABSTRACT

Arbutin is a hydroquinone derivative that has been found in species of several plant families. Within the genus *Origanum* the formation of arbutin is polymorphic, with arbutin present in considerable amounts (*O. dubium* 20.8 ± 15.3 mg/g; wild *O. majorana* 51.3 ± 15.4 mg/g, cultivated *O. majorana* 40.6 ± 11.2 mg/g), minor amounts (*O. microphyllum* 0.1 ± 0.1 mg/g, wild *O. onites* 0.3 ± 0.1 mg/g, cultivated *O. onites* 0.1 ± 0.1 mg/g, *O. saccatum* 0.1 ± 0.1 mg/g, *O. solymicum* 0.4 ± 1.0 mg/g) or completely absent (*O. husnucan-baseri*, *O. syriacum*, *O. vulgare*). Whereas the most important commercial oregano species (*O. onites* and *O. vulgare*) contain no or only minor amounts of arbutin, marjoram (*O. majorana*) has considerably high amounts. The high variability of arbutin in *O. majorana* would allow a selection into cultivars with high arbutin content and low arbutin varieties. In a segregating F_2-generation of a species crossing between *O. majorana* (high content of arbutin) and *O. vulgare* ssp. *vulgare* (free of arbutin), the presence of arbutin followed a Mendelian segregation of 3:1, indicating that only one gene is responsible for the polymorphism of arbutin in the genus *Origanum*. The absence of arbutin in *O. vulgare* ssp. *vulgare* or *O. syriacum* would even enable the breeding of marjoram with no arbutin at all.

1. Introduction

Arbutin (4-hydroxyphenyl-β-D-glucopyranoside) is a hydroquinone derivative that consists of a phenol molecule with a glucose moiety in the *para*-position. Arbutin has been found in species of several plant families, for example in the Rosaceae (*Pyrus communis* L. (Cui, Nakamura, Ma, Li, & Kayahara, 2005)), Lamiaceae (*Origanum majorana* L. (Assaf, Ali, & Makboul, 1987)), Myrothamnaceae (*Myrothamnus flabellifolia* Welw. (Suau, Cuevas, Valpuesta, & Reid, 1991)) and Ericaceae (e.g. *Vaccinium* spp. (Saario, Koivusalo, Laakso, & Autio, 2002; Wha, Sang, Soon, Kuk, & Won, 1999) or *Arctostaphylos uva-ursi* L. (Parejo, Viladomat, Bastida, & Codina, 2002)). Although in plants the content of arbutin can reach considerable amounts (up to 25% of the dry weight in leaves of *M. flabellifolia* (Bianchi et al., 1993; Suau et al., 1991) or up to 17% in the widely used *A. uva-ursi* (Hoppe, 1975; Hänsel, Keller, Rimpler, & Schneider, 1992; Parejo et al., 2002)) its physiological and ecological functions are still under discussion. As it is present in plant taxa capable of withstanding extreme low temperatures or extended drought, arbutin is thought to play an important role in resistance to such environmental stress (Hincha, Oliver, & Crowe, 1999; Oliver, Hincha, Tsvetkova, Vigh, & Crowe, 2001; Oliver et al., 2002). In *Pyrus* spp. hydroquinone formation from arbutin was found to be involved in fireblight resistance (Hildebrand, Powell, & Schroth, 1969; Smale & Keil, 1966).

In cosmetic preparations arbutin is widely used to lighten the skin (Lin, Chiang, Lin, & Wen, 2008; Parvez, Kang, Chung, & Bae, 2007). Arbutin is also well known for its diuretic and urinary anti-infective properties and the arbutin-rich leaves of *A. uva-ursi* (bearberry) are internally used for moderate inflammatory conditions of the urinary tract and bladder (Yarnell, 2002). In both cases the active principle is hydroquinone, a metabolite of arbutin. Arbutin, however, could also exhibit adverse effects, as its metabolites showed hepatotoxic, nephrotoxic, mutagenic and carcinogenic potentials in animal studies (Nowak, Shilkin, & Jeffrey, 1995; Peters, Jones, Monks, & Lau, 1997; Shibata et al., 1991). Furthermore, a hypothesis was published linking phenol and hydroquinone as causal factors for leukaemia (McDonald, Holland, Skibola, Duramad, & Smith, 2001). For this reason the therapeutic use of *A. uva-ursii* is always restricted to a short period.

The genus *Origanum* comprises 43 species (Ietswaart, 1980; Skoula & Harborne, 2002), mainly distributed in the Eastern Mediterranean region. Due to their high content of essential oil, species of the genus have traditionally been collected for centuries, for the flavouring of traditional dishes as well as for several purposes in traditional medicine. Today two aromatic qualities, marjoram and oregano, are commercially traded and widely used all over the world as popular herbs. The four closely-related species of section Majorana (*O. onites* L., *O. syriacum* L., *O. majorana* L. and *O. dubium* Boiss.), are amongst the most important species of the

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genus. Carvacrol-rich *O. onites* (Turkish oregano) possesses the typical oregano flavour and is, beside *O. vulgare* L., one of the most traded and consumed *Origanum* species. In 1999, Turkey exported more than 7500 tons of oregano, more than 80% of the exported plant material derived from *O. onites* (Baser, 2002). *O. syriacum* (Israeli oregano) is not extensively traded on the world market but is a crop of local importance as it is a basic ingredient of za'atar which has traditionally been used throughout the Arab world for the seasoning of meats and vegetables (Fleisher & Fleisher, 1988). *O. majorana* is cultivated for the production of marjoram in several European and African countries (Sarlis, 1994). Carvacrol-rich *O. dubium* (White oregano) is, due to its high essential oil content, the preferred plant material for the industrial production of essential oregano oil in Turkey (export volume > 30 tons per year) (Baser, 2002).

Within the genus *Origanum* the formation of arbutin seems to be polymorphic, with arbutin present in considerable amounts (*O. majorana*) or completely absent (*O. vulgare*) (Assaf et al., 1987; Kraus, Koch, & Hoffstetter-Kuhn, 1996). So far there is no information available whether the occurrence of arbutin is a special characteristic of *O. majorana* or whether other *Origanum* species contain this compound. The aim of this study was to give a first outlook about the intra-generic and intra-specific variability of arbutin in the genus *Origanum*. Due to their commercial importance and the affiliation of a species rich in arbutin a special focus was given to section Majorana (*O. onites*, *O. syriacum*, *O. majorana* and *O. dubium*). Additionally, one population of *O. microphyllum* (Bentham) Vogel and single plant individuals of *O. husnucan-baseri* Duman, Ayataç et Duran, *O. saccatum* Davis and *O. solymicum* Davis were analysed. Moreover, samples of commercial marjoram and oregano were included in the analysis. To study the segregation pattern and to discuss possible approaches for the breeding of arbutin-low or even arbutin-free marjoram, an inter-specific crossing between arbutin-rich marjoram (*O. majorana*) and arbutin-free oregano (*O. vulgare* ssp. *vulgare*) was analysed.

2. Material and methods

2.1. Plant material

For the investigation of intra-generic and intra-specific variability four populations of *O. majorana* L. (maj1 = seeds from a natural population on Cyprus; cultivars 'Erfo', 'G' and 'Miraz' = seeds from a commercial source), one population of *O. microphyllum* (Bentham) Vogel (seeds collected from a natural population in Crete) and one population of *O. onites* L. (seeds from a commercial source) were grown in the greenhouse of the Institute for Applied Botany, University of Veterinary Medicine, Vienna. Additional plants of *O. syriacum* L., *O. onites* L., *O. majorana* L., *O. dubium* Boiss., *O. saccatum* Davis, *O. solymicum* Davis and *O. husnucan-baseri* Duman, Ayataç et Duran were collected in Syria (May 2006, beginning to full bloom), Turkey (June 2006, beginning to full bloom), Cyprus (July 2006, full bloom) and Greece (October 2007, seed ripening), from their natural habitats. Details about their geographic origin, the identification number of the sampled populations and the number of individuals investigated are provided in Table 1. The species were identified by following the identification key in the latest taxonomic revision of the genus *Origanum* (Ietswaart, 1980). For the delimitation of *O. dubium* and *O. majorana* the Flora of Cyprus (Ietswaart, 1985) was used as a second reference. Voucher specimens representing wild and greenhouse populations were deposited in the Herbarium of the Institute for Applied Botany and Pharmacognosy, University of Veterinary Medicine, Vienna. The plants grown in the greenhouse were harvested in full bloom and dried at 35 °C in a drying chamber (Memmert, Schwabach, Germany). The plant material collected from the natural populations was dried at room temperature.

The samples of commercial marjoram and oregano (usually made up of plant material cut into small pieces) were purchased from local markets or supermarkets in several European countries. The geographical origin of the commercial plant material (as indicated on the packaging) is provided in Table 2. An assignment of the plant material was performed on the basis of intact calyces (according to Ietswaart, 1980) that were isolated from the samples. The commercial oreganos from Italy, Peru, Slovenia, Tunisia and those two of unknown origin were identified as *O. vulgare*. In Turkish oregano calyces with a shape typical for *O. onites* were present. According to calyx shape, sensorial quality and with the knowledge about the geographical origin, the oregano sample from Cyprus was identified as *O. dubium*.

The three bearberry samples were obtained from Viennese pharmacies.

For the inheritance study a segregating F_2-generation was established by the selfing of a naturally occurring hybrid between the species *Origanum majorana* L. and *Origanum vulgare* L. ssp. *vulgare* detected in a population survey of oregano (Marn, Novak, & Franz, 1999; Novak, Gimplinger, & Franz, 2002). The hybrid nature was unambiguously identified by the shape of the calyx (Novak et al., 2002). The plants were grown in the greenhouse and 106 individuals were harvested at the beginning of bloom and dried at 35 °C in a drying chamber.

2.2. Extraction

Leaves and flowers of each plant were separated from the stem and ground to a fine powder in a micro hammer mill (Culatti, Zürich, Switzerland). For the extraction of the traded samples of marjoram and oregano a representative quantity of each sample was ground.

For TLC analyses 0.5 g of the powder were extracted with 5 ml methanol:water (50:50) under reflux for 15 min. The extracts were filtered immediately through a 'fast' folded filter (Whatman Ltd., Springfield Mill, England) and 0.2 ml of a 9.5% lead acetate solution were added to the filtrate. After a second filtration the extracts were used for TLC (Kraus et al., 1996).

For HPLC analyses 0.05 g of powdered plant material were extracted with 25 ml methanol:water (50:50) for 30 min at 25 °C in an ultrasonic water bath (Parejo, Viladomat, Bastida, & Codina, 2001; the methanol:water ratio was modified). The extracts were filtered through a microfilter with 2 μm pore size (Minisart RC 25, Sartorius, Göttingen, Germany).

2.3. TLC

The reference substance was 10 mg of arbutin (Roth, Karlsruhe, Germany) dissolved in 10 ml methanol. A stationary phase of HPTLC, silica gel 60 F254 nm (Merck, Darmstadt, Germany) with a mobile phase of acetic acid ethyl ester/methanol/water (77:13:10) was used. Detection was with dibromchinochlorimide with inspection in visible light; R_f-value of arbutin: 0.47 (Kraus et al., 1996). Absence or presence of arbutin was scored as 0 or 1, respectively.

2.4. HPLC

HPLC analysis was carried out on a Waters modular system (626 pump, in-line degasser AF, photodiode array detector PDA996; Waters, Milford, MA) using a Symmetry C18 column (5.0 μm, 4.6 × 150 mm). The mobile phase was water:methanol (95:5) at a flow rate of 2 ml/min. The injection volume was 10 μl and UV detection was at 220 nm. The concentration of arbutin was deter-

Table 1
Identification number and geographical origin of the natural populations investigated (pop. = population; s.l. = sea level (m); *n* = number of samples; unkn. = unknown geographical position; TR = Turkey; CY = Cyprus; GR = Greece; SYR = Syria). Mean value (MV), standard deviation (STD), minimum (min.) and maximum (max.) value of arbutin (mg/g dry mass) in the populations analysed (n.d. = not detected; tr. = trace, <0.1 mg/g arbutin).

Species	Geographical position					Arbutin (mg/g dry mass)				
	Pop.	Origin	s. l.	N	E	*n*	MV	STD	Min.	Max.
O. dubium	dub1	TR	104	36°29′47.2″	32°07′13.5″	12	37.3	8.8	25.8	52.4
	dub2	TR	598	36°30′12.0″	32°10′08.2″	5	46.7	16.2	23.0	66.0
	dub3	TR	1037	36°33′02.3″	32°17′56.1″	2	57.8	1.5	56.8	58.9
	dub4	TR	738	36°30′09.3″	32°11′02.5″	9	49.3	10.2	31.8	64.8
	dub5	TR	9	36°37′44.2″	31°45′36.7″	3	31.1	4.3	26.2	34.2
	dub6	TR	unkn.	unkn.	unkn.	3	11.8	7.0	7.5	19.9
	dub7	TR	135	36°05′56.8″	32°32′02.8″	1	23.2	–	–	–
	dub8	TR	135	36°05′56.8″	32°32′02.8″	1	15.1	–	–	–
	dub9	TR	145	36°06′14.0″	32°34′55.5″	8	18.9	5.1	11.2	25.6
	dub10	TR	145	36°06′14.0″	32°34′55.5″	3	22.3	5.6	17.1	28.2
	dub11	TR	209	36°05′54.4″	32°34′59.3″	9	16.5	7.6	5.0	32.2
	dub12	TR	253	36°01′57.6″	32°46′12.4″	3	13.8	2.9	11.4	17.0
	dub13	TR	102	36°05′30.5″	32°55′14.6″	11	15.6	5.5	6.5	24.1
	dub14	TR	28	36°20′40.4″	32°12′42.9″	10	18.5	5.2	10.0	26.3
	dub15	TR	155	37°10′09.1″	31°11′32.2″	1	17.5	–	–	–
	dub16	TR	38	36°58′08.7″	31°12′24.5″	1	37.2	–	–	–
	dub17	CY	153	35°05′31.2″	32°32′31.8″	16	9.8	4.7	5.0	25.2
	dub18	CY	15	35°08′45.6″0	32°31′58.5″	15	7.2	2.9	n.d.	11.1
	dub19	CY	638	35°01′19.1″	32°34′49.8″	13	9.8	3.4	5.3	16.1
O. husnucan-baserii	husnu1	TR	1345	36°32′52.9″	32°20′50.1″	1	n. d.	–	–	–
O. majorana	maj1	CY	801	34°49′39.3″	32°49′43.6″	15	58.7	18.6	11.1	90.3
	maj2	CY	unkn.	unkn.	unkn.	12	51.3	13.9	30.3	73.4
	maj3	CY	801	34°49′39.3″	32°49′43.6″	13	62.4	23.0	36.1	118.0
	maj4	CY	164	34°58′36.4″	32°28′23.0″	10	43.3	12.7	25.6	68.4
	maj5	CY	unkn.	unkn.	unkn.	7	50.9	9.6	36.0	67.4
	maj6	CY	547	34°55′43.6″	32°26′32.5″	5	43.9	13.2	33.2	65.4
	maj7	CY	278	34°56′59.3″	32°29′26.8″	15	50.3	9.1	35.9	66.3
	maj8	CY	436	34°54′25.5″	32°33′42.0″	12	44.0	6.7	29.5	52.8
	maj9	CY	596	34°48′56.9″	32°39′47.5″	11	48.1	13.3	27.6	65.3
O. microphyllum	mic1	GR	unkn.	unkn.	unkn.	27	0.1	0.1	tr.	0.5
O. onites	oni1	TR	5	36°50′01.7″	30°35′01.0″	7	1.9	0.3	1.4	2.3
	oni2	TR	362	36°35′38.5″	30°28′23.3″	9	1.1	1.2	0.1	3.0
	oni3	TR	112	36°44′19.6″	30°32′55.5″	12	0.1	0.0	n. d.	0.1
	oni4	TR	880	36°59′18.5″	30°28′05.1″	23	0.1	0.0	n. d.	0.1
	oni5	TR	135	36°05′56.8″	32°32′02.8″	3	0.1	0.1	n. d.	0.1
	oni6	TR	135	36°05′56.8″	32°32′02.8″	28	n. d.	–	–	–
	oni7	TR	35	36°59′18.3″	31°12′25.4″	5	0.1	0.0	n. d.	0.1
	oni8	TR	134	37°05′06.2″	31°13′51.0″	6	0.1	0.0	n. d.	0.1
	oni9	TR	108	37°07′49.7″	31°12′24.3″	1	0.10	–	–	–
	oni10	TR	787	37°15′55.8″	31°12′48.8″	6	2.0	3.7	0.1	9.4
	oni11	TR	817	37°16′24.3″	31°12′50.2″	18	0.1	0.0	n. d.	0.1
	oni12	TR	799	37°16′00.3″	31°12′48.2″	10	0.1	0.0	n. d.	0.1
	oni13	TR	138	37°03′14.0″	31°14′41.5″	2	0.1	0.0	0.1	0.1
	oni14	TR	87	37°01′15.4″	31°14′27.8″	1	0.1	–	–	–
	oni15	TR	23	36°58′53.6″	31°12′23.3″	6	0.1	0.0	0.1	0.1
	oni16	GR	9	36°51′05.5″	22°39′16.2″	7	n. d.	–	–	–
	oni17	GR	10	36°40′13.3″	23°01′47.5″	4	n. d.	–	–	–
O. saccatum	sac1	TR	1137	36°31′48.5″	32°14′12.8″	7	0.1	0.0	n. d.	0.1
	sac2	TR	1037	36°33′02.3″	32°17′56.1″	1	n. d.	–	–	–
O. solymicum	sol1	TR	362	36°35′38.5″	30°28′23.3″	6	0.4	1.0	n. d.	2.5
O. syriacum	syr1	TR	598	36°30′04.2″	36°44′46.0″	13	n. d.	–	–	–
	syr2	SYR	unkn.	36°09′27.4″	36°30′10.2″	2	n. d.	–	–	–
	syr3	SYR	300	35°49′53.2″	36°17′54.2″	1	n. d.	–	–	–
	syr4	SYR	525	35°49′53.7″	36°15′51.0″	8	n. d.	–	–	–
	syr5	SYR	347	35°42′40.0″	36°05′58.3″	4	n. d.	–	–	–
	syr6	SYR	523	35°47′16.6″	36°02′12.0″	8	n. d.	–	–	–
	syr7	SYR	538	35°20′06.5″	36°06′38.3″	5	n. d.	–	–	–
	syr8	SYR	860	35°20′57.2″	36°08′55.3″	6	n. d.	–	–	–
	syr9	SYR	937	35°05′49.7″	36°12′16.0″	5	n. d.	–	–	–
	syr10	SYR	575	34°59′46.7″	36°11′45.9″	5	n. d.	–	–	–
	syr11	SYR	270	34°47′09.7″	36°09′28.9″	6	n. d.	–	–	–

mined using a calibration curve established with seven concentrations of arbutin from 10 to 300 μg/ml.

2.5. Statistical analyses

Statistical analyses were performed with SPSS 14.0 (SPSS Inc., Chicago, IL, USA). The inheritance pattern was evaluated using a chi-square test.

3. Results and discussion

3.1. Intra- and inter-specific variability of arbutin in the genus origanum

Eight species of the genus *Origanum* were analysed for the presence and variability of arbutin (Table 2; mean values of natural populations are provided in Table 1). No arbutin could be detected

Table 2
Mean value (MV), standard deviation (STD), minimum (Min.) and maximum (Max.) value of arbutin (mg/g dry mass) in natural populations of eight *Origanum* species, potted cultivars of *O. majorana* and *O. onites*, as well as traded marjoram, oregano and bearberry (CY = Cyprus, TR = Turkey, ET = Egypt, I = Italy, SLO = Slovenia, TN = Tunisia, GR = Greece, PE = Peru, unkn. = unknown geographical origin). Commercial marjoram was identified as *O. majorana*. The commercial oreganos from Italy, Peru, Slovenia, Tunisia and those two of unknown origin were identified as *O. vulgare*; the Turkish oregano samples were identified as *O. onites*. The oregano from Cyprus was identified as *O. dubium*.

	Arbutin (mg/g dry mass)				
	n	MV	STD	Min.	Max.
O. dubium	126	20.8	15.3	tr.	66.0
CY	44	8.9	3.9	tr.	25.2
TR	82	27.1	15.3	4.9	66.0
O. husnucan-baserii	1	n. d.	–	–	–
O. majorana	100	51.3	15.4	11.1	118.0
O. microphyllum	27	0.1	0.1	n. d.	0.5
O. onites	148	0.3	0.1	n. d.	9.4
GR	11	n. d.	–	–	–
TR	137	0.3	0.9	n. d.	9.4
O. saccatum	8	0.1	0.1	n. d.	0.1
O. solymicum	6	0.4	1.0	n. d.	2.5
O. syriacum	63	n. d.	–	–	–
O. majorana cultivars	69	40.6	11.2	24.2	75.3
'Erfo'	24	40.3	9.4	26.4	64.1
'G'	21	47.1	14.6	26.8	75.3
'Miraz'	24	35.2	5.0	24.2	46.6
O. onites cultivar					
'Greek oregano'	38	0.1	0.1	n. d.	0.3
Traded marjoram	9	18.6	12.2	1.9	40.4
ET	4	23.9	3.7	19.0	27.9
I	1	1.9	–	–	–
SLO	1	12.1	–	–	–
TN	1	13.0	–	–	–
unkn.	2	22.1	25.9	3.8	40.4
Traded oregano	17	2.0	6.1	n. d.	24.8
I	5	5.3	10.9	n. d.	24.8
PE	1	n. d.	–	–	–
SLO	2	n. d.	–	–	–
TN	1	n. d.	–	–	–
TR	5	0.1	0.3	n. d.	0.7
CY	1	7.3	–	–	–
unkn.	2	n. d.	–	–	–
Traded bearberry	3	94.5	20.7	71.1	110.6

in *O. syriacum* and *O. husnucan-baseri*. *O. onites* (n.d. –9.4 mg/g), *O. microphyllum* (n.d. –0.5 mg/g), *O. saccatum* (n.d. –0.1 mg/g) and *O. solymicum* (n.d. –2.5 mg/g) principally possess the ability to synthesise arbutin but do not accumulate this compound in high concentrations. High amounts of arbutin were detected in the two closely-related species *O. dubium* (tr. –66.0 mg/g) and *O. majorana* (11.1–118.0 mg/g). In *O. dubium* arbutin was significantly higher in the populations from Turkey (27.1 mg/g) than in the populations from Cyprus (8.9 mg/g). In Turkish *O. dubium* high amounts of arbutin were especially obvious in the populations dub1 (37.3 mg/g), dub2 (46.7 mg/g), dub3 (57.8 mg/g), dub4 (49.3 mg/g) and dub5 (31.1 mg/g) that were located in the Taurus Mountains east of Alanya. With the exception of dub16 (37.2 mg/g) in the other populations of *O. dubium* from Turkey the average amount of arbutin was significantly lower and ranged between 11.8 mg/g (dub6) and 23.2 mg/g (dub7). The three populations of *O. dubium* collected in Cyprus were comparatively low in arbutin with an average amount of 7.2 mg/g (dub18) to 9.8 mg/g (dub19). In natural and cultivated populations of *O. majorana* the mean value of arbutin ranged between 35.2 mg/g (*O. majorana* cv. 'Miraz') and 62.4 mg/g (maj3). No significant difference could be found between the wild populations from Cyprus (51.3 mg/g) and the three commercial cultivars of this species (40.6 mg/g) that were grown potted in the greenhouse for one vegetation period under standard conditions. In this investigation the overall highest arbutin concentrations were found in a natural population of *O. majorana* (maj3 with 62.4 mg/g, maximum value 118.0 mg/g).

3.2. Oregano and marjoram – a source of hydroquinone in human diet?

Oregano is the common name for a number of carvacrol-rich plant species of different genera that have been used all over the world as popular spices. Only some of them are also of commercial interest and today most of the industrially processed and traded oregano derives either from plant material of *O. vulgare* and *O. onites* ('European oregano') or from plant material of *Lippia graveolens* HBK and *L. berlandieri* Millsp. (both Verbenaceae, 'Mexican oregano') (Kintzios, 2002).

Seventeen commercially traded oregano samples of different producing countries and trademarks were included in our arbutin analyses (Table 2; 'traded oregano'). No arbutin at all was present in oregano samples deriving from Peru, Slovenia and Tunisia. In Italian oregano the arbutin content ranged from non-detectable (three of the five samples analysed) up to 24.8 mg/g. According to intact plant calyces that were found in the samples, the plant material of Italian origin was assigned to *O. vulgare*, a species that was supposed to be free of arbutin. Possibly in the two arbutin-containing samples plant material of *O. vulgare* was mixed with plant material of an arbutin-rich species. However, from the present results it cannot be excluded that the widespread and rather heterogeneous species *O. vulgare* might be polymorphic for arbutin. The five oregano samples of Turkish origin were mainly made up of plant material from *O. onites*. Interestingly only one sample of commercially traded Turkish *O. onites* possessed trace levels of arbutin (0.7 mg/g) whereas in nearly all wild populations of Turkish *O. onites* small quantities of this compound were present (Table 1). Arbutin was also found in a commercial oregano sample that was purchased from a small market in Cyprus. The oregano plant material of Cypriot origin was supposed to derive from *O. dubium*, an *Origanum* species that is endemic to Cyprus and the adjacent parts of Turkey and that is usually of less importance for the industrial production of oregano. Three such 'non-commercial' *Origanum* species that are mostly collected for the direct sale on local markets or for home requirements were included in this study. *O. syriacum*, distributed in parts of the Near East, is the basic ingredient of za'atar and of great local commercial importance throughout the Arab region. Syrian populations of this species were found to be free of arbutin. *O. microphyllum*, an endemic to Crete, has traditionally been collected and locally sold on markets (Skoula, personal communication); it seems to be polymorphic and individuals exhibit either no or small amounts of the compound under study. *O. dubium*, distributed in Cyprus and Southern Turkey, was one of the two *Origanum* species that were found to accumulate high amounts of arbutin. In Cyprus *O. dubium* is collected in wild populations or occasionally cultivated in home gardens and the dried plant material is widely used in the preparation of traditional food preparations (dried herbs) and for a number of purposes in traditional medicine (water infusions, essential oils; Della, Paraskeva-Hadjichambi, & Hadjichambis, 2006). An exposure of the consumer to arbutin depends on the preparation of the plant material. The substance is water soluble and can therefore be found in hot and cold infusions. An exposure to arbutin is also given when *O. dubium* is consumed as herb (see below).

The presence of arbutin in marjoram (plant material from *O. majorana*) has been described before (Assaf et al., 1987). Assaf et al. (1987) isolated arbutin and free hydroquinone from an *O. majorana* cultivar from Egypt and estimated arbutin contents of 0.41–0.45% and 0.14%, respectively (two different methods were

Table 3
Segregation ratio of arbutin in an inter-specific hybrid between *O. majorana* and *O. vulgare*.

O. majorana:*O. vulgare*	Abbrev.	Suggested segregation	Observed segregation	Expected segregation	χ^2	*P*
Present:Absent	A:a	3:1	79:27	79.5:26.5	0.013	0.91

used for their estimation). In natural populations of *O. majorana* in Cyprus as well as in populations of three different commercial cultivars we detected arbutin concentrations that were 10-fold higher (11.1–118.0 mg/g; Table 1). In traded marjoram of different geographical origin and trademarks the arbutin content ranged from 1.9 mg/g to 40.4 mg/g (Table 2, 'traded marjoram'). The highest arbutin content of traded marjoram that was found in a marjoram sample of unknown geographical origin is similar to the mean arbutin value we have detected in plant material of three different *O. majorana* cultivars (40.6 mg/g, Table 2, '*O. majorana* cultivars') that were grown in pots in the glass-house for one vegetation period. From the present results it cannot clearly be deduced why the arbutin content of the traded marjoram samples is in most cases lower than the mean arbutin content in natural populations of *O. majorana* from Cyprus or in our potted populations of the *O. majorana* cultivars. As arbutin is thought to play a role in the plants' response to environmental stress (Hincha et al., 1999; Oliver et al., 2001, 2002) genetic factors as well as ecological conditions of the producing area might play a role. It would also be possible that arbutin degrades to some degree when harvested plant material is stored for a longer period.

Marjoram is normally used in amounts of 0.2–0.3 g dried plant material per dish (the estimation is based on traditional Austrian dishes). With a mean value of about 2% arbutin in traded marjoram the actual intake would be between 4 and 6 mg arbutin per dish, which is approximately half of the figure Blaut et al. (2006) were assuming for a serving of pear of 180 g. Following the calculative approach of Blaut et al. (2006) for the resorption of hydroquinone originating from arbutin in the colon further on, hydroquinone is completely released within 1–4 h. However, some open questions remain that will possibly reduce the proposed figure of hydroquinone released (Schindler et al., 2002). Most individuals daily ingest significant quantities of arbutin and/or hydroquinone with a number of common foods and drinks, e.g. 0.001–0.01 mg arbutin per g wheat product, 0.004–0.015 mg arbutin per g pear, 0.0001 mg arbutin per g coffee or tea, 0.0005 mg free hydroquinone per g red wine, 0.0001 mg free hydroquinone per g broccoli (Deisinger, Hill, & English, 1996). The regular intake of small amounts of an arbutin-rich aromatic plant may not be seen as critical. Nevertheless, the regular consumption of oregano and marjoram might significantly contribute to the daily hydroquinone balance of individuals.

3.3. Inheritance of arbutin in an inter-specific hybrid between *O. majorana* and *O. vulgare*

Arbutin was found to be polymorphic in the segregating F_2-generation of a species hybrid between *O. majorana* and *O. vulgare*. The segregation pattern followed exactly the expected ratio of 3:1 (Table 3) indicating a single gene being responsible for the arbutin polymorphism. In plants that are free of arbutin one step of the arbutin biosynthesis, e.g. the glycosylation step (Arend, Warzecha, & Stöckigt, 2000) might be blocked. It would also be possible that not the biosynthesis itself is blocked but the accumulation of arbutin in the vacuole. Tonoplast-localized sucrose transporters (Sauer, 2007) are responsible for the transfer of arbutin into the vacuole. It was found that some sucrose transporters (e.g. AtSUC9 from *Arabidopsis thaliana* (Sivitz et al., 2007)) accept arbutin, while others (e.g. LjSUT4 from *Lotus japonicus* (Reinders, Sivitz, Starker, Gantt, & Ward, 2008)) do not transport this compound. Plants that do not accumulate arbutin could lack transporter proteins that accept arbutin.

The large arbutin variability present in *O. majorana* from natural populations would enable a disruptive selection into two directions. As the potential arbutin content in *O. majorana* was found to be between 5% and 10% (Table 2) marjoram selections with high arbutin content could serve as a viable natural source for arbutin. *A. uva-ursi* is listed as a rare or endangered plant species in many countries (ECPGR, 2002) but large amounts of bearberry are still collected from the wild. For the commercial production of marjoram *O. majorana* has been cultivated for at least two centuries and a major advantage of marjoram would be its well-proven, sustainable and controlled production in the field. Low arbutin varieties of *O. majorana* could be used in the food sector, although natural variability will allow only genotypes with a minimum of probably 1–3% arbutin and no selection for arbutin-free marjoram. Inter-specific crossings between *O. majorana* and arbutin-free *O. vulgare* (or *O. syriacum*), followed by subsequent backcrosses with *O. majorana*, could open a way to breed marjoram cultivars free of arbutin.

4. Conclusion

Arbutin, present in species of a number of plant families, can be regarded as an undesirable substance in the human diet. Within the genus *Origanum* the formation of arbutin was found to be polymorphic, with high amounts of arbutin present in *O. dubium* as well as in wild and cultivated *O. majorana*. The regular consumption of plant material from these two species might significantly contribute to the daily hydroquinone balance of individuals.

O. dubium is locally collected from wild populations, for personal use or for trade in local markets. *O. majorana* is among the most important *Origanum* species and is, for the production of marjoram, commercially cultivated all over the world. Because in the genus *Origanum* only one gene seem to be responsible for the arbutin polymorphism, classical breeding techniques could open a way to breed marjoram cultivars free of arbutin.

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PART 3

Tracing factors that influence the qualitative and quantitative essential oil composition in *Origanum*

Oregano or Marjoram? The γ-terpinene synthase affects chemotype formation in the genus *Origanum*

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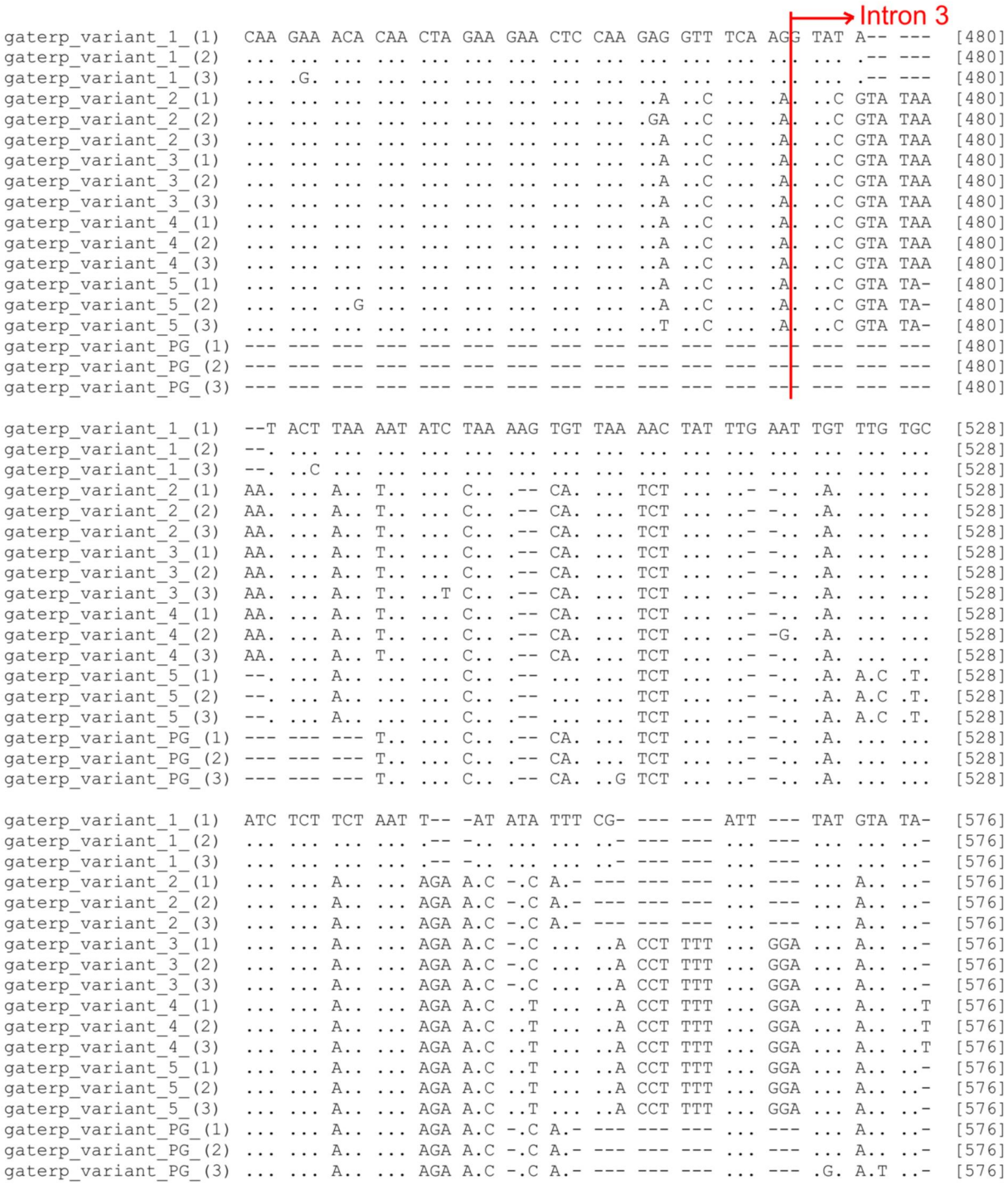

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Abstract

The enzyme γ-terpinene synthase has a key position in the biosynthesis of essential oil compounds in the genus *Origanum*. It catalyses the cyclization of GPP to γ-terpinene giving rise to the 'cymyl'-pathway and the accumulation of γ-terpinene, *p*-cymene, carvacrol and related compounds in a plant. A comprehensive sequence analysis of a part of the γ-terpinene synthase gene (intron 2, exon 3 and intron 3) in *O. dubium*, *O. majorana*, *O. onites*, *O. syriacum* (section Majorana) and *O. vulgare* (section Origanum) revealed six different variants of the gene that were distinguishable according to sequence characteristics in intron 3. The formation of 'cymyl'-compounds was found to be associated with the presence of variants 1 and/or 3 and/or 4 of the γ-terpinene synthase gene. In plants lacking these gene variants 'cymyl'-compounds were completely absent or present in trace amounts only. The alternative 'sabinyl'-chemotype (*O. majorana*) as well as the linalool-chemotype (Turkish *O. dubium* and *O. onites*) could originate in a plants' disability to produce γ-terpinene.

Key words

Origanum, γ-terpinene synthase, essential oils, chemotype, oregano, marjoram

Introduction

Terpenes are the largest and most widespread class of secondary metabolites in plants and more than 20.000 different terpene compounds have been identified to date (Connolly and Hill, 1991). Terpenes are produced by a wide variety of plants and are primary components of resins and essential oils. Up to 100 different terpenes were isolated from individual plants (Harborne and Turner, 1984), the unique terpene composition of each taxon is mainly controlled by a set of mono-, sesqui- and diterpene synthases which catalyze the transformation of the respective terpenoid precursor (GPP, FPP or GGPP) to the parent structures of each type. Recent research provided many insights into enzymatic principles of terpene biosynthesis and molecular biology of terpene synthases (TPS). All plant TPS seem to be evolutionary related and share common reaction mechanisms and conserved structural and sequence characteristics (Bohlmann et al., 1998; Trapp and Croteau, 2001; Aubourg et al., 2002; Christianson, 2006; Tholl, 2006). Numerous TPS have been isolated and characterized (Cseke et al., 1998; Wise et al., 1998; Schnee et al., 2002) and more than hundred TPS genes are known today. However, only scarce information is available about the chromosomal and genomic organisation as well as the evolution of individual TPS genes. There is also restricted knowledge about the molecular mechanisms that are regulating terpene production in general and terpene variation within single species.

The enzyme γ-terpinene synthase has a key position in the biosynthesis of essential oil compounds in the genus *Origanum*. It catalyses the cyclization of GPP to γ-terpinene (Poulose and Croteau, 1978) giving rise to the so-called 'cymyl'-pathway and the accumulation of γ-terpinene, *p*-cymene, carvacrol, thymol and other related compounds in a plant (Figure 1). 'Cymyl'-compounds are character compounds for a number of *Origanum* species, e. g. *O. dubium* Boiss., *O. onites* L. and *O. syriacum* L. (Skoula and Harborne, 2002), three of the four species of section Majorana. Beside the widespread 'cymyl'-chemotype two other main essential oil chemotypes are present in section Majorana. *O. majorana* L. usually accumulates large amounts of 'sabinyl'-compounds (Fischer et al., 1987; Novak et al., 2008). In Turkish populations of *O. onites* and *O. dubium* 'cymyl'-chemotypes are mixed with almost pure linalool-chemotypes (Baser et al., 1993). These two alternative chemotypes are exceptional as 'cymyl'-compounds are nearly absent whereas compounds deriving from another independent metabolic pathway ('sabinyl'-pathway or linalool-pathway, respectively, Figure 1) are accumulated in large amounts. Until now it remained unclear why the 'cymyl'-pathway is blocked in the two alternative chemotypes. As all compounds of the 'cymyl'-pathway are not present the γ-terpinene synthase gene could be responsible for the essential oil polymorphism.

The aim of this study was to learn more about the γ-terpinene synthase gene in the four species of section Majorana. We sequenced a large part of the gene in an accession of *O. syriacum* to gain information about the genomic organisation of the gene in the genus *Origanum*. A part of the γ-terpinene synthase gene was cloned and sequenced in accessions of *O. dubium*, *O. onites*, *O. majorana*, *O. syriacum*, *O. vulgare* and *Thymus vulgaris* to compare the γ-terpinene synthase gene of

closely and distantly related species. Accessions with different essential oil chemotypes were included in the analyses to verify whether the γ-terpinene synthase is involved in the essential oil polymorphism in section Majorana.

Material and Methods

Plant material and DNA extraction

Individual plants of *O. dubium* Boissier, *O. majorana* L., *O. onites* L. and *O. syriacum* L. were collected during excursions to Greece, Turkey, Cyprus and Syria. One accession of *O. syriacum* (SP3) was obtained from the Institute of Field Crops Newe Ya'ar Research Center, Israel. Additional plant material of cultivated *O. majorana* and *Thymus vulgaris* was taken from plants grown from commercial seeds in the greenhouse of the University of Veterinary Medicine, Vienna. Details about the geographic origin as well as identification number of the samples investigated are given in Table 1. Species were identified by following the key of the taxonomic revision of Ietswaart (1980). In case of *O. majorana* s. l. the Flora of Cyprus (Ietswaart, 1985) was used as a second reference to distinguish between *O. dubium* and *O. majorana* s. str. Voucher specimens are kept at the herbarium of the Institute for Applied Botany, University of Veterinary Medicine, Vienna. Genomic DNA was extracted from young, silica gel dried leaves using a modified CTAB extraction procedure (Doyle & Doyle, 1990).

Amplification and sequencing of the γ-terpinene synthase (gaterp) gene of O. syriacum

Primers were developed with Primer Express 2.0 software (Applied Biosystems, Foster City, USA) on the basis of two gaterp gene sequences that were isolated from *O. vulgare* (GenBank accession numbers CS498356.1 and CS498357.1). The following primer-combinations were used to amplify bps 52 to 1745 of the gene (forward and reverse): gaterp52F (5'-GTCAACAACATTGGCGTGAGA-3') and gaterp592R (5'-CTTCGTAAAGCTGCAATACGTCTTT-3'), gaterp465F (5'-CAGACTCCTCA-GACTGCACGG-3') and gaterp910R (5'-CGGCGAGGTTTGAATTAGTCC-3'), gaterp759F (5'-GGCGAGGTGGTTCTTAGATGC-3') and gaterp1366R (5'-GAGAGATAATAGTAGGAGATGAT-ATTGAAACTTT-3'), gaterp1299F (5'-CGCCAACCCTAGCAGAATACC-3') and gaterp1745R (5'-GTTATGAATCTCTGAGTGCTGAACG-3'). The amplification reactions were performed on a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, USA) in 20 µl volumes with the following reaction components: 0.5 µl template DNA (1-50 ng), 18 µl 1.1xReddyMixTM PCR Master Mix (2.5 mM $MgCl_2$) (ABGene, Epsom, UK), 0.5 µl DMSO, 0.2 µl ddH_20 and 0.4 µl (400 nM) of each primer (Invitrogen, Carlsbad, USA). One cycle of amplification with 1 min at 95 °C, 1 min at 58 °C and 2 min at 72 °C were preceded by 35 cycles of amplification with 1 min at 95 °C, 1 min at 58 °C and 2 min at 72 °C by a 3 min denaturation step at 95 °C and followed by an additional 7 min at 72 °C.

All PCR products were checked on 1.4 % agarose gels before being purified with EXO1 and SAP (Fermentas, Burlington, Canada) according to the manufacturer's instructions. Sequencing of both

strands was performed using BigDye Terminators (Applied Biosystems, Foster City, USA) and primers from the original amplifications. The sequences were generated with an ABI 3130x automated sequencer (Applied Biosystems, Foster City, USA) and edited using Chromas Vers. 2.24 (Technelyseum, Tewantin, Australia).

Amplification, cloning and sequencing of a part of the gaterp gene

A part of the gaterp gene was amplified using the primers gaterp465F and gaterp910R. PCR-amplification and sequencing was performed according to the procedure described above.

Nearly all of the directly sequenced PCR products resulted in ambiguous sequences characterized by the appearance of few to many polymorphic sites and/or noise or divergence of the sequence after/to a specific point. In order to isolate intra-individual diverging gamma-terpinene synthase copies, PCR products were subsequently cloned into the pGEM-T easy-cloning vector system (PROMEGA, Madison, USA). Plasmid DNAs were reamplified from 10 to 40 clones for each individual using the original gaterp primers and the same PCR conditions described above. After being checked on 1.4 % agarose gels the PCR products were cleaned with EXO1 and SAP. Purified plasmid DNAs were sequenced in one direction (primer gaterp465F) according to the methods described for the direct sequencing of PCR products.

Sequence analyses

The sequences were aligned using MEGA 4 (Tamura et al., 2007) with subsequent manual correction. Variable positions in the data matrices were checked against the original sequence chromatogram files. To localise the placement of introns gaterp of SP3 was aligned with the cDNA sequences OvTPS2-d06-01 and OvTPS2-f02-04 (GenBank sequences CS498356.1 and CS498357.1).

For the analyses of the gaterp clones the software programs G2CEF and EUKDIS (Goeker and Grimm, 2008) were used. In a first step the clones of each plant accession were manually inspected and classified according to their structure in intron 3. From the aligned sequence data for each sub-group of each accession the relative character frequencies were computed (program G2CEF, FRQ character transformation). In a second step sub-group to sub-group distances were computed from this character matrix (program EUKDIS, distance method: Euclidean distances). A split graph was computed on the resultant distance matrix with SplitsTree 4.8 (Huson and Bryant, 2006) by using the Neighbor-Net algorithm.

Essential Oil analyses

Extraction of plant material (leaves and flowers) as well as GC and GC-MS analyses was performed as described in Lukas et al. (2009). Chemotype classification of samples was done according to the proportions of cymyl-, sabinyl- and acyclic compounds in their essential oils. (Skoula and Harborne, 2002).

Results

Structure of the γ-terpinene synthase gene in Origanum syriacum (SP3)

To identify the genomic organisation of the γ-terpinene synthase gene a large proportion of the gene (bp 52 to 1745 of the 1785 bp containing ORF) was sequenced for one accession of *O. syriacum*. Accession SP3 was chosen for this work as its intra-individual gaterp sequence variability was restricted to a small number of ambigous bases and direct sequencing of the gene was possible. The resultant sequence comprised 2249 bp and included six introns (97, 83, 104, 93, 92 and 92 bp long) (Figure 2). According to its genomic structure (six introns, seven exons) gaterp of *Origanum* can be classified as class III-type TPS gene (Trapp and Croteau, 2001).

The gaterp protein sequence from *O. syriacum* (SP3) was aligned with the gaterp protein sequence of *O. vulgare* (OvTPS2-d06-061 and OvTPS2-f02-04) (Figure 2) and the percentage of identity was calculated for the region comprising residues 18 to 579. In this region gaterp of SP3 shares 99.1 % AS identity with OvTPS2-d06-01 and 99.2 % AS identity with OvTPS2-f02-04.

Diversity and phylogenetic relationships of cloned gaterp sequences

The cloned and sequenced gene region comprises the complete intron 2, exon 3, intron 3 and a small part of exon 4 of the gaterp gene. A total of 444 gaterp clones from 30 accessions of *Origanum dubium* (8), *O. majorana* (4), *O. onites* (10), *O. syriacum* (3), *O. vulgare* (4) and *Thymus vulgaris* (1) were generated for the analyses. The number of clones per individual plant ranged between 8 and 31. Three accessions of cultivated *O. majorana* were directly sequenced and included in the alignment. The length of the cloned sequences ranged from 290 to 582 base pairs (bp), the alignment of all gaterp sequence positions resulted in a matrix of 628 characters. Intron 2 accounted for bp 1 to 118, intron 3 for bp 505 to 617 of the alignment. A number of clones of accessions of *O. dubium*, *O. onites* and *O. syriacum* lacked bp 246 (located in intron 2) to bp 523 (located in exon 3) indicating the presence of a non-functional gaterp pseudogene. 408 of the 628 sequence characters were variable, 267 of them were potentially parsimony-informative.

In most accessions investigated high intra-individual gaterp sequence heterogeneity was found. Initial phylogenetic analyses including intron and exon sequence data revealed clusters of clones that did not correspond to taxonomic entities but mainly to sequence characteristics that were present in intron 3 (data not shown). On closer inspection of the sequence alignment in the clones six different nucleotide patterns became clearly obvious by their indel structure and although point mutations characterized species or individuals the overall structure of the six patterns was conserved in plant individuals belonging to species of section Majorana and to the distantly related species *O. vulgare* (section Origanum) but they were not found in the accession of *Thymus vulgaris* that was chosen as outgroup species. In order to simplify the illustration of phylogenetic relationships the clones of each plant accession were manually inspected and grouped according to their indel structure in intron 3. For each of these sub-groups the relative character frequencies were calculated from the aligned sequences and the resulting character matrix was used to compute distances between the sub-groups. In the resulting

network (Figure 3) two distinct clusters were obvious. Intron pattern 1 (all sub-groups representing cloned sequences that exhibited intron pattern 1) is clearly separating whereas intron patterns 2, 3, 4 and 5 seem to be more closely related. Within the latter cluster intron pattern 5 has a somehow distant position whereas intron patterns 2, 3 and 4 cluster closely together. Intron patterns 3 and 4 are not well separated and one sub-group that represents sequences with intron pattern 4 of an accession of *O. syriacum* (SP3) clusters together with sub-groups that represent cloned sequences that exhibited intron pattern 3. The putative pseudogene (PG) seems to derive from gaterp with intron pattern 2. Within the clusters described no clear grouping according to taxonomic entities was observed, however, sub-groups representing sequences that were isolated from *O. vulgare* appear always slightly separated from sub-groups that are based on clones that were isolated from the four species of section Majorana.

Appearance of the intron 3 patterns in individual plants

As the strongest phylogenetic signal came from the sequence characteristics that were found in intron 3 their presence/absence in *O. dubium*, *O, majorana*, *O. onites*, *O. syriacum* and *O. vulgare* was analysed (Table 1). In clones from accessions of *O. dubium* all five intron patterns were found. The co-occurrence of pattern 1 and 4 was frequent in this species and was present in plants collected in Cyprus (SR1028, SR1054 and SR1200, in the latter one accompanied by pattern 5) and Turkey (SR603 and SR684). Two Turkish accessions (SR532 and SR540) exhibited clones with pattern 2 and additionally possessed the putative pseudogene. From one Turkish accession of this species (SR745) clones with pattern 1, 2 and 3 were isolated together with the pseudogene. *O. majorana* was the only species in this investigation that possessed only one intron pattern, intron pattern 5 that was infrequent in the other species. This pattern was found in three wild accessions of *O. majorana* as well as in three different commercial cultivars of this species. In *O. onites* from most accessions clones that exhibited pattern 1 and pattern 3 were isolated, sometimes accompanied by clones with pattern 2 and/or pattern 4 or pattern 5, respectively. Two Turkish accessions of this species (SR498 and SR 528) were exceptional as only clones with pattern 2 were found together with clones that exhibited the pseudogene characteristics. In the species *O. syriacum* pattern 4 was the main pattern in the clones. From one accession (SR326) additionally clones with pattern 1 were isolated, in a second (SR438) also clones with the pseudogene characteristics were present. In the species *O. vulgare* in the four accessions analysed different combinations of intron patterns were detected. In SR3 only clones with pattern 3 were found. Accession d-06-01 exhibited clones with pattern 1 and 3, accession f-02-04 clones with pattern1, 2 and 5. From accession SR472 clones with pattern 2 and 5 were isolated.

Correlation of intron 3 patterns with chemotypes of individual plants

Surprisingly, in accessions of the four species of section Majorana the presence or absence of specific patterns in intron 3 is correlated with the presence or absence of certain monoterpenes in the essential oils of the respective accession (Table 1). All plant individuals of *O. majorana* and cultivated *O. majorana* exhibited exclusively clones with pattern 5 and their essential oil was mainly made up of 'sabinyl'-compounds (e. g. *cis*-sabinene hydrate, *cis*- and *trans*-sabinene hydrate acetate) but lacked

typical compounds of the 'cymyl'-pathway (e. g. p-cymene, γ-terpinene, thymol, carvacrol) and acyclic compounds (e. g. linalool, linalool acetate). From two accessions of *O. onites* (SR498 and SR 528) and *O. dubium* (SR532 and SR540) only clones with pattern 2 were isolated together with clones that indicate the presence of a gaterp pseudogene. These accessions were exceptional as they produced large amounts of the acyclic compound linalool (70 – 80 % of their essential oils) whereas 'cymyl'-compounds and 'sabinyl'-compounds were not detected or were found in traces only. As soon as intron pattern 1 and/or 3 and/or 4 were present in a plant the essential oils contained 'cymyl'-compounds. In most cases (with the exception of SR745 and SR792) the 'cymyl'-compounds were the main essential oil compounds whereas 'sabinyl'-compounds and acyclic compounds were not detected or were found in traces only. In the species *O. vulgare* the situation was similar and in the essential oils of a plant 'cymyl'-compounds were only present when gaterp with intron pattern 1 and/or 3 and/or 4 was isolated.

Discussion

Intra-individual and intra-specific gaterp sequence variability

High intra-individual gaterp variability was observed and in most accessions the number of clones sequenced corresponded to the number of different clones. This would lead to the conclusion that in *Origanum* gaterp is encoded by a veritable multigene family. Nevertheless, cloning is known to be error-prone and to some degree the sequence variability that was found in the clones may be due to Taq polymerase errors and artificial recombination during the process of PCR and cloning (e. g. Keohavong and Thilly 1989; Cronn et al., 2002). As sequence mutations that are based on true genetic variation can not unambigously be distinguished from such artefacts, an interpretation of intra-individual gaterp diversity, without further analysis that address the gaterp gene copy number, is difficult. However, when considering the conspicious sequence patterns that were obvious in intron 3 there is strong evidence for at least six different gaterp variants, either alleles or separate genes, in the genus *Origanum*. Alignment characteristics indicated allelic variation and the presence of more than three gaterp genes can be assumed.

Up to now, only limited information is available about the chromosomal organisation of terpene synthases. In *Arabidopsis thaliana* Aubourg et al. (2002) discovered six putative monoterpene synthases that were localised on chromosome 2, chromosome 3 (three of them tandemly organised) and chromosome 4 (two of them tandemly organised). In the monoterpene synthase cluster on chromosome 3 they found evidence for recent gene duplication. The five intact gaterp variants of the genus *Origanum* exhibited a high degree of sequence identity in exon 3. Probably all six variants derive from one locus that comprises a small number of clustered, tandemly organized gaterp genes that originated from gene duplication.

The gaterp variants with intron pattern 1, 2, 3 and 5 were found in the four closely related species of section Majorana as well as in the distantly related species *O. vulgare*. As *O. syriacum*, *O. onites* and

O. vulgare are considered to be ancient species in the genus *Origanum* (Ietswaart, 1980) these variants must have been present from the early beginnings of the genus. Little genetic divergence was observed between clones of a variant, even between clones of distantly related species.

Within section Majorana gaterp variants or combinations of gaterp variants were not restricted to one of the taxonomic entities but within the four species certain variants or their combinations seem to be more abundant. This is especially visible in *O. majorana* that possessed exclusively the variant that is based on intron pattern 5. In *O. dubium*, for example, the combination of gaterp with intron pattern 1 and 4 was found in five of the eight accessions analysed. Hybridisation is a frequent phenomenon in the genus *Origanum* and could be responsible for the complex combinations of variants in individual plants. Within section Majorana the most complex mixtures of gaterp variants were found in *O. onites* and *O. dubium*, two species that are suspected to hybridize. *O. onites* seem to be basically equipped with gaterp that exhibit intron patterns 1 and 3 but additionally possessed the variant with intron pattern 4 that was frequently found in *O. dubium* and *O. syriacum*. One accession of *O. dubium* showed gaterp with pattern 3 that seems to derive from the species *O. onites*. In accessions of both species the gaterp variant with intron pattern 2 was present that did occur neither in *O. majorana* nor in *O. syriacum*.

Occurrence of gaterp variants - correlation with main chemotypes in the species of section Majorana

Three main essential oil chemotypes are present in the four species of section Majorana. Large quantities of 'cymyl'-compounds (mainly γ-terpinene and carvacrol) are responsible for the well-known, pungent oregano flavour whereas in sweet marjoram 'sabinyl' compounds (mainly cis-/trans-sabinene and cis-sabinene hydrate acetate) represent the bulk of the essential oils and acyclic phenolic compounds are predominant in the linalool type. Three independent pathways seem to be responsible for the accumulation of the respective main compounds from their common precursor GPP (Poulose and Croteau, 1978; Croteau, 1987; Pichersky et al., 1995), but only restricted knowledge exists about the genetic background responsible for this essential oil variability. From the results of this investigation it can be deduced that a plants' equipment with γ-terpinene synthase gene variants might play a crucial role. The accumulation of 'cymyl'-compounds was always associated with the occurrence of gaterp variants 1 and/or 3 and/or 4. When solely gaterp variant 2 (in combination with the putative pseudogeneous variant 6) or variant 5 were isolated from a plant, 'cymyl'-compounds were completely absent or present in trace amounts only. As only a part of the γ-terpinene synthase gene was sequenced and gene expression as well as biochemical data is not available the background for the observed genotype/chemotype correlation remains unclear. With variant 6 the presence of a putative gaterp pseudogene in *Origanum* was already demonstrated. Gaterp variant 2 and 5 could also be affected by sequence mutations interfering with transcription or enzyme structure. Already single nucleotide changes may greatly reduce the activity of the encoded enzyme as it was shown for a sesquiterpene synthase of *Zea mays* (Köllner et al., 2004). However, it would also be possible that

variant 2 or 5 represent intact genes that are simply not expressed in leaves and flowers of mature plants.

In most accessions that exhibited variants 1 and/or 3 and/or 4 ‘cymyl’-compounds represented the bulk of the essential oils and compounds from the ‘sabinyl’- and linalool-pathway were (almost) absent. Nevertheless, the presence of one or more of these gaterp variants in a plant did not consistently result in an almost pure ‘cymyl’-chemotype. Four accessions (SR745, SR792, d06-01 and f02-04) that carried one or more of the ‘active’ variants produced mixed essential oil chemotypes containing ‘cymyl’-compounds and linalool or ‘sabinyl’-compounds, respectively. Two of these exceptional individuals (SR792 and d06-01) carried the same gaterp variants as accessions that exhibited the oregano-typical ‘cymyl’-chemotype (SR1299 and SR1372). This indicates that beside molecular variation of the γ-terpinene synthase gene other factors control the qualitative composition of the *Origanum* essential oils. Further investigations are needed to learn more details about the complex regulatory mechanism responsible for the chemical polymorphism observed.

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Table 1: Species, identification number and geographical origin (comm. = commercial origin; unkn. = geographical coordinates or elevation unknown) of the accessions studied. Appearance of gaterp gene variants in *O. dubium*, *O. majorana*, *O. onites*, *O. syriacum* and *O. vulgare* (figures in brackets refer to the number of clones that exhibited the respective intron pattern) and percentages of 'cymyl'-compounds, 'sabinyl'-compounds and linalool in the essential oils of the accessions studied (S = 'sabinyl'-compounds, C = 'cymyl'-compounds, L = linalool; + 5 % - 10 %; ++ 10 % - 50 %, +++ > 50 %).

		geographical origin				gaterp variant						EO chemotype		
species	ID	country	N	S	s. l.	1	2	3	4	5	6 (PG)	S	C	L
O. dubium	SR540	TU	36°56'9.2"	31°28'34.1"	unkn.	-	X (8)	-	-	-	X (4)	-	-	+++
	SR532	TU	"	"	"	-	X (12)	-	-	-	X (7)	-	-	+++
	SR603	TU	36°29'47.2"	32°07'13.5"	104 m	X (1)	-	-	X (12)	-	-	-	+++	-
	SR684	TU	36°09'4.1"	32°25'28.4"	20 m	X (4)	-	-	X (11)	-	-	-	+++	-
	SR745	TU	36°01'57.6"	32°46'12.4"	253 m	X (1)	X (3)	X (3)	-	-	X (5)	-	+	+++
	SR1028	CY	35°05'31.2"	32°32'31.8"	153 m	X (2)	-	-	X (12)	-	-	-	+++	-
	SR1054	CY	"	"	"	X (4)	-	-	X (11)	-	-	-	+++	-
	SR1200	CY	35°01'42.9"	32°36'50.3"	878 m	X (1)	-	-	X (8)	X (1)	-	-	+++	-
O. majorana	H44	CY	unkn.	unkn.	unkn.	-	-	-	-	X (27)	-	+++	-	-
	SR849	CY	35°04'54.1"	32°17'36.9"	unkn.	-	-	-	-	X (16)	-	+++	-	-
	SR998	CY	34°58'36.4"	32°28'23.0"	164 m	-	-	-	-	X (15)	-	+++	-	-
	SR1087	CY	34°55'43.6"	32°26'32.5"	547 m	-	-	-	-	X (14)	-	+++	-	-
O. majorana cult.	Malta	comm.	-	-	-	-	-	-	-	X	-	+++	-	-
	Mira2/3	comm.	-	-	-	-	-	-	-	X	-	+++	-	-
	Obi	comm.	-	-	-	-	-	-	-	X	-	+++	-	-
O. onites	SR498	TU	36°56'9.2"	31°28'34.1"	unkn.	-	X (7)	-	-	-	X (1)	-	-	+++
	SR505	TU	"	"	"	X (8)	X (1)	X (3)	-	-	-	-	+++	-
	SR512	TU	"	"	"	X (6)	-	X (3)	-	X (2)	-	-	+++	-
	SR528	TU	"	"	"	-	X (21)	-	-	-	X (10)	-	-	+++
	SR691	TU	36°05'56.8"	32°32'02.8"	135 m	X (6)	-	X (9)	-	-	-	-	+++	-

		geographical origin				gaterp variant						EO chemotype		
species	ID	country	N	S	s. l.	1	2	3	4	5	6 (PG)	S	C	L
	SR724	TU	"	"	"	X (6)	-	X (3)	X (5)	-	-	-	+++	-
	SR792	TU	37°16'24.3"	31°12'50.2"	817 m	X (2)	X (5)	X (3)	X (1)	-	-	-	+	+++
	SR1299	GR	36°51'05.5"	22°39'16.2"	9 m	X (4)	X (1)	X (6)	X (1)	-	-	-	+++	-
	SR1339	GR	36°40'13.3"	23°01'47.5"	10 m	X (6)	X (2)	X (4)	-	-	-	-	+++	-
	SR1372	GR	37°43'49.9"	22°45'22.8"	unkn.	X (5)	-	X (7)	-	-	-	-	+++	-
O. syriacum var. unkn.	SP3	IS	unkn.	unkn.	unkn.	-	-	-	X (31)	-	-	-	+++	-
O. syriacum var. *bevanii*	SR326	SY	36°30'04.2"	36°44'46.0"	598 m	X (2)	-	-	X (8)	-	-	-	+++	-
	SR438	SY	34°47'09.7"	36°09'28,9"	270 m	-	-	-	X (2)	-	X (5)	-	+++	-
O. vulgare	d-06-01	unkn.	unkn.	unkn.	unkn.	X (6)	-	X (9)	-	-	-	++	++	-
	f-02-04	unkn.	unkn.	unkn.	unkn.	X (1)	X (5)	-	-	X (8)	-	++	++	-
	SR3	VP	42°49'00.8"	9°25'36.3"	0 m	-	-	X (9)	-	-	-	-	+++	-
	SR472	AU	unkn.	unkn.	unkn.	-	X (2)	-	-	X (6)	-	-	-	-
Thymus vulgaris	-	comm.	-	-	-	-	-	-	-	-	-	unkn.	unkn.	unkn.

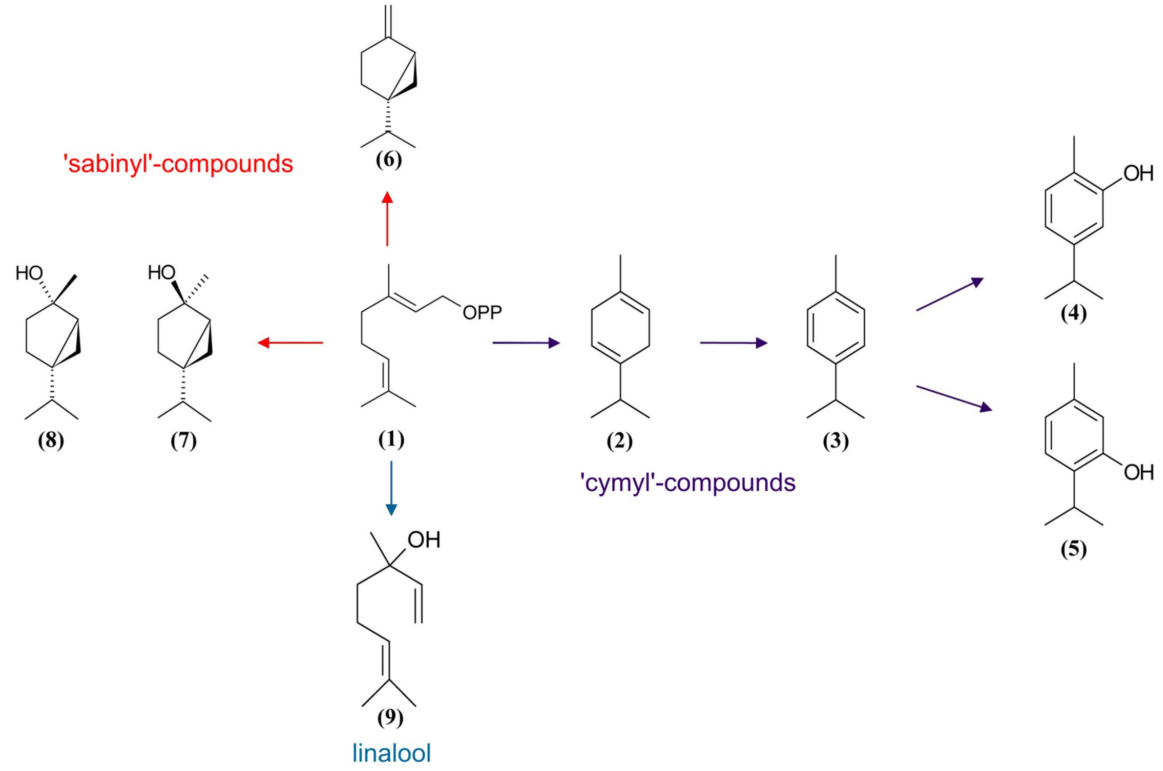

Figure 1: The biosynthetic pathways that are predicted to be responsible for the production of 'cymyl'-compounds, 'sabinyl'-compounds and linalool from their common precursor ((1)…GPP (geranylpyrophosphate), (2)…γ-terpinene, (3)…*p*-cymene, (4)…carvacrol, (5)…thymol, (6)…sabinene, (7)…*cis*-sabinene hydrate, (8)…*trans*-sabienen hydrate, (9)…linalool).

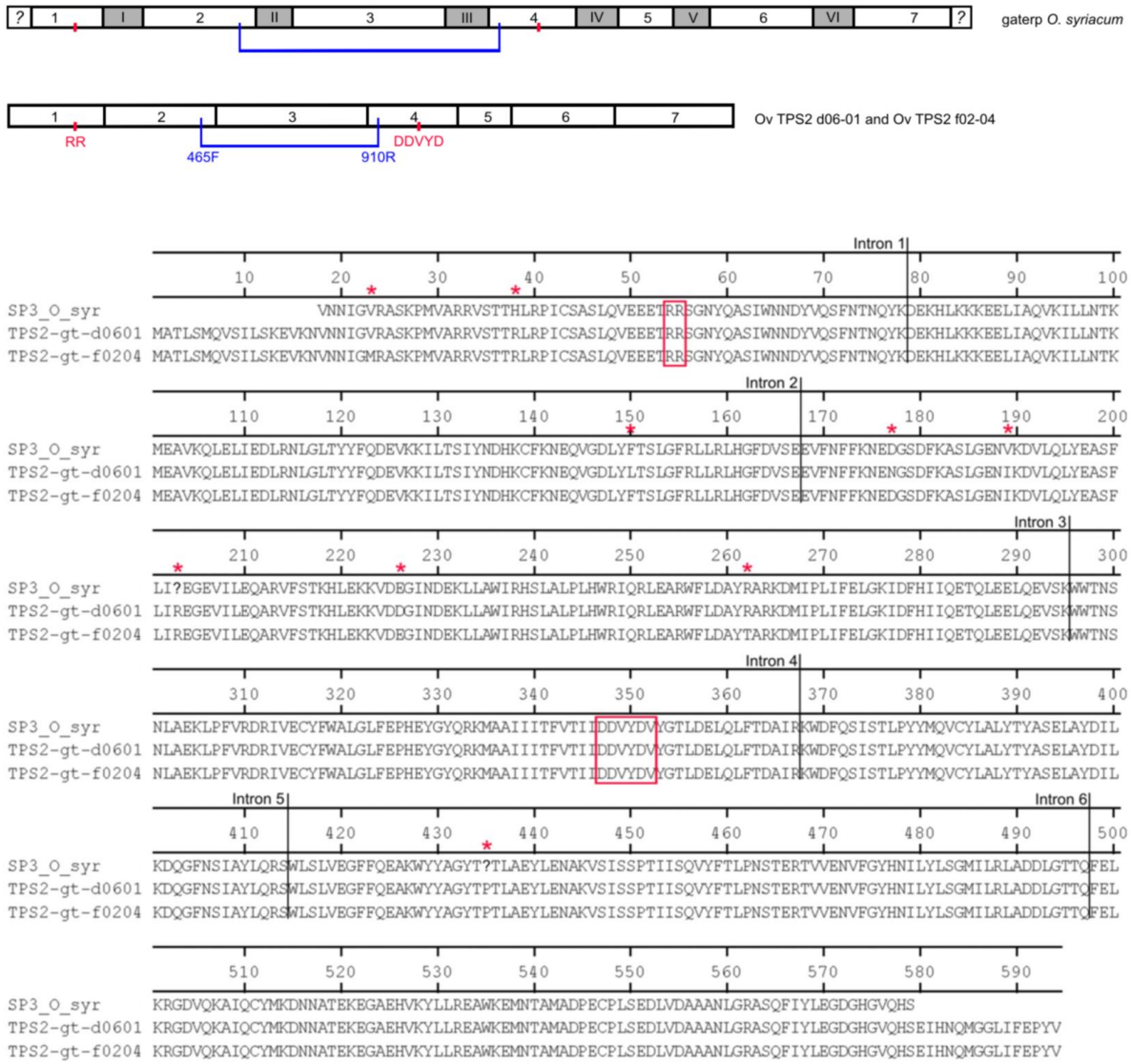

Figure 2: Genomic structure of the gaterp gene in *Origanum syriacum* (SP3). Question marks refer to the non-sequenced part of the gene. Light areas with Arabic numerals indicate exons, dark areas with Roman numerals indicate introns. Box sizes refer to the length of exons and introns. The cloned gaterp region is indicated by the blue bracket (465F to 910R). The two motifs, conserved in all plant terpene synthases (RR and DDxxD), are indicated in red (top). Amino Acid Sequence Alignment of the γ-terpinene synthase of *O. syriacum* with γ-terpinene synthase isolated from *O. vulgare* (OvTPS2 d06-01 and OvTPS2 f02-04). Asterisks refer to heterogeneous AS sequence positions (bottom).

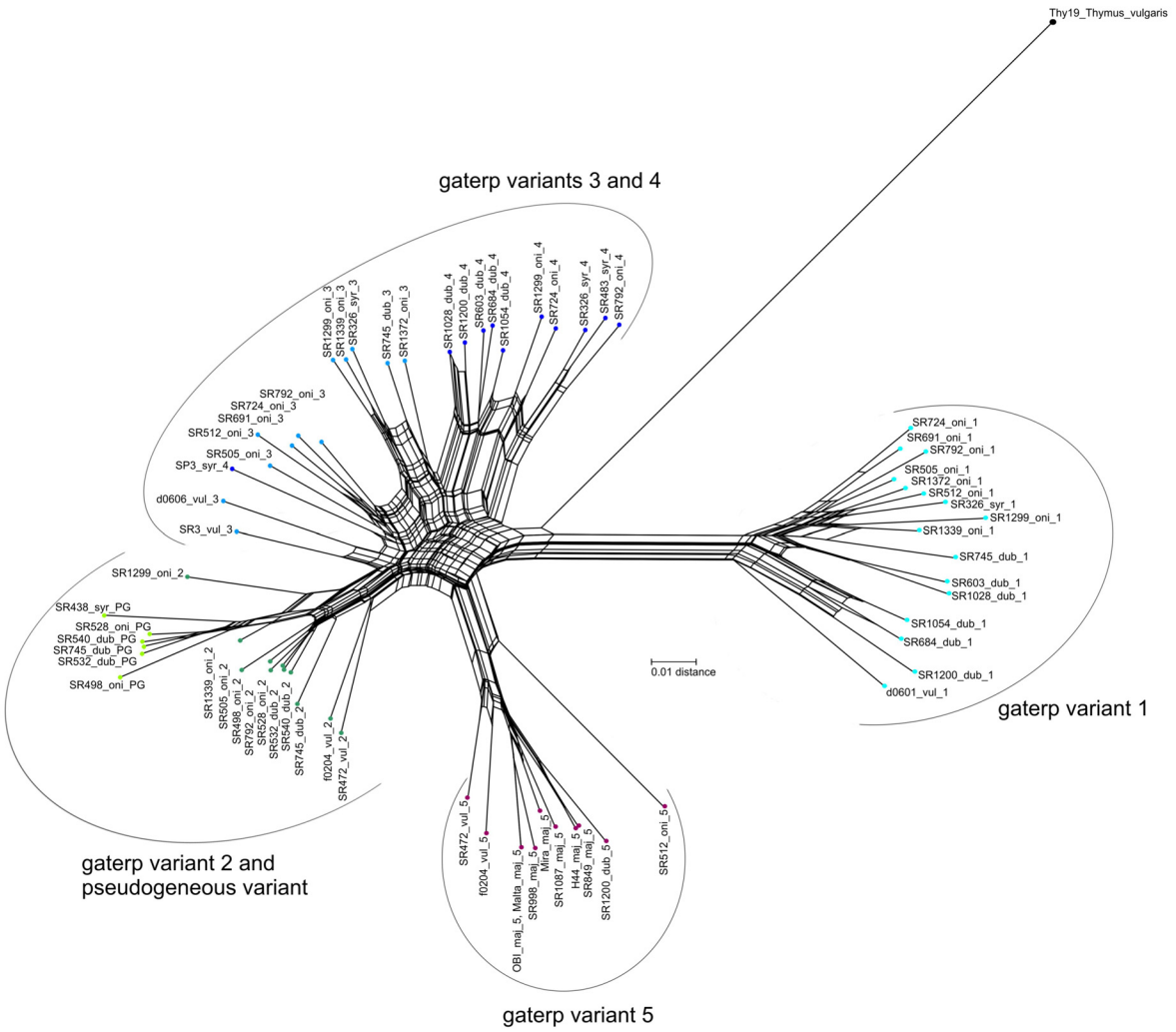

Figure 3: Neighbor-Net splits graph of gaterp from *O. dubium* (dub), *O. majorana* (maj), *O. onites* (oni), *O. syriacum* (syr), *O. vulgare* (vul) and *Thymus vulgaris*. Arabic numerals (1-5) refer to the five different gaterp variants, PG indicates the pseudogeneous gaterp variant.

ANNEX TO PART 1

Identification and characterization of simple sequence repeat markers from a glandular *Origanum vulgare* expressed sequence tag

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PERMANENT GENETIC RESOURCES

Identification and characterization of simple sequence repeat markers from a glandular *Origanum vulgare* expressed sequence tag

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Abstract

Oregano (*Origanum vulgare*) and marjoram (*Origanum majorana*) are two sensorial distinct spices within the genus *Origanum* (Lamiaceae). Simple sequence repeat (SSR) markers were developed from expressed sequence tags (ESTs) of essential oil glands of *O. vulgare*. Thirteen EST-SSR loci were evaluated using 20 individual plants of *O. vulgare* and 19 plants of *Origanum majorana*. The number of alleles per locus ranged from one to four. All loci developed from *O. vulgare* successfully cross-amplified in *O. majorana*.

Keywords: EST, marjoram, oregano, *Origanum majorana* L., *Origanum vulgare* L., SSR

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The genus *Origanum* (Lamiaceae) contains two important aromatic plants with different sensorial qualities, marjoram and oregano. The aromatic quality of marjoram is to be found in one species in the section *Majorana* only (*Origanum majorana*). In contrast to marjoram, the quality of oregano arises from many different species, even from other genera of the Lamiaceae and other plant families. The best qualities of oregano, however, are coming from different subspecies of *Origanum vulgare*, from *O. onites* and *O. syriacum* (Baser *et al*. 1993).

In both, marjoram and oregano, an essential oil containing mono- and sesquiterpenes is responsible for their use as aromatic plants. The bicyclic monoterpene *cis*-sabinene hydrate and its acetate derived from the biosynthetical 'sabinyl' pathway are responsible for marjoram's quality, while the phenolic monoterpene carvacrol, arising from the 'cymyl pathway' (Skoula *et al*. 1999), is the typical compound of oregano. Recently, in an assessment to study terpene synthases, an expressed sequence tag (EST) library was developed from epidermal gland cells that are responsible for the production and storage of mono- and sesquiterpenes (Crocoll *et al*. 2007).

The development of SSR markers is often time-consuming and labour-intensive. Therefore, ESTs are an ideal starting point to develop simple sequence repeat (SSR) markers quickly (Scott 2001). Epidermal glands of *Origanum vulgare* were separated from leaves and 2031 ESTs were generated from these glands (Crocoll *et al*. 2007). Repeats were identified with TANDEM REPEATS FINDER (Benson 1999). Primer pairs were designed using PRIMER EXPRESS 2.0 (Applied Biosystems). The SSR primers were tested on 20 individual plants from a population of oregano (*Origanum vulgare* ssp. *hirtum*), cultivated from seeds collected in the wild, and on 19 individual plants from an inbreeding line of marjoram (*Origanum majorana*) originating from a marjoram hybrid development (Novak *et al*. 2002).

The DNA amplifications were carried out in 15 μL reactions containing template DNA, 0.6 μM forward and reverse primers, 1.5 mM $MgCl_2$, 100 μM dNTPs, 1× *Taq* buffer (1× reaction buffer BD (Solis BioDyne) and 0.6 U of *Taq* HOT FIREPol polymerase (Solis BioDyne). The polymerase chain reaction (PCR) cycling profile started with an initial denaturation at 95 °C for 15 min followed by 35 cycles of 1 min at 95 °C, 1 min at 59 °C and 2 min at 72 °C, with a final extension step of 9 min at 72 °C. PCR products were analysed using silver-stained 10% polyacrylamide gels.

In total, 47 repeats were identified in the EST-library (2.3%). No primers could be developed for 15 repeats. Of the remaining repeats, 13 showed polymorphisms in oregano and were also evaluated in marjoram.

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Table 1 Characteristics of 13 EST-SSR markers, observed heterozygosity (H_O), expected heterozygosity (H_E) and fixation index (F) of oregano (*Origanum vulgare* ssp. *hirtum*, *n* = 20) and marjoram (*Origanum majorana*, *n* = 19)

				Oregano				Marjoram			
Locus	Primer sequence	Repeat motif	Allele size (bp)	No. of alleles	H_O	H_E	F	No. of alleles	H_O	H_E	F
OR09	F: TTGAAGCATTGTTGGAGGTAGATG R: TCCCAACTAGGGAGAAATGTGC	$(TTTTTC)_4(T)_5(TTTTTC)_1$	148–177	2	0.25	0.40	0.37	1	0.00	0.00	—
OR10	F: TTTGCTCCGACATCTTCAACC R: AGCCTGCTGTGTTTGGATCAG	$(ACC)_1ATC(ACC)_4$	113–118	2	0.50	0.48	–0.04	2	0.05	0.05	–0.03
OR12	F: GCCCCTGCAGTGACTCCTAC R: AAAAAGGCTTCGGACTCGATC	$(AG)_7G(AG)_3$	119–123	2	0.15	0.14	–0.08	3	0.40	0.33	–0.22
OR13	F: GAGAGAATCCAAGCCTCCGC R: TGAAGGAGTCCGATGTTGACG	$(AAC)_7AGC(AAC)_1$	135–144	3	0.55	0.51	–0.09	2	0.05	0.05	–0.03
OR14	F: TGTTTGGTGGAAACCGATCC R: AGACGACGAGCTCCAATAACG	$(GAT)_8$	83–92	3	0.45	0.49	0.07	2	0.05	0.05	–0.03
OR27	F: TCAGAAACAATGAAGGCCGC R: CCGTACAGGTCAAACACCGG	$(CCT)_6$	100–119	3	0.25	0.31	0.18	1	0.00	0.00	—
*OR32**	F: TCTTGCCAATTTATGCGTGTTC R: GAAACAAGCATCTTTTCCTGAATTC	$(AG)_6GG(AG)_2GA(AG)_5GG(AG)_1$	108–112	2	0.00	0.54	1.00	2	0.05	0.05	–0.03
OR40	F: GCCCAAGGACATCCAACTTG R: CAACTGAACACCTCCCACAATG	$(GGT)_4GTT(GGT)_1$	122–131	3	0.45	0.54	0.16	3	0.50	0.61	0.18
OR44	F: TCAAGGGTAGAGCTGCTGCAG R: GCTTTACGGAGGAAGAATGGG	$(GAT)_3GAA(GAT)_4$	148–156	2	0.15	0.14	–0.08	1	0.00	0.00	—
OR64	F: TCCCGCCTTCAAGAAATGAC R: AGAGAGCACGTTGATGAACCG	$(AAG)_1A_3(AAG)_6A$	74–81	3	0.60	0.67	0.10	3	0.40	0.36	–0.11
OR75	F: CAAGAAGAATAACGGAGGAGCAG R: TGGAGAATTTCTGATGCTCGG	$(GCA)_6$	88–100	4	0.40	0.43	0.07	2	0.25	0.29	0.13
OR77	F: TGAAGTCAGTTTGGATGATGGTG R: GTCACGTATGGAATGCACGG	$(GGT)_6$	115–120	2	0.30	0.26	–0.15	2	0.35	0.44	0.20
*OR81**	F: GAAGTTCGCCGAGGCTCTC R: CAAAGCACAAGAAAATACAATAGCAC	$(CTTTT)_3AT_8(CTTTT)_1TT(CTTTT)_1$	147–157	3	0.20	0.42	0.52	1	0.00	0.00	—

*Markers deviating significantly from the Hardy-Weinberg equilibrium.

Observed and expected heterozygosities as well as the fixation index were calculated with GENALEX 6 (Peakall & Smouse 2006). Hardy–Weinberg equilibrium and genotypic disequilibrium between pairs of microsatellites were calculated with GENEPOP version 3.4 (Raymond & Rousset 1995; Markov chain Monte Carlo parameters: 10 000 dememorization steps, 100 batches and 5000 iterations per batch). Sequential Bonferroni correction (Rice 1989) was applied for all multiple tests.

The number of alleles for oregano ranged between two and four (Table 1). The observed and expected heterozygosities ranged from 0.00 to 0.60, and from 0.14 to 0.67, respectively. No linkage between all loci could be observed. The fixation index indicated for all but two loci (*OR32*, *OR81*) random mating. The two exceptional loci had high positive values of 1.00 and 0.52, respectively, possibly indicating undetected null alleles. Significant deviations from Hardy–Weinberg equilibrium were observed for the same two loci (*OR32* and *OR81*) and both loci showed a deficiency of heterozygotes.

All oregano loci cross-amplified in marjoram. Only five loci were polymorphic with one to three alleles and observed and expected heterozygosities ranging from 0.25 to 0.50, and from 0.29 to 0.61, respectively. As in oregano, no linkage between the loci could be observed. The fixation index indicated random mating for all loci. A significant deviation from Hardy–Weinberg equilibrium existed for *OR40*. No deficiencies of heterozygotes were observed.

The 13 microsatellite loci from *O. vulgare* present a first set of SSR markers for the genus *Origanum*. The SSR markers presented here may also be useful in other closely related Lamiaceae like mint, thyme, sage and savory which are valuable medicinal and aromatic plants. Because many of these species are still collected from the wild in tremendous quantities (Gilbert 1997), the markers can be used to control the conservation of genetic resources. Further applications are breeding (Franz & Novak 1997) and evolutionary studies of the genus *Origanum* and related Lamiaceae.

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A Strategy to Setup Codominant Microsatellite Analysis for High-Resolution-Melting-Curve-Analysis (HRM)

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Methodology article

A Strategy to Setup Codominant Microsatellite Analysis for High-Resolution-Melting-Curve-Analysis (HRM)

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Abstract

Background: High resolution melting curve analysis (HRM) is a technique that measures exactly the decreasing fluorescence of intercalating dye in the process of dissociation of double stranded DNA. The measurement is immediately following PCR in a one-step, closed-tube method. The shape of the melting curve depends on the GC content, length and sequence of the amplicon. Hence it is a powerful, fast and cheap method to detect Single Nucleotide Polymorphisms (SNPs) and other mutations.

Results: Here we present a strategy to set up microsatellite analysis for HRM including the correct assignment of heterozygous samples by comparative analysis and artificial mixtures of samples. The approach is demonstrated on two Simple Sequence Repeat (SSR) loci of different complexity in the genus *Origanum*. Following this strategy all alleles of our sample sets could be classified correctly.

Conclusion: HRM can be used in microsatellite analysis and other codominant marker systems implementing a protocol of comparative melting curve assignment with artificial mixtures of samples to overcome difficulties in correctly assigning heterozygous samples. The method is faster, more sensitive and cheaper than standard protocols for microsatellite analysis.

Background

High resolution melting curve analysis (HRM) has been introduced several years ago, extending the possibilities of the analysis of DNA melting curves, a standard diagnostic feature in qPCR [1], towards a sensitive method in detecting mutations. The main field of application is the reliable and fast determination of SNPs [2], where the sensitivity of the method has already been widely demonstrated [3]. It is also used for assessment of DNA methylation [4]. HRM also proved applicable for larger sequence aberrations such as Internal Tandem Duplications (ITDs) [5]. It was used for the detection of unknown mutations in a region of low complexity [6] and recently also for analysis of microsatellites [7].

HRM can be seen as an offspring from qPCR technology, where fluorescent dyes are used to detect the quantity of double stranded DNA during the PCR. At the end of the PCR, the fluorescence signal level is high, due to the high amount of dsDNA with intercalated fluorescence dye accumulated in the process. Primer dimers and other non-specific products can subsequently be detected in most qPCR instruments by melting the dsDNA and a stepwise measurement of the decreasing fluorescence. The distance

between T_m values allows the discrimination of the targeted amplicon and non-specific products [1]. Technological progress facilitated a decrease of the size of temperature steps between fluorescence measurement points in the melting process, generating more data and thus drawing a high resolution curve of the level of fluorescence. The HRM curves obtained are highly characteristic for each amplicon and depend on GC content, amplicon length and sequence [8].

Variation in microsatellite loci is often visualized by electrophoresis on polyacrylamide gels, which is principally sensitive only to amplicon-length. Gel-electrophoresis is not only time-consuming, but may also lead to problems in interpretation due to stutter bands. The nowadays common method of microsatellite analysis with multiplex PCR and capillary electrophoresis affords the use of labeled primers, which have to be optmized for multiplex PCR. The problem of stutter peaks also occurs and the method is still quite costly offside routine analysis with well known organisms and markers. Above this it requires at least one additional step following PCR.

Here we describe a strategy to develop a co-dominant microsatellite marker system in diploid organisms using HRM with the advantages of visualization immediately following PCR, low costs and higher sensitivity than electrophoretic detection systems.

Microsatellite primers for *Origanum vulgare*, which have also cross-amplified in other species of the genus *Origanum* [9] are used to demonstrate the strategy.

Methods

PCR and HRM were performed on a RotorGene 6500 (Corbett Research Pty Ltd, Sydney, Australia) with a HRM-module, the results were analysed using the RotorGene 6000 series software, Version 1.7.65. To obtain interrun comparability standard samples were used in every run.

DNA was extracted from dried leaves of *Origanum* using a modified CTAB extraction protocol [10]. The samples represent a subset from a study on natural hybridizations in the section *Majorana* of the genus *Origanum* (data in preparation). The individuals tested were taken from populations in southern Turkey, sample set 1 consists of 33 individuals of *Origanum onites* (from nearby Manavgat, province of Antalya), sample set 2 comprises 27 individuals of *Origanum majorana* (same location). Sample set 3 is a mixed set of 22 individuals from a total of 11 populations from Southern Turkey (*Origanum onites* and *Origanum majorana*).

The 10 μl PCR reaction volume contained 5.6 μl H_2O dest., 0.4 Units Taq HOT FIREPol® polymerase, 1 μl Buffer B (Solis BioDyne, Tartu, Estonia), 1.4 μl MgCl2 (25 mM), 0.2 μl DMSO (14.08 M), 0.1 μl dNTP-mix (10 mM), 0.1 μl of each Primer (10 μM) and 0.6 μl fluorescent dye BEBO 36 μM (TATAA Biocenter [11]). 1 μl DNA solution was added to each reaction, containing between 0.25 and 0.8 ng/μl DNA. The mixed samples also contained 1 μl DNA solution with the two compounds at a ratio of 1:1 or 1:2. All reactions were done in duplicate.

The PCR cycling started with an initial phase of 15 min (for the Taq HOT FIREPol® polymerase) at 95°C, then 40 cycles of 10 s at 95°C, 20 s at 60°C and a 20 s elongation step at 72°C. High resolution melting was carried out immediately following PCR from 70°C to 90°C at steps of 0.05°C, each step with a 1 s hold.

For the locus SSR214 the forward primer is 5'-TGTTTGGTGGAAACCGATCC-3', the reverse primer is 5'-AGACGACGAGCTCCAATAACG-3', and the repeat motif is GAT. For the locus SSR244 the forward primer is 5'-TCAAGGGTAGAGCTGCTGCAG-3', the reverse primer is 5'-GCTTTACGGAGGAAGAATGGG-3', and the repeat motif is (GAT)3GAA(GAT)4. [9]

For verification 20 samples were selected for sequencing. Their PCR products were checked on 1.4% agarose gels before being purified with EXO1 and SAP (Fermentas GmbH, St. Leon-Rot, Germany) according to the manufacturer's instructions. Sequencing of both strands was performed using BigDye Terminators (Applied Biosystems, Brunn am Gebirge, Austria) and primers from the original amplifications. The sequences were generated with an ABI 3130x automated sequencer (Applied Biosystems, Brunn am Gebirge, Austria) and edited using Chromas Vers. 2.24 (Technelyseum, Tewantin, Australia).

Observed and expected heterozygosities as well as the fixation index (an index ranging between -1 and +1, values close to zero indicate random mating), were calculated with the software Microsatellite analyzer MSA 4.05 [12].

Results

Strategy to setup codominant microsatellite-analysis

In order to demonstrate the usability of HRM for analysing microsatellites, two microsatellites were chosen from a set of recently published sequences [9], one with low complexity (three alleles), and the other more complex with seven alleles in the sample sets.

At both loci HRM curves showed significant variation, the various curve shapes could be grouped into single inflection point graphs and graphs with two inflection points. Considering the length of the amplicons used (92 bp and 148 bp, respectively), PCR products of homozygous samples should have only one melting domain [13] and thus

one inflection point. Consequently an additional melting domain indicates the presence of an additional PCR product. Since *Origanum* is a diploid organism, heterozygous samples should theoretically have two different DNA-strands in a ratio of 1:1 of the amplicons. The PCR products then consist of four different double-stranded DNAs (dsDNAs), A_1A_1, A_2A_2, A_1A_2, A_2A_1, the latter two being called heteroduplexes [14]. The heteroduplex dsDNA shows an imperfect binding site of the strands where the additional repetition(s) of the microsatellite occur in one allele. This lowers the melting temperature (T_m) of the heteroduplexes compared to the related homoduplexes. Therefore, heterozygous samples have two inflection points, and the inflection point at the lower melting temperature is caused by the heteroduplexes, while the second inflection point is caused by homoduplexes. Following this rule, homozygous samples can easily be classified by their curve shape (and between each other by their melting temperature). As mentioned above, interrun comparability was obtained by reference samples of each curve form. Although identical genotypes showed a high congruency (Figure 1), the classification of heterozygous samples by their HRM curve, however, can not be given immediately, because the curve shape does not allow an immediate assignment of the alleles present, as e.g. in size-based electrophoresis. Depending on the alleles present, a comparative identification is nevertheless possible and can be performed according to the flowchart presented in Figure 2.

Classification of heterozygous samples is possible based on the fact that artificial sample mixtures of two different homozygous samples will result in exactly the same curve shape like the heterozygous samples consisting of the two alleles of the two homozygous samples in the artificial mixture (Figure 3). If the heterozygous sample curve is not congruent with one of the curves of the artificial homozygous sample mixtures, no homozygous sample was found so far for one or for both allele(s) of the heterozygous sample. In this case, the heterozygous sample can be mixed consecutively with all the homozygous samples. If the curve shape of one of these curves lies exactly between the curve of the heterozygous and the respective homozygous sample, one of the alleles in the heterozygous sample is identical to the allele in the homozygous sample (Figure 4). The reason is that the PCR product of the mixed sample contains then the same dsDNAs like the heterozygous sample, only in a different quantitative composition. Given the other case that the heterozygous sample does not contain the allele of the homozygous sample, the PCR product of the artificial mix will contain two additional heteroduplexes of the allele, the total

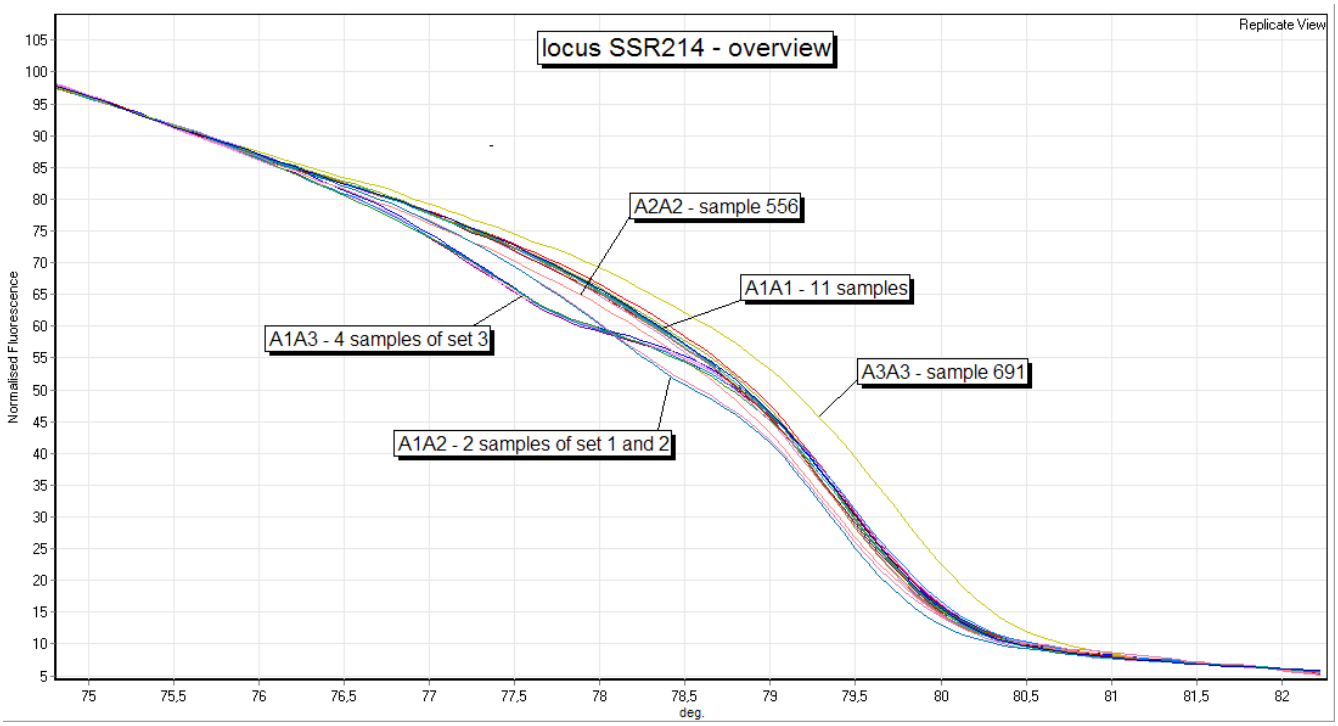

Figure 1
Overview of all curve forms appearing in locus SSR214. Melting curves of the homozygous genotypes A1A1, A2A2, A3A3 and of the heterozygous genotypes A1A2 and A1A3. The genotype A2A3 does not occur in the samples analyzed.

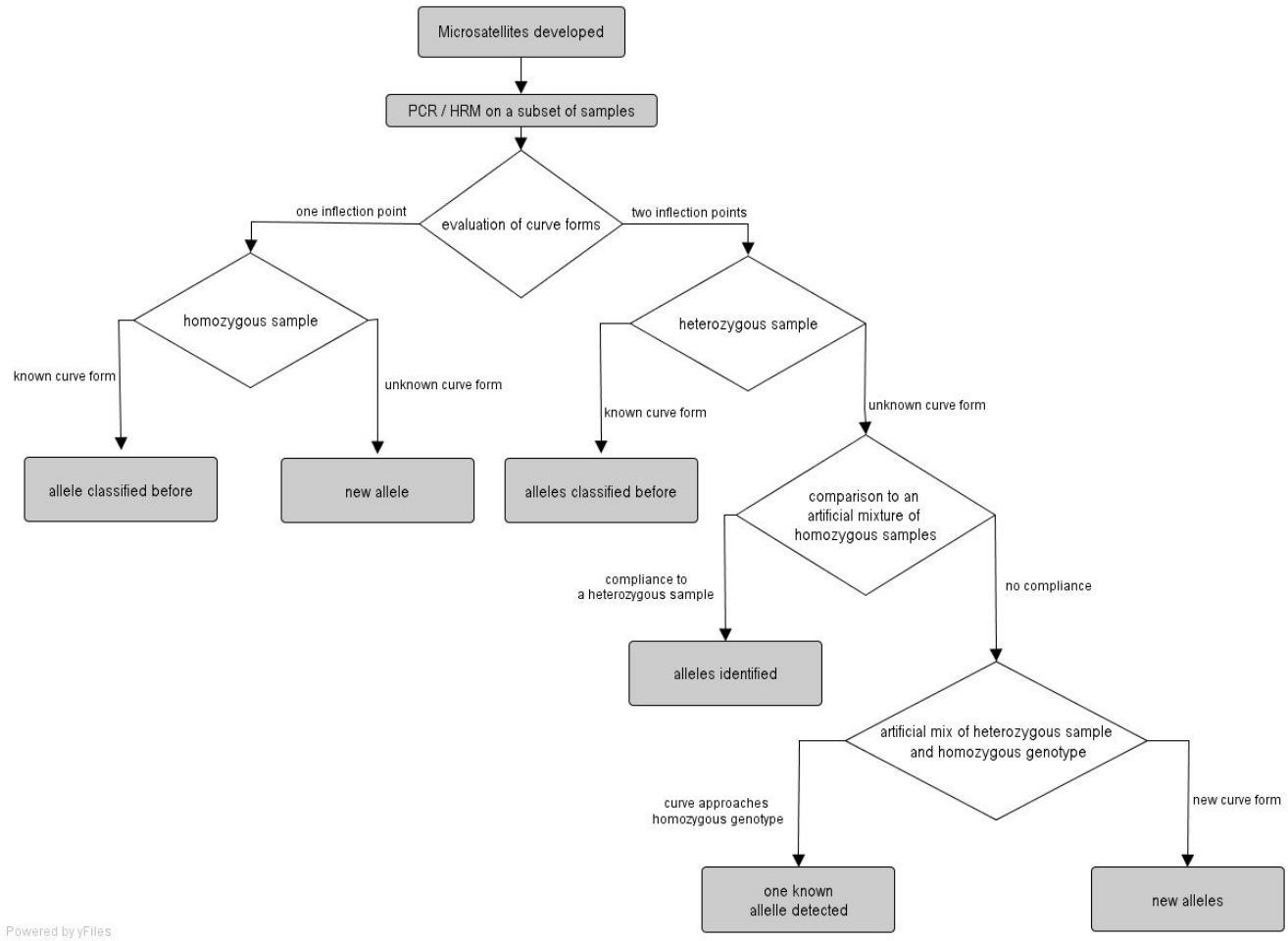

Figure 2
Flowchart outlining the approach to identify the alleles.

number of alleles in the mix being three, and the number of different amplicons being six. The presence and comparatively higher ratio of heteroduplexes versus homoduplexes will then lead to a curve shift that differs significantly from the heterozygous sample. Therefore the presence of a known allele in an unidentified heterozygous sample can be determined. If the curves of the artificial heterozygous/homozygous mixtures do not converge to any of the homozygous curves, the heterozygous sample consists of two new alleles.

With this approach the characteristics of the two loci in the sample sets of *Origanum sp*. were elaborated.

Results of allele analysis

Table 1 gives a summary of the calculated population genetic data. These results are consistent with those of former studies based on gel electrophoresis [9] and therefore confirm the correctness of HRM analysis.

At locus SSR214 all 3 alleles are present in homozygous and heterozygous individuals. Therefore both heterozygous genotypes occurring (A1A2, A1A3) could be classified by comparison to mixed samples of the homozygous individuals. Allele A3 appears in the mixed sample set 3 only. An overview of all curve forms for this locus is given in Figure 1. Figure 5 shows the melting curves of 9 samples of three different genotypes to clarify the precision of HRM analysis. Eight samples representing different curve forms were sequenced. These sequences confirmed HRM analysis. (data not shown)

At locus SSR244 the HRM curves revealed seven different heterozygous genotypes, but only three different homozygous genotypes. Four alleles were found which do not occur in homozygous genotypes in the samples tested. These alleles could be correctly assigned with artificial mixes of the respective heterozygous sample and each homozygous genotype available as shown on an example

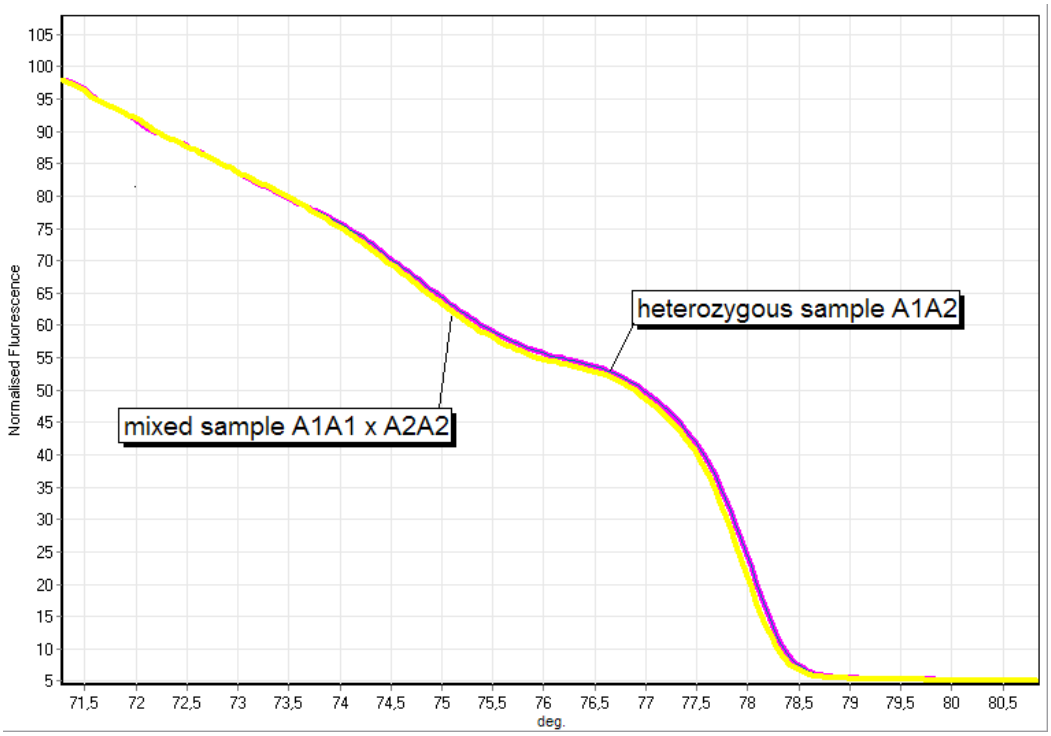

Figure 3
Comparative identification of heterozygous samples. Melting curves of a heterozygous sample A1A2 and an artificial mix between the two homozygous samples A1A1 and A2A2 in a ratio of 1:1 at the locus SSR244.

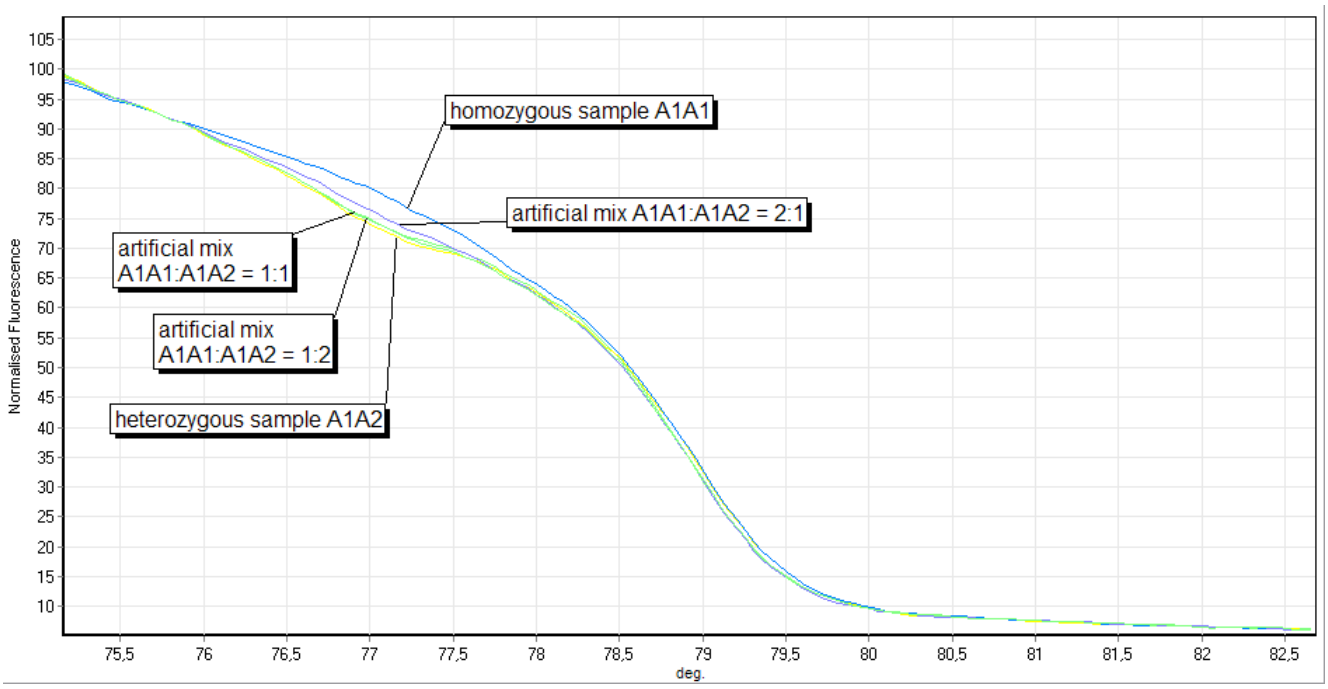

Figure 4
Artificial mixes of samples at locus SSR214. Locus SSR214: Melting curves of a heterozygous sample A1A2, a homozygous sample A1A1 and artificial mixes of both in the ratio 1:2, 1:1 and 2:1.

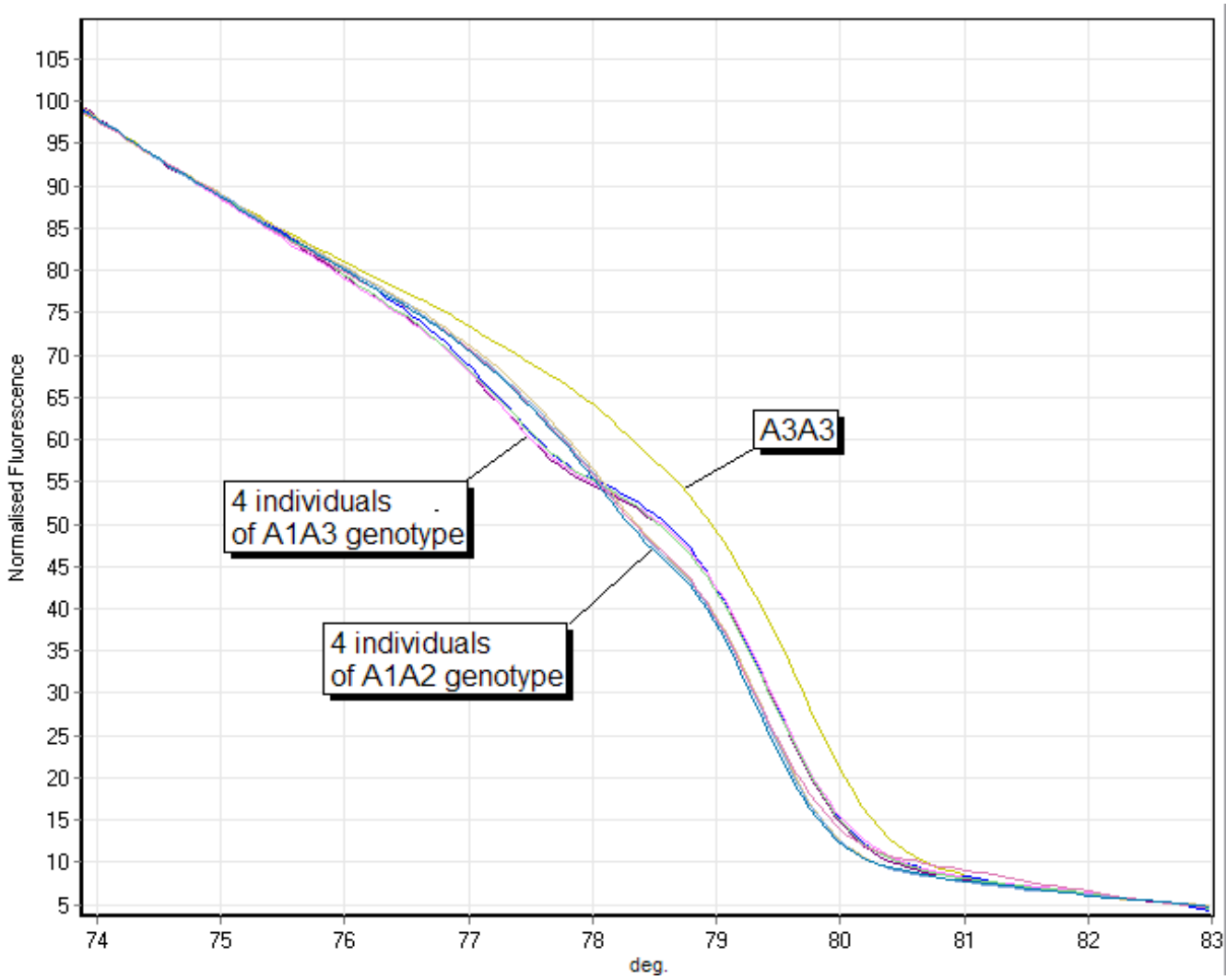

Figure 5
Precision of detection of various curve shapes and genotypes. Locus SSR214: Melting curves of one homozygous sample A3A3 and 4 individual samples of A1A3 and A1A2, respectively.

Table 1: Results of microsatellite allele analysis

	Locus	N	Na	Ho	He	F
Sample set 1	***SSR214***	33	2	0.091	0.088	-0.04
	SSR244	33	5	0.364	0.409	0.11
Sample set 2	***SSR214***	27	2	0.481	0.439	-0.11
	SSR244	27	3	0.556	0.451	-0.25
Sample set 3	***SSR214***	22	2	0.136	0.201	0.32
	SSR244	22	3	0.364	0.638	0.43

Sample sizes (N), number of alleles (Na), observed (Ho) and expected (He) heterozygosity, and fixation index (F) of the three sample sets

in Figure 6 at samples of the genotypes A3A3 and A3A5. For verification of HRM results at this locus 12 samples of various curve forms were sequenced and confirmed HRM results.

Discussion

The experiment demonstrated that the strategy to setup codominant microsatellite analysis worked perfectly well. With known and thoroughly tested microsatellite markers of grapevine HRM was recently implemented by MacKay et al[7]. In species or taxa where no such markers exist yet, like *Origanum*, the lack of certified reference DNA and the uncertainty of alleles present makes an elaborated development strategy for melting curve analysis necessary. To overcome the difficulties with correctly assigning hetero-

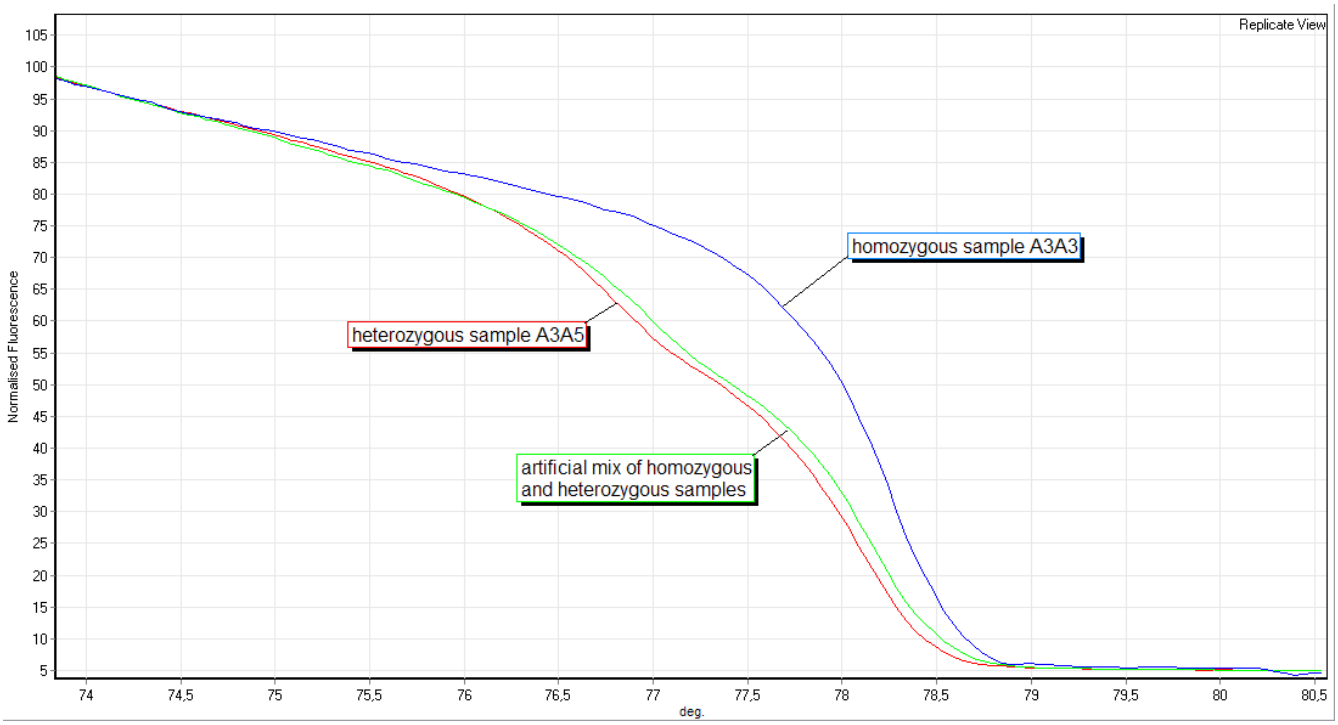

Figure 6
Artificial mixes of samples at locus SSR244. Locus SSR244: The HRM curve of an artificial mixture of a homozygous (sample 514, genotype A3A3) and a heterozygous sample (513, genotype A3A5) and the curves of both constituents in the same run.

zygous samples, the artificial mixes proposed proved to be accurate. The bottleneck in the setup of such a comparative analysis of dissociation curves is the number of different alleles in a given subset of samples. Therefore the sample size to setup this approach has to be increased when the number of heterozygous genotypes is high in order to correctly identify alleles, since the presence of at least some homozygous individuals is necessary to follow this approach. Considering this fact together with the comparative inexpensiveness and ease of use, HRM can be classified as a method with a mid-range capacity. With the ongoing advances in PCR-technology and miniaturization [15], HRM has the capacity to develop into a high throughput method.

Concerning the number of alleles in a population, there is no limit for analysis with HRM as long as a sufficient number of homozygous controls is available to mix with. The only case, which is not entirely resolvable with mixes is the occurrence of two rare alleles in a heterozygous genotype never occurring in a homozygous form. Sequencing such genotypes as well as unexpected genotypes can fill this gap.

As recently shown [16] interrun comparability of melting profiles is also manageable without reference samples in each run, which of course expands the number of samples that can be tested in one run. Furthermore a database utility of melting plots is already envisaged by MacKay et al [7].

To further increase sample throughput, a low number of alleles would allow sample pooling and could enable even higher throughputs with today's standard equipment. For correct classification of pooled sample melting curves it would be necessary to run mixed samples of known genotypes as calibrators or to re-run samples with ambiguous curves individually. A slightly modified strategy could be used to setup the analysis of organisms with higher ploidy levels. Here, quantitative variation between heterozygous and homozygous samples ('curve convergings') could be used to assign the correct allele composition.

HRM also reveals additional mutations in putative microsatellite loci, which can be seen as a great advantage in comparison to electrophoretic analysis, since the alleles of imperfect microsatellites often can not be correctly classified by their length, due to electrophoretic homoplasy of additional SNPs [17]. Additional mutations may also occur quite frequently in the flanking regions of microsatellites. In this aspect melting curve analysis has a great potential in the study of loci involv-

ing both SSR and SNPs [18], expanding the observed polymorphisms and thus lead to deeper insights into population structures [19].

Conclusion

With the implementation of a systematic comparison with artificial mixes of samples – as shown herein – HRM becomes a useful tool for the fast, reliable and cheap analysis of codominant markers. The high sensitivity is a major advantage over electrophoretic analysis when unknown mutations occur. Hence HRM has the potential to substitute other techniques in microsatellite analysis.

Authors' contributions

EM carried out the experimental work, developed the presented method and drafted the manuscript. BL collected the samples and did experimental preliminary work. JN participated in the design of the experiment and drafted the manuscript. All authors read and approved the final manuscript.

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Oregano: A Source for Peroxisome Proliferator-Activated Receptor γ Antagonists

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Oregano: A Source for Peroxisome Proliferator-Activated Receptor γ Antagonists

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Peroxisome proliferator-activated receptors (PPARs) are drug targets for several perturbations of metabolic syndrome, defined as the coexistence of obesity, hyperglycemia, hypertension, and hyper/dyslipidemia. In this study, PPAR activation by oregano (e.g., *Origanum vulgare*) and its components was tested. Oregano extracts bind but do not transactivate PPARγ, and binding affinity differs among different oregano extracts. The extracts contain PPARγ antagonists (e.g., quercetin, luteolin, rosmarinic acid, and diosmetin), selective PPARγ modulators (e.g., naringenin and apigenin), and PPARγ agonists (e.g., biochanin A). Oregano extract and isolated compounds in the extract antagonize rosiglitazone-mediated DRIP205/TRAP220 recruitment to PPARγ, pointing to oregano extracts as putative food supplements for weight reduction. Rosmarinic acid and biochanin A, PPARα agonists, may ameliorate the lipid profile. By endothelial nitric oxide synthase activation, oregano extract could prevent atherosclerosis. The results warrant further investigation of oregano extract for its potential to prevent and ameliorate metabolic syndrome and its complications.

KEYWORDS: Metabolic syndrome; *Origanum vulgare*; PPARγ; diabetes; eNOS; rosmarinic acid

INTRODUCTION

Because of the proliferation of a high-calorie, high-fat intake, sedentary lifestyle, obesity has become a worldwide epidemic. It is associated with an increased risk of developing metabolic syndrome, the coexistence of the metabolic perturbations hyperglycemia, hypertension, hyper/dyslipidemia, and central obesity. Cardiovascular morbidity and mortality are more prevalent among patients with this syndrome (*1*).

The peroxisome proliferator-activated receptor (PPAR) isoforms α, γ, and δ are nuclear receptors that play a role in ameliorating the perturbations of metabolic syndrome. After activation, they form a heterodimer with retinoid-X receptors, which can bind to the peroxisome proliferator-response element and promote or decrease the transcription of target genes (*2*). PPARγ is mainly expressed in adipose tissue and has been used as a drug target for treating type 2 diabetes (*2*). Full PPARγ activators, such as the thiazolidinediones currently used for treating type 2 diabetes, have several side effects, including promoting weight gain. Selective PPARγ modulators (SPPARγMs) show antidiabetic action without weight gain because of their mechanism involving only selective cofactor recruitment. Adipogenic cofactors, including DRIP205/TRAP220 and TIF2, are not recruited (*3*). PPARγ antagonists have been shown to exert antiobesity and antidiabetic actions (*4*). The expression of PPARα is mainly in the liver, brown fat, kidney, heart, and skeletal muscle. PPARα activation stimulates the expression of lipoprotein lipase and apolipoprotein A-V, an activator of lipoprotein lipase; it also stimulates the reduction of hepatic apolipoprotein C-III, an inhibitor of lipoprotein lipase, which leads to lower triglyceride levels in chylomicrones and in very low-density lipoprotein particles. Furthermore, PPARα activation leads to an elevated high-density lipoprotein cholesterol level by increased hepatic apolipoprotein A-I and -II expression and to a promotion of cholesterol efflux from cells to high-density lipoprotein by stimulating expression of the ATP-binding cassette A1 transport protein (*2*). PPARδ is ubiquitously expressed. At the cellular level, its activation increases fatty acid oxidation and energy expenditure in the muscles and in adipocytes and lipogenesis in the liver and reduces glucose output in the liver. In vivo, the lipoprotein profile, triglyceride levels, and insulin sensitivity are improved. PPARδ is further suggested to be a drug target in the treatment of obesity (*5*). All three PPAR subtypes play a role in ameliorating athero-

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sclerotic status primarily via inhibition of inflammation (*2, 5*). Another mechanism for improving atherosclerosis is activation of endothelial-derived nitric oxide (NO) by ameliorating endothelial dysfunction (*6*).

Natural ligands of the PPARs are fatty acids and their derivatives, such as eicosanoids. The synthetic ligands comprise the group of thiazolidinediones for PPARγ, fibrates for PPARα (*2*), and GW501516 or L-165041 for PPARδ (*5*).

To best of our knowledge, until now, oregano (e.g., *Origanum vulgare*) has not been described as a PPAR ligand, but the hypoglycemic, hypotensive, and hypolipidemic activities of oregano extracts and its compounds have been reported. Oregano and rosmarinic acid, a main phenolic compound, inhibit pancreatic amylase and α-glucosidase, and the components caffeic acid, rosmarinic acid, protocatechuic acid, quercetin, luteolin, and luteolin 7-*O*-glucoside inhibit α-glucosidase (*7*−*10*). Inhibition of both enzymes causes a decrease in the absorption of carbohydrates, resulting in a slower and lower rise in blood glucose. The oregano compounds caffeic acid, chlorogenic acid, and rosmarinic acid also have been reported to decrease glucose production in rat hepatocytes, by an inferred mechanism of induced glucokinase mRNA expression (*11*). Management of hypertension could arise from angiotensin I-converting enzyme inhibition by oregano extract (*9*). Salvianolic acid B has been further reported to have a hypotensive effect because of angiotensin I-converting enzyme inhibitory properties (*12*). In this study, various oregano extracts were examined as potential sources of PPARγ, -α, and -δ ligands or activators.

MATERIALS AND METHODS

Reagents. Solvents, dimethylsulfoxide (DMSO), formic acid, trifluoroacetic acid, apigenin, arbutin, biochanin A, carvacrol, β-caryophellene, *p*-coumaric acid, *p*-cymene, diosmetin, diosmin, eriodictyol, α-humulene, hyperoside, isoquercetrin, isovitexin, limonene, linalool, kaempferol, luteolin, naringenin, naringin, ocimene, peonidin chloride, protocatechuic acid, quercetin, rosmarinic acid, rutin, taxifolin, thymol, ursolic acid, vanillic acid, and vitexin were obtained from Sigma-Aldrich (St. Louis, MO). Salvianolic acid B was purchased from Chromadex Inc. (Santa Ana, CA), and chrysoeriol was purchased from Roth (Karlsruhe, Germany). Rosiglitazone, WY14643, and GW501516 were purchased from Cayman Chemicals (Ann Arbor, MI). Oregano dried leaves, powder, or plant extracts were purchased from different suppliers such as Fuchs (Dissen, Germany), Kotany (Wolkersdorf, Austria), Galke (Gittelde, Germany), Exxentia (Madrid, Spain), Naturex (Avignon, France), Hamburger Gewürzmühle (Hamburg, Germany), Paul Bruns GmbH (Hamburg, Germany), McCormick (United States), Edora (Kleinostheim, Germany), Vivatis Pharma (Hamburg, Germany), Pharmalink (Leichlingen, Germany), Los Chileros de Nuevo Mexico (Santa Fe, NM), Geelawson (London, Great Britain), and Pfannenschmidt (Hamburg, Germany). The black U96 microwell plate was obtained from Nunc (Roskilde, Denmark). The Polar Screen PPARγ Competitive Assay was purchased from Invitrogen Corp. (Carlsbad, CA).

The following were obtained from the stated suppliers: Dulbecco's minimum essential medium (DMEM) for the transactivation assay from Biochrom (Berlin, Germany), fetal calf serum (FCS) from HyClone (Logan, UT), SuperFect from Qiagen (Germantown, MD), and the Dual Glo Luciferase Assay System from Promega (Madison, WI). Acidic ion-exchange resin Dowex 50 W, 200−400 mesh, was purchased from Sigma. 1.1x ReddyMix PCR Master Mix (2.5 mM $MgCl_2$) was purchased from ABgene (Surrey, United Kingdom), primers were obtained from Invitrogen (Lofer, Austria), EXO1 and SAP were purchased from Fermentas (St. Leon-Rot, Germany), and BigDye 3.1 Terminators were obtained from Applied Biosystems (Applera Austria Handels GmbH, Brunn am Gebirge, Austria).

Identification of Plant Materials. Morphological analyses were performed for all samples of small-cut plant material by assessment of calyx morphology. A representative amount of calyces was compared with calyx figures provided in the current taxonomic reference for the genus (*13*) and with calyces of reference herbarium specimens kept at the Institute for Applied Botany and Pharmacognosy (University of Veterinary Medicine, Vienna, Austria).

DNA Analyses. Sequence analyses were performed for all samples included in the study. DNA was extracted from a representative amount of ground leaves, powders, or dried extracts using a modified CTAB extraction following the protocol described in ref *14*. For DNA amplification, two different procedures were used for the different plant preparations. For the fine-cut plant materials and the powders, the nuclear ITS (internal transcribed spacer) region and a part of the nuclear DXS (1-deoxy-D-xylulose 5-phosphate synthase) gene were amplified using universal primers for ITS (ITS5, 5′-GGAAGGAGAAGTCGTAACAAGG-3′; and ITS4, 5′-TCCTTCCGCTTATTGATATGC-3′) (*15, 16*) and EST-derived primers for DXS (DXS24F1, 5′-GGAAGCAAGGTGGCGATTC-3′; and DXS390R1, 5′-GAAACAACAGTCCCTGCGATATG-3′) (*17*). The amplifications were performed on a GeneAmp PCR System 9700 (Applied Biosystems) in 20 μL volumes with the following reaction components: 0.5 μL of template DNA (1−50 ng), 18 μL of 1.1x ReddyMix PCR Master Mix (2.5 mM $MgCl_2$), 0.5 μL of DMSO, 0.2 μL of ddH_20, and 0.4 μL (400 nM) of each primer. For ITS, 35 cycles of amplification with 1 min at 95 °C, 1 min at 55 °C, and 1 min at 72 °C were preceded by a 3 min denaturation step at 95 °C and followed by an additional 7 min at 72 °C. For DXS, 35 cycles of amplification with 1 min at 95 °C, 1 min at 60 °C, and 1 min at 72 °C were preceded by a 3 min denaturation step at 95 °C and followed by an additional 7 min at 72 °C.

Because of the low amounts of extractable DNA for the dried extracts, a nested polymerase chain reaction (PCR) approach for sequence analyses of the ITS region was chosen. In a first PCR, the primers 1406F (5′-TGTACACACCGCCCGT-3′) and 307R (5′-TTGGGCTGCATTCCCA-3′) (*18*) were used as external primers. The amplifications were performed in 15 μL volumes with the following reaction components: 0.5 μL of template DNA (original DNA extract diluted 1:10 with ddH_2O), 13 μL of 1.1x ReddyMix PCR Master Mix (2.5 mM $MgCl_2$), 0.75 μL of DMSO, 0.15 μL of ddH_20, and 0.3 μL (400 nM) of each primer (35 cycles of amplification with 1 min at 94 °C, 1 min at 58 °C, and 2 min at 72 °C, preceded by a 3 min denaturation step at 94 °C and followed by an additional 7 min at 72 °C). The amplification products of the first PCR were cleaned with EXO1 and SAP according to the supplier's instructions and then diluted 1:500 with ddH_2O. A second PCR reaction was carried out with 0.5 μL of this dilution (instead of genomic DNA) with primers ITS4 and ITS5 as nested primers. The PCR conditions were the same as described above.

For sequence analyses, all PCR products were checked on 1.4% agarose gels before being cleaned with EXO1 and SAP. Cycle sequencing was performed in forward and reverse reactions using BigDye 3.1 Terminators and primers from the original amplifications. The sequences were generated with an Applied Biosystems 3130x automated sequencer and edited using Chromas Vers. 2.24 (Technelyseum, Tewantin, Australia).

Because of the small amounts of plant material in the dried extracts, DNA extraction from such plant preparations was sensitive to contamination. Therefore, these samples were extracted and sequenced in duplicate to allow a validation of the results. Blank samples (respectively, nontemplate controls) were included in each extraction procedure and PCR to ensure that no laboratory contamination had occurred.

For identification of the plant samples, an alignment of the sample sequences and reference sequences from a broad range of *Origanum* species and one *Lippia* species (*17*) was conducted with Mega 4 (*19*). Species identification was done on the basis of percent homology with the reference sequences. All sequences have been deposited in GenBank, and accession numbers are listed in **Table 1**.

Extraction and Preparation of the Plants/Plant Powders. A total of 100 mg dry powder of oregano dried leaves or extract were suspended in 1 mL of DMSO for 24 h at room temperature. The suspension was stirred on a magnetic stirrer. After 24 h, the suspension was clarified by centrifugation for 1 h at 13000 rpm. The clear supernatant was used for further analysis.

Table 1. Tested Oregano Materials, Country of Origin, Species According to Supplier Specifications and as Determined by Morphological and DNA Analysis (ITS and/or DXS Gene), the IC_{50} and Equivalent Rosiglitazone Concentration Values of the Oregano Extracts as Determined by the Competitive PPARγ Ligand Binding Assay (LBA), IC_{50} Value and the Equivalent GW9662 Concentration of the Extracts as Determined by PPARγ TR-FRET Antagonist Screening and the Results of GAL4-PPARα, -β, and -γ Transfection Assays

			species (DNA analysis)			PPARγ LBA		TR-FRET	
no.	plant/extract/ standardization	species (acc. to supplier)	species (microscopic analysis)	ITS gene	DOXYP gene	country	IC_{50} (μg/mL)	eq IC_{50} (nmol/g)	IC_{50} (μg/mL)	eq IC_{50} (nmol/g)
1	leaves	*O. onites*	*O. onites*	*O. onites*	*O. onites*	Turkey	1.6 ± 0.3	75025	6.2 ± 0.7	1737
2	leaves	*O. onites*	*O. onites*	*O. onites*	*O. onites*	Turkey	1.8 ± 0.3	66202	2.2 ± 0.2	4910
3	leaves		*O. vulgare*	*O. vulgare*	*O. vulgare*	Albany, Turkey, Greece	3.4 ± 1.3	35057	17.8 ± 1.7	606
4	leaves	*O. onites*	*O. onites*	*O. onites*	*O. onites*	Turkey	3.4 ± 0.3	35805	1.4 ± 0.4	7937
5	extract, >1% rosmarinic acid	*O. vulgare*		*O. majorana*[a]	ND	China	5.3 ± 0.7	22483	2.0 ± 0.2	5395
6	extract 5:1	*O. vulgare*		ND	ND	China	6.8 ± 0.9	17553	2.1 ± 0.4	5035
7	flower					Austria	10.9 ± 1.2	10982		
8	extract 10:1	*O. vulgare*		ND	ND	China	14.0 ± 1.3	8550	23.5 ± 3.9	460
9	leaves	*O. vulgare*		*O. onites*	*O. onites*	Turkey	15.1 ± 3.6	7931	88.7 ± 36.3	121
10	extract 5:1	*O. vulgare*		*O. onites*	ND	China	16.8 ± 2.0	7149		
11	extract 5:1	*O. vulgare*		ND	ND	China	16.9 ± 2.5	7117		
12	extract, >2.5% rosmarinic acid	*O. vulgare*		ND	ND	Bulgaria	20.8 ± 2.6	5771		
13	extract 15:1	*O. vulgare*		ND	ND	China	22.1 ± 1.7	5423	11.9 ± 1.5	903
14	extract 20:1	*O. vulgare*		ND	ND	Spain	23.6 ± 3.3	5081	10.5 ± 1.6	1025
15	extract 10:1	*O. vulgare*		ND	ND	China	27.8 ± 3.5	4314		
16	leaves	*O. vulgare*	O. vulgare	*O. vulgare*	*O. vulgare*		28.6 ± 4.3	4202		
17	leaves	*O. vulgare*	O. vulgare	*O. vulgare*	*O. vulgare*	Albany, Turkey, Greece	29.2 ± 3.9	4010		
18	leaves		*O. vulgare*	*O. vulgare*	*O. vulgare*	Albany, Turkey, Greece	23.6 ± 2.5	5076		
19	extract 15:1	*O. vulgare*		*O. onites*	ND	Spain	32.8 ± 6.9	3664		
20	extract 20:1	*O. vulgare*				Spain	35.3 ± 6.9	3399		
21	leaves	*O. onites*		ND	*O. onites*	Turkey	35.5 ± 4.1	2940		
22	leaves	*O. onites*	*O. onites*	*O. onites*	*O. onites*	Turkey	41.5 ± 8.3	2890		
23	leaves		*O. vulgare*	*O. vulgare*	*O. vulgare*	Spain	41.7 ± 9.2	2874		
24	extract	*O. vulgare*		*O. onites*	ND	Turkey	49.9 ± 3.8	2403		
25	leaves			*Lippia graveolens*	ND	Mexico	52.9 ± 4.6	2270		
26	extract 4:1			*O. onites*	ND		57.0 ± 13.0	2106		
27	extract	*O. vulgare*		*O. onites*	ND	Turkey	65.7 ± 10.0	1826		
28	extract 15:1	*O. vulgare*		ND	ND	China	66.3 ± 134.9	1809		
29	extract	*O. vulgare*		ND	ND	Bulgaria	66.9 ± 6.7	1793		
30	extract			*O. onites*	ND	Turkey	70.2 ± 25.0	1709		
31	extract, >1% rosmarinic acid	*O. vulgare*		ND	ND	Balkans	75.0 ± 7.5	1600		
32	leaves			ND	ND		76.9 ± 7.7	1561		
33	extract 15:1	*O. vulgare*		ND	ND		95.5 ± 23.4	1256		
34	leaves		ND	ND	ND	Austria	100 ± 10.6	1199		
35	extract 4:1	*O. vulgare*		*O. onites*	ND	China	134.1 ± 16.4	895		
36	extract, >1% rosmarinic acid			ND	ND	Turkey	330 ± 71	364		
37	extract 4:1	*O. vulgare*		*O. onites*	ND	China	388 ± 72	309		
38	extract 5:1	*O. vulgare*		ND	ND	Spain	ligand			

[a] Putative hybrids of *O. vulgare* with one species of the *Origanum* section Majorana. ND, not determinable.

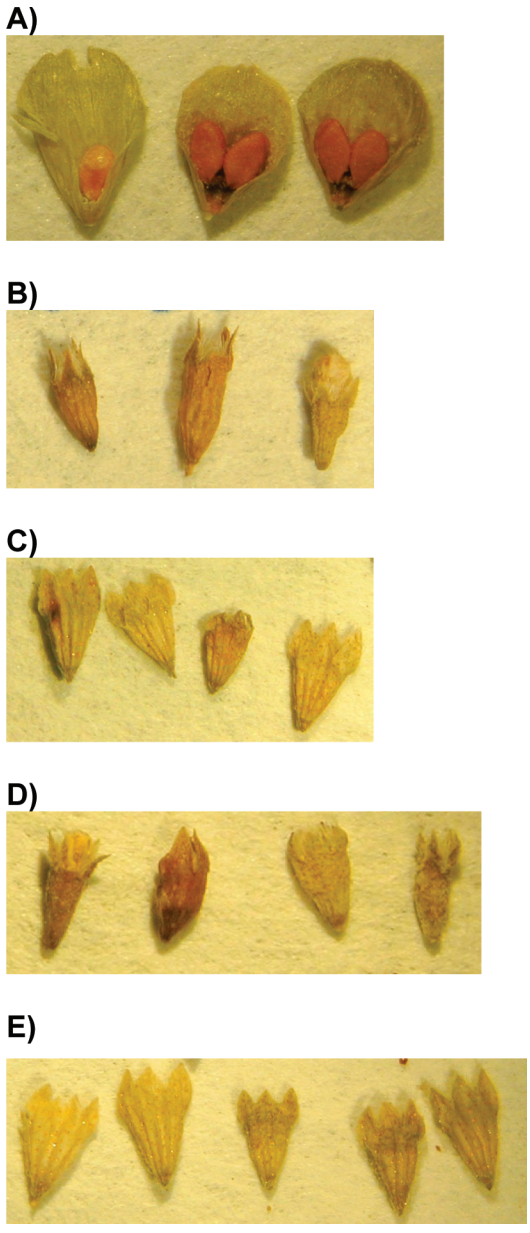

Figure 1. Examples of calyces isolated from *Origanum* small-cut plant materials [(**A**) *O. onites* and (**B**−**E**) *O. vulgare*]. (**A**) Extract 1, (**B**) extract 3, (**C**) extract 16, (**D**) extract 18, and (**E**) extract 23.

Polar Screen PPAR Competitive Assay. A ligand-binding competitive assay was performed using PolarScreen PPARγ Competitor Assay, Green, according to the manufacturer's protocol. Briefly, the PPARγ ligand binding domain (LBD) and the fluorescent PPARγ ligand (Fluoromone PPARγ Green) were combined to form a PPARγ-LBD/fluoromone PPARγ Green complex with a high polarization value. Test compounds in various concentrations competed for binding to PPARγ. Ligands displaced the fluorescent ligand, releasing the fluoromone into the solution to tumble rapidly during its fluorescence lifetime, causing a low polarization value. If substances did not bind to PPARγ and displace the fluorescent ligand from the complex, the polarization value remained high. The change in polarization value was used to determine the relative affinity of test compounds for the PPARγ-LBD. Fluorescence polarization measurements were obtained using a Genios Pro plate reader (Tecan, Crailsheim, Germany) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. The PPARγ agonist rosiglitazone was used as the standard. The maximal polarization value of rosiglitazone was defined as 100%.

Time-Resolved Fluorescence Resonance Energy Transfer (TR-FRET) Coactivator Assay. Antagonist screening was performed with the Lantha Screen TR-FRET PPARγ coactivator assay in antagonist mode according to the manufacturer's protocol. In brief, different concentrations of various test compounds, the PPARγ LBD tagged with glutathione-*S*-transferase (GST), and a mixture of the fluorescein-labeled coactivator peptide, terbium-labeled anti-GST antibody, and rosiglitazone (final concentration, 250 nM) were combined. Binding of an agonist (in this case rosiglitazone) to PPARγ resulted in a conformational change and coactivator peptide TRAP220/DRIP-2 recruitment. If the fluorescently labeled coactivator peptide and the terbium-labeled anti-GST antibody bound to the PPARγ GST tag came into close proximity, energy was transferred from the terbium label to the fluorescein label, which was detected as emission at 520 nm. Certain concentrations of PPARγ antagonists displaced rosiglitazone from PPARγ, and the emission signal at 520 nm decreased. The fluorescence intensity was measured using the Genios Pro plate reader at an excitation wavelength of 340 nm, emission wavelengths of 520 and 495 nm, a delay time of 100 μs, and an integration time of 200 μs. The emission ratio 520:495 nm was determined. This value indicated the extent of coactivator splitting from PPARγ and thus the antagonistic activity of the substances.

Plasmids. The pGAL4-hPPARγ-LBD, the pGAL4-hPPARα-LBD, and the pGAL4-hPPARδ-LBD expression plasmids, which express chimeric proteins containing the GAL4 DNA binding domain fused to the human PPAR LBD (*20*), was provided by Prof. Staels (Institut Pasteur, University of Lille, France). The luciferase reporter plasmid pFR-Luc containing a firefly luciferase gene controlled by five repeats of GAL4-binding element was purchased from Stratagene (La Jolla, CA). The Renilla control plasmid (pRL-tk), which encodes *Renilla* luciferase, was obtained from Promega.

Transactivation Assay. One day before transfection, NIH-3T3 cells (German Collection of Microorganisms and Cell Cultures, Accession. No. 59) were seeded at a density of 1×10^4 cells per well. Cells were cotransfected with 30 ng of pGAL4-hPPARγ-LBD, 60 ng of pGAL4-hPPARα-LBD, or 30 ng of pGAL4-hPPARδ-LBD expression plasmid, 300 ng of pFR-Luc reporter plasmid, and 15 ng of pRL-TK using the SuperFect transfection reagent. After transfection, the medium was changed, and cells were incubated with standard or test substances dissolved in DMSO for 24 h. The DMSO content of the DMEM did not exceed 0.3%. Luciferase assays were performed using the Dual Glo Luciferase Assay System (following the manufacturer's manual). Firefly luminescence correlating to transactivation efficiency and Renilla luminescence indicating transfection efficiency were measured with the Genios Pro plate reader in luminescence mode. The firefly-to-Renilla ratio was calculated and normalized to the DMSO control. Rosiglitazone (PPARγ agonist), WY14643 (PPARα agonist), and GW501516 (PPARδ agonist) were used as positive controls. The efficiency of these substances was defined as 100%.

Curve Fitting. Data for binding affinity, transactivational activity, and antagonistic activity were processed in the following way: Relative polarization values, transactivational efficiencies, and emission ratio (referred to the reference substance) were plotted against the concentration of test compounds. Curve fitting was performed using a logistic dose−response model (eq 1) of the software Table Curve 2D software (Jandel Scientific, Erkrath, Germany), a nonlinear equation using a Levenberg−Marquard algorithm (*21*).

$$y = a + \frac{b}{1 + (c/x)^d} \qquad (1)$$

where a equals the baseline, b is the difference between the plateau of the curve and the baseline, and c is the transition center of the curve, which is the concentration that causes 50% efficiency (ligand potency). d is the transition zone and is a measure of positive or negative cooperativity.

The concentration of the test compounds that results in a half-maximal decrease of polarization value, half-maximal transactivational efficiency, or half-maximal decrease of emission ratio is the IC_{50}/EC_{50} value of the test compound. This value indicates PPARγ binding affinity, transactivational activity, or antagonistic activity.

Statistics. The competitive assay was performed in duplicate, the transactivation assay was performed in quadruplicate, and the TR-FRET

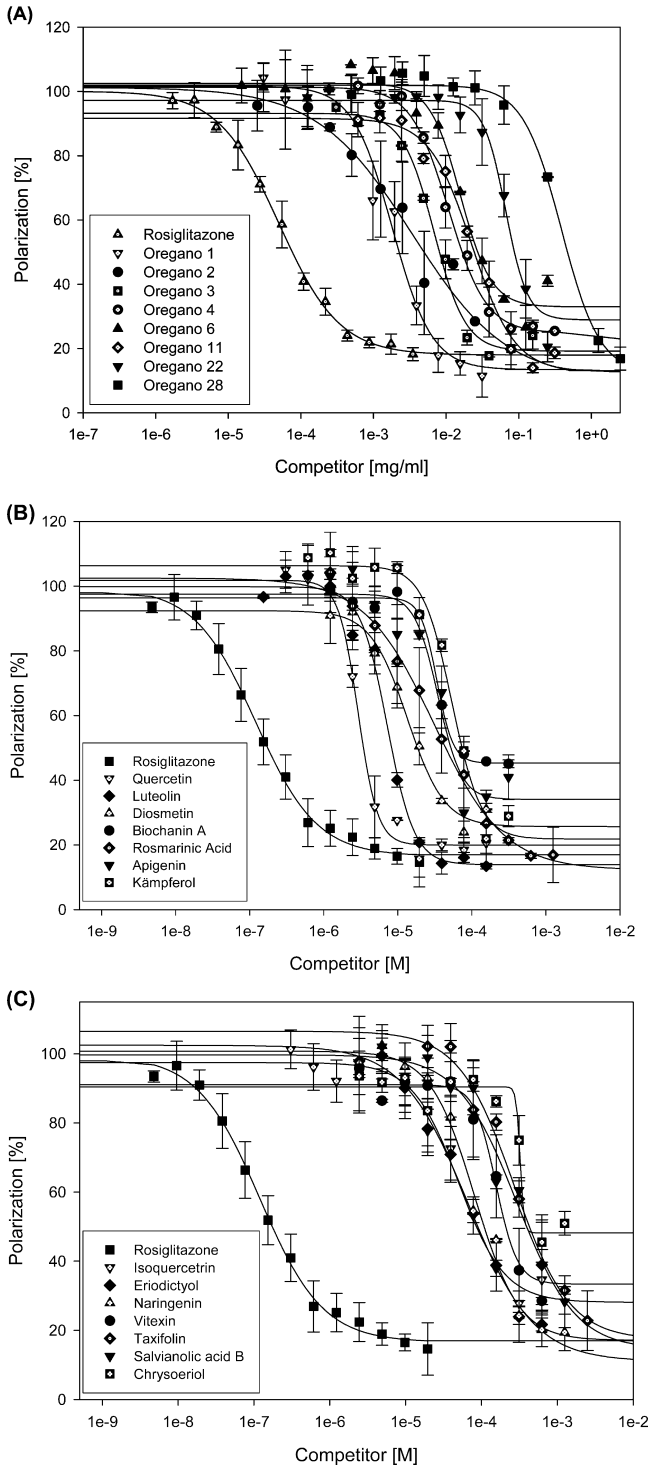

Figure 2. (**A**) Logistic dose−response curves for various oregano extracts, determined using the polar screen PPARγ competitive assay; (**B**) curves for rosiglitazone (standard), quercetin, luteolin, rosmarinic acid, diosmetin, biochanin A, apigenin, and kämpferol; and (**C**) curves for rosiglitazone (standard), isoquercetrin, eriodictyol, naringenin, vitexin, salvianolic acid B, and chrysoeriol.

PPARγ coactivator assay was performed in triplicate. Each assay was performed on independent days, and intervariation was in the same range as intravariation.

Determination of Equivalent Rosiglitazone Concentration. The equivalent rosiglitazone concentration corresponds to the theoretical concentration of rosiglitazone in the extract resulting in the same binding affinity. Rosiglitazone was used as the reference substance. The equivalent IC_{50} value was calculated by dividing the IC_{50} value of rosiglitazone by the IC_{50} value of the plant extracts or isoflavones (eq 2):

$$IC_{50}\,(\text{nmol/g}) = \frac{IC_{50(\text{rosiglitazone})}\,(\text{nmol/L})}{IC_{50(\text{extract})}\,(\text{g/L})} \qquad (2)$$

eNOS Assay: Cell Cultures and Treatments. Human umbilical vein endothelial cells (HUVECs) were harvested enzymatically with type I A collagenase (1 mg/mL), as previously described (*22*), and maintained in phenol red-free DMEM, containing HEPES (25 mM), heparin (50 U/mL), endothelial cell growth factor (50 ng/mL), L-glutamine (2 mM), antibiotics, and 10% FBS. Before each experiment, HUVECs were kept for at least 48 h in DMEM containing 10% steroid-deprived FBS. All experiments were performed on confluent monolayers of endothelial cells. Before every experiment investigating rapid, nontranscriptional effects (up to 30 min treatments), HUVECs were serum starved in DMEM containing no FBS for 8 h before treatment to avoid the confounding effects of serum. Whenever an inhibitor was to be used, we added the compound 30 min before the treatments.

eNOS activity was determined based on the conversion of [^{3}H]arginine to [^{3}H]citrulline in endothelial cell lysates. Converted citrulline was separated by unconverted arginine using the acidic ion-exchange resin Dowex 50 W, 200−400, as described (*23*). Extracts incubated with the eNOS inhibitor, *N*-nitro-L-arginine methyl ester (1 mM), served as the blank.

NO production was determined by a modified nitrite assay using 2,3-diaminonaphtalene, as described (*23*). Fluorescence of 1-(*H*)-naphtotriazole was measured with excitation and emission wavelengths of 365 and 450 nm, respectively. Standard curves were constructed with sodium nitrite. Nonspecific fluorescence was determined in the presence of NG-monomethyl-L-arginine (3 mM).

High-Performance Liquid Chromatography−Electrospray Ionization−Mass Spectrometry (HPLC-ESI-MS) Analysis. Separation for a HPLC-MS was carried out with a Finnigan Surveyor Plus HPLC system (ThermoFinnigan, San Jose, CA) connected with a Luna C18(2) column (3 μm, 100 mm × 2 mm, Phenomenex, Torrance, CA) and a security guard cartridge (Phenomenex). The mobile phase consisted of solvent A (5% acetonitrile, 0.1% formic acid) and solvent B (acetonitrile, 0.1% formic acid). The gradient profile was as follows: from 0−5 min, 100% B; 5−30 min, from 0 to 17.5% A; 30−55 min from 17.5 to 50% A; 55−65 min, 50% A; 65−75 min, 90% A; 75−78 min, 100% B; 78−88 min, 90% A; and 88−90 min, 100% C. Mass analyses were performed with an LTQ ion trap mass spectrometer (ThermoFinnigan) equipped with an electron spray ionization source as previously described (*24*).

RESULTS

Commercial sources of different oregano plant material were purchased. To ensure the identity as oregano and further characterize these materials, morphological and DNA analyses were performed. The results of the morphological and DNA analyses are summarized in **Table 1**. Most of the traded oregano in the "old world" is derived from plant material of the species *O. vulgare* and *Origanum onites* (*25*). Because these two species show very different calyx characteristics, this morphological feature is an appropriate tool for determining samples of small-cut plant material and to distinguish the "Mexican oregano," which derives mainly from *Lippia* sp. (Verbenaceae) (*25*). Out of 38 samples, nine could be analyzed morphologically, and the other samples were extracts. Five samples were identified as *O. vulgare* and four as *O. onites* (**Figure 1**).

The results of the DNA analyses were in accordance with the morphological analyses. Out of 38 analyzed samples, sequences were only obtained with 20 samples. Thirteen were identified as *O. onites*, five as *O. vulgare*, one as *Lippia* sp., and one as *O. majoranum*. The success of sequence analyses was correlated with the particular preparation of the plant

Table 2. IC_{50} Values of Substances Occurring in Oregano, Determined by Competitive PPARγ LBA, the IC_{50} Value of Oregano Compounds Determined by TR-FRET Assay in Antagonist Mode, and the EC_{50} Value and Efficiency (%) of Oregano Compounds, Determined by Chimeric GAL4-PPARγ, GAL4-PPARα, or GAL4-PPARδ Transfection Assay

		PPARγ				PPARα		PPARδ	
		LBA	TR-FRET	transactivation		transactivation		transactivation	
substance	CAS no.	IC_{50} (μM)	IC_{50} (μM)	EC_{50} (μM)	efficiency[d] (%)	EC_{50} (μM)	efficiency (%)	EC_{50} (μM)	efficiency (%)
rosiglitazone	122320-73-4	0.12 ± 0.01		0.21 ± 0.05	100.0				
GW9662	22978-25-2		0.013 ± 0.002						
WY 14643	50892-23-4					10.5 ± 1.4	100.0		
GW 501516	317318-70-0							0.0005 ± 0.0001	100.0
quercetin	849061-97-8	3.0 ± 0.2	3.7 ± 0.4	ND[e]		ND		ND	
luteolin	491-70-3	7.2 ± 0.6	4.9 ± 0.4	ND		ND		ND	
diosmetin	520-34-3	13.5 ± 2.0	antagonist[b]	ND		ND		ND	
biochanin A	491-80-5	23.7 ± 6.2	ND	32.1 ± 6.4	26.5	23.6 ± 2.1	54.7	ND	
rosmarinic acid	20283-92-5	32.4 ± 4.0	43.6 ± 4.2	ND		43.0 ± 5.3	19.4	ND	
apigenin	520-36-5	40.9 ± 9.1	antagonist	active[c]	15.7	ND		ND	
kämpferol	520-18-3	47.6 ± 8.2	32.9 ± 4.0	ND					
isoquercetrin	21637-25-2	49.9 ± 8.8	antagonist	ND		ND		ND	
eriodictyol	552-58-9	66.6 ± 11.9	16.1 ± 8.1	ND		ND		ND	
naringenin	67604-48-2	81.0 ± 10.3	35.3 ± 15.2	79.5 ± 9.0	16.4	ND		ND	
vitexin	3681-93-4	155.7 ± 18.7	30.6 ± 1.4	ND		ND		ND	
taxifolin	480-18-2	275.4 ± 40.4	75.7 ± 41.8						
salvianolic acid B	115939-25-8	ligand[a]	antagonist	ND		ND		ND	
chrysoeriol	491-71-4	ligand	ND	ND					
carvacrol	499-75-2	ligand	antagonist						
naringin	10236-47-2	ligand	ND						
thymol	89-83-8	ligand							
limonene	5989-27-5	ligand							
peonidinCl	134-01-0	ligand							
ursolic acid	77-52-1	ligand							
apigenin-7-glycoside	578-74-5	ligand							
p-coumaric acid	501-98-4	ligand							
vanillic acid	121-34-6	ligand							
isovitexin	29702-25-8	ND							
hyperoside	482-36-0	ND							
arbutin	497-76-7	ND							
protocatechuic acid	99-50-3	ND							
linalool	78-70-6	ND							
β-caryophellene	87-44-5	ND							
ocimene	13877-91-3	ND							
caffeic acid	331-39-5	ND							
diosmin	520-27-4	ND							
p-cymene	99-87-6	ND							
α-humulene	6753-98-6	ND							

[a] Compounds that bound to PPARγ lacking saturation of the logistic dose−response curve were described as ligands. [b] Compounds antagonizing rosiglitazone-mediated coactivator recruitment in the TR-FRET assay without reaching a plateau for the minimum value were described as antagonists. [c] If transactivation was observed (firefly luciferase value ≥1.5-fold the DMSO control), but no EC_{50} value could be determined, compounds were described as active. [d] The efficiency describes the maximal induction referred to rosiglitazone using the chimeric GAL4-PPAR assay. [e] For some substances, no binding or transactivation was observed (ND, not determinable) up to a concentration of 2.5 (LBA) or 0.3 mM (transactivation assay).

materials. DNA extraction from leaves and powders and subsequent sequencing is a standard procedure, and the two different DNA markers used allowed an unambiguous determination of the respective plant material. Because of the tiny amounts of plant residuals and therefore extractable DNA, work with extracts is much more sensitive. Dealing with traces of DNA amplification of the DXS region was not possible; thus, amplification of the ITS region was successful only for some of the dried extract samples (**Table 1**).

The binding affinity of oregano extract and its compounds for PPARγ was determined by a competitive assay based on the ability of the samples to displace the above-described fluorescent PPARγ ligand. Data were approximated by logistic dose−response curves, and potency values were derived. A great variety of oregano extracts was assayed to get a first insight how the PPARγ binding affinity varied with different influences like species, growing area, and climatic influences. The PPARγ affinity of the different samples varied in a wide range from an IC_{50} value of 1.6 ± 0.3 to 500 μg/mL (**Table 1** and **Figure 2A**). At first glance, one cannot conclude that a certain species was a higher PPARγ binder.

Polyphenolic compounds, typically present in oregano, were also tested (*26, 27*). A total of 23 different compounds that bind to the receptor could be identified. The most potent substances where quercetin (IC_{50} = 3.0 μM), luteolin (IC_{50} = 7.2 μM), rosmarinic acid (IC_{50} = 32.4 μM), and diosmetin (IC_{50} = 13 μM) (**Figures 2 B**,**C** and **Table 2**). Some compounds did not bind to PPARγ up to a concentration of 0.025 M: isovitexin, hyperoside, arbutin, protocatechuic acid, linalool, β-caryophellene, ocimene, α-humulene, *p*-cymene, caffeic acid, and diosmin.

For determination of transactivation properties, the extracts were assayed by transfection assay. The transactivation assay was performed by cotransfection of NIH-3T3 cells with pGAL4-hPPARγ-LBD, pGAL4-hPPARα-LBD, or pGAL4-hPPARδ-LBD and a luciferase reporter plasmid. Transiently transfected cells were incubated with substances at a concentration range from 3×10^{-4} to 1×10^{-6} M and plant extracts up to a concentration of 0.3 mg/mL. Logistic dose−response curves

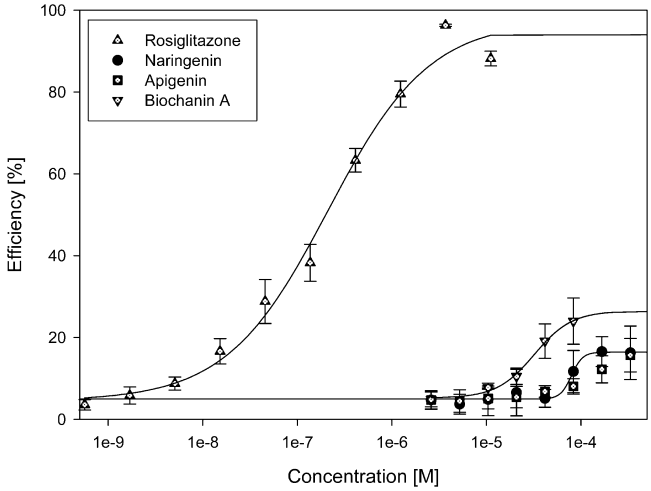

Figure 3. Logistic dose−response curves for rosiglitazone, biochanin A, naringenin, and apigenin, determined by chimeric GAL4-PPARγ transfection assay.

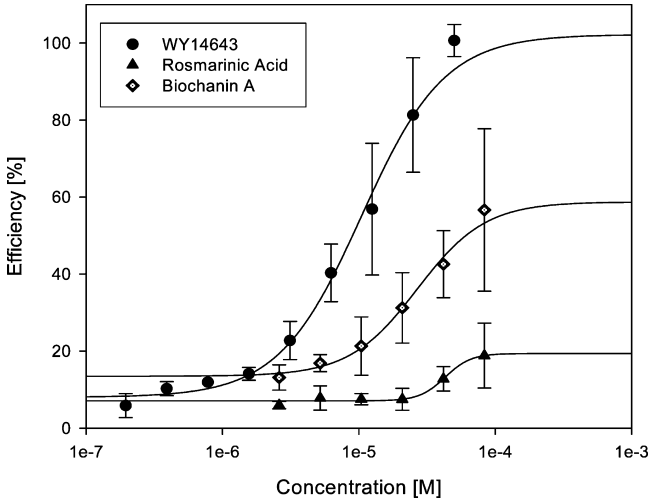

Figure 4. Logistic dose−response curves for WY14643, biochanin A, and rosmarinic acid, determined by chimeric GAL4-PPARα transfection assay.

were calculated as described above, and activity and potencies were derived from the fitted parameters.

The oregano compounds naringenin and apigenin showed moderate PPARγ transactivational activity with respective EC_{50} values of 79.5 and 201.1 μM and a respective maximal activation of 16.4 and 15.7% as compared to rosiglitazone (**Figure 3** and **Table 2**). Biochanin A was a potent PPARγ transactivator (EC_{50} = 32.1 μM; maximal activity of 26.5%). Various oregano extracts and the compounds rosmarinic acid, quercetin, isoquercetrin, vitexin, salvianolic acid B, chrysoeriol luteolin, diosmetin, eriodictyol, and taxifolin did not show PPARγ transactivational activity. Oregano extract and some of the oregano compounds exerted cytotoxic activity. This activity was especially high for luteolin, quercetin, and diosmetin, beginning at a concentration of 10 μM.

For PPARα transactivation assays, WY14643, a known PPARα agonist, served as the control substance (EC_{50} = 10.5). Rosmarinic acid, the main phenolic compound of oregano extract, was a weak transactivator (EC_{50} = 43.0 μM), and biochanin A was a moderate transactivator (EC_{50} = 23.6 μM) (**Figure 4** and **Table 2**). The maximum activity (as compared to the activity of WY14643, defined as 100%) was 19.4% for rosmarinic acid and 54.7% for biochanin A. No significant

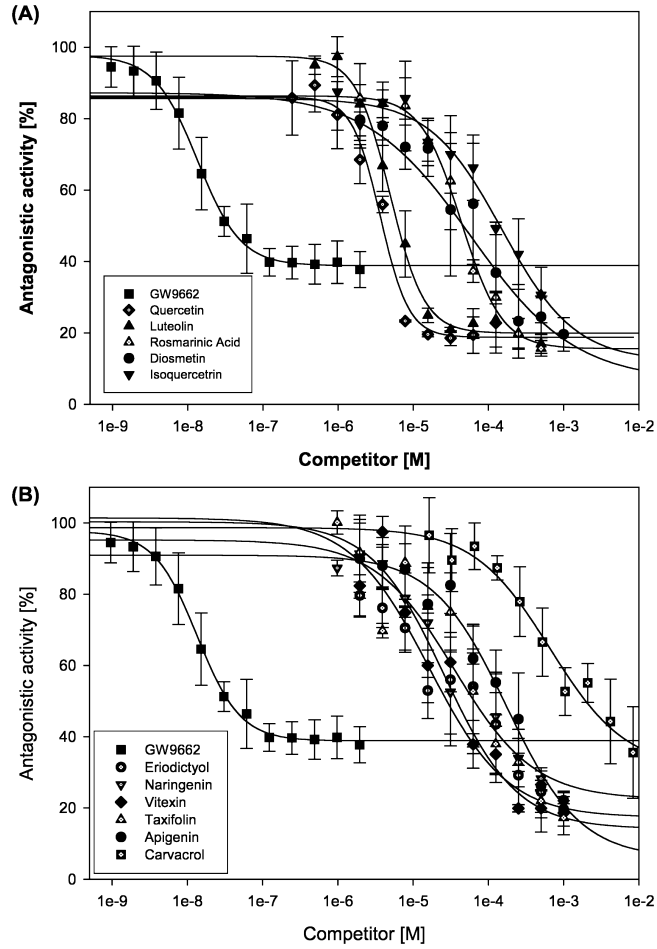

Figure 5. Logistic dose−response curves for (**A**) GW9662, quercetin, luteolin, rosmarinic acid, diosmetin, biochanin A, and isoquercetrin and (**B**) eriodictyol, naringenin, apigenin, vitexin, taxifolin, and carvacrol, determined by the TR-FRET assay in antagonist mode.

transactivational activity was observed for vitexin, naringenin, apigenin, eriodictyol, and isoquercetrin. Luteolin, quercetin, and diosmetin did not activate PPARα but showed high cytotoxic activity. Different oregano extracts and the abundant compound salvianolic acid also did not transactivate PPARα.

Various oregano extracts and the oregano compounds quercetin, luteolin, rosmarinic acid, diosmetin, biochanin A, isoquercetrin, naringenin, eriodictyol, apigenin, vitexin, and salvianolic acid B did not transactivate PPARδ.

With TR-FRET assay, the antagonistic activity of oregano compounds and extracts was analyzed. The reference substance GW9662 antagonized rosiglitazone-mediated TRAP220/DRIP-2 coactivator recruitment with an IC_{50} of about 14 nM. Luteolin (IC_{50} = 4.9 μM), isoquercetrin (IC_{50} = 15.1 μM), quercetin (IC_{50} = 3.7 μM), vitexin (IC_{50} = 30.6 μM), rosmarinic acid (IC_{50} = 43.6 μM), eriodictyol (IC_{50} = 16.1 μM), taxifolin (IC_{50} = 75.7 μM), naringenin (IC_{50} = 35.3 μM), diosmetin, salvianolic acid, apigenin, and carvacrol showed significant antagonistic activity in this assay (**Figure 5** and **Table 2**). The IC_{50} value of the oregano extracts ranged from 1.4 (extract 4) to 88.7 (extract 9, **Table 1**).

Endothelial NO synthase (eNOS) activation is also a good indication for vascular health. This was assayed by exposure of umbilical vein endothelial cells to oregano extract. A concentration-dependent increase in NO release with a maximum of approximately 20-fold induction as compared to negative control in the cell culture medium, which was

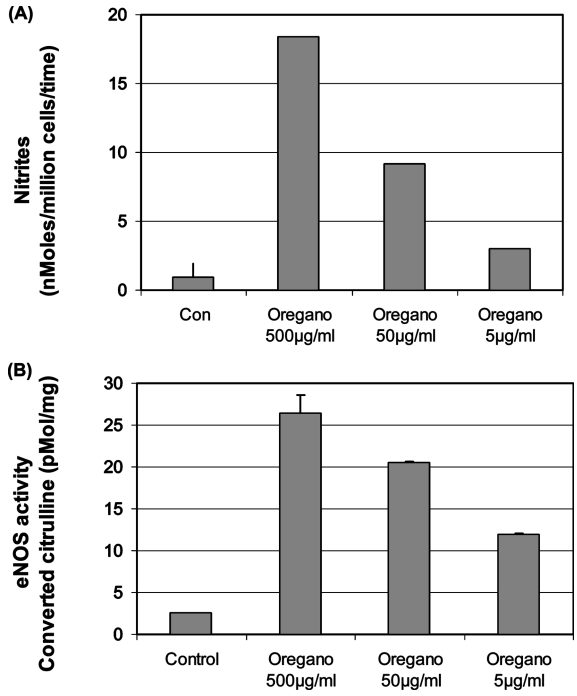

Figure 6. Influence of oregano extract on (**A**) nitrites formation and (**B**) endothelial nitric oxide synthase (eNOS) activity in human endothelial cells.

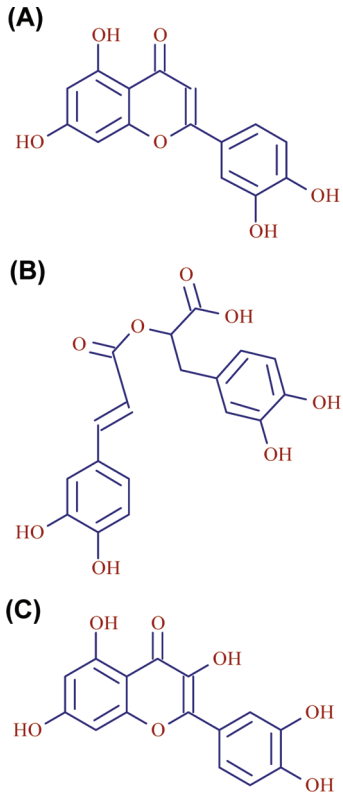

Figure 7. Structures of (**A**) luteolin, (**B**) rosmarinic acid, and (**C**) quercetin.

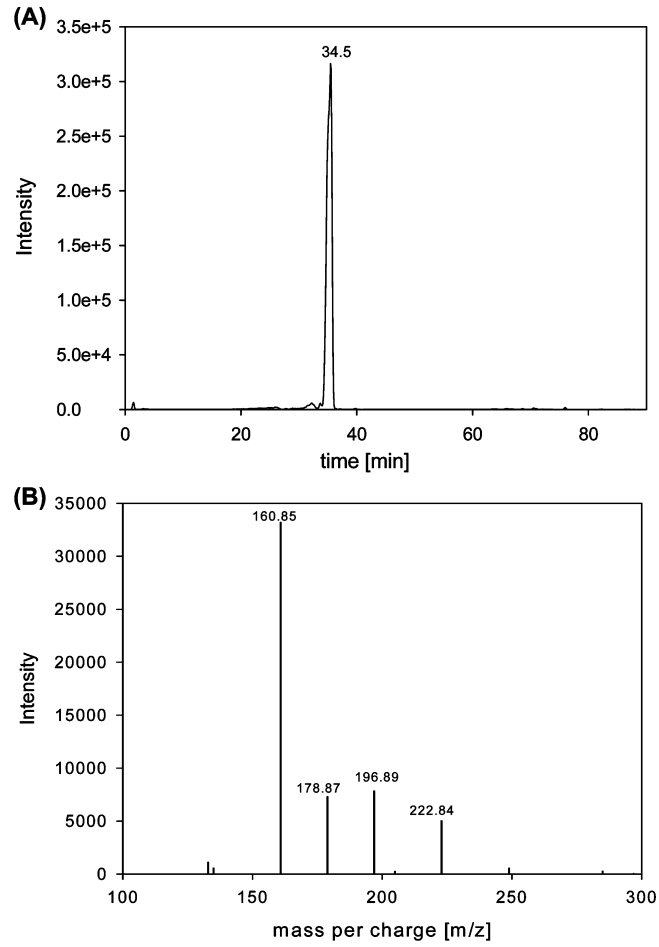

Figure 8. (**A**) MS/MS chromatogram of the mass −1 of rosmarinic acid, 359.3 and (**B**) spectrum of the MS/MS experiment from the precursor mass of 359.0.

Table 3. Retention Time (T_R) and Mass Spectrometry (MS) Data for Standards, Determined by HPLC-MS[a]

substance	T_R (min)	$[M - H]^-$ (*m*/*z*)	fragment ions (*m*/*z*)
quercetin	37.8	301.0	178.8, 150.8, 272.9
rosmarinic acid	31.8	359.0	160.8, 178.8, 196.8, 222.9
luteolin	38.3	285.0	240.9, 198.9, 174.87
biochanin A	53.3	283.1	267.9
isoquercetrin	25.1	463.0	301.9
apigenin	42.1	271.1	224.9, 148.9, 200.1
naringenin	43.1	271.3	150.8, 176.9, 124.9
taxifolin	26.0	303.1	284.9, 176.9, 124.9
apigetrin	32.8	431.2	269.0
chrysoeriol	44.4	299.1	285.1
rutin	27.2	609.1	343.0, 300.9, 270.9
naringin	32.2	579.1	459.0, 270.9, 313.0
kämpferol	44.1	284.9	150.8, 256.9, 240.9
diosmetin	42.0	299.1	283.9
apigenin-7-glycoside	32.8	431.2	268.9
salvianolic acid B	38.9	717.0	518.9, 536.9,321.0
eriodictyol	35.6	287.0	268.9, 242.9, 150.8
diosmin	33.5	607.0	298.9, 283.9

[a] With full MS mode, molecule ions $[M - H]^-$ are derived; in MS/MS mode, fragment patterns are derived, which serves for identification of a specific substance.

associated with a parallel activation of eNOS reaching a maximum of approximately 10-fold induction (**Figure 6**), was observed.

To determine the occurrence of compounds in oregano, the extract with the highest PPARγ-binding affinity (extract no. 1) was further analyzed by HPLC-MS. With the use of authentic standards, certain compounds could be identified as rosmarinic acid, quercetin, luteolin, biochanin A, isoquercetrin, apigenin, naringenin, taxifolin, apigetrin, chrysoeriol, rutin, naringin, kämpferol, diosmetin, apigenin-7-glycoside, salvianolic acid B, eriodictyol, and diosmin (**Figures 7** and **8** and **Tables 3** and **4**). Rosmarinic acid was the most abundant phenolic compound in the oregano extract. The MS/MS spectrum of rosmarinic acid (MS-1 = 359.3 g) is shown in **Figure 6** with the most abundant

Table 4. Retention Time (T_R) and MS Data for the Main Compounds Present in *O. onites* (Oregano Extract 1)[a]

T_R (min)	$[M - H]^-$ (*m*/*z*)	fragment ions (*m*/*z*)	compound identified
53.0	283.1	268.0	biochanin A
38.0	301.2	178.8, 150.8, 272.9	quercetin
32.4	359.3	160.8, 178.8, 196.8, 222.9	rosmarinic acid
24.8	463.4	301.9	isoquercetrin
39.0	285.2	240.9, 198.9, 174.87	luteolin
43.0	271.3	150.8, 176.9, 124.9	naringenin
25.9	303.2	284.9, 176.9, 124.9	taxifolin
32.6	431.4	269.0	apigetrin
26.25	299.3	285.1	glycoside of chrysoeriol
27.0	609.5	343.0, 300.9, 270.9	rutin
39.5	717.6	518.9, 536.9,321.0	salvianolic acid B
36.7	287.3	268.9, 242.9, 150.8	eriodictyol
33.6	607.0	298.9, 283.9	diosmin
44.4	284.9	150.8, 256.9, 240.9	kaempferol
42.2	299.1	298.9, 283.9	diosmetin
42.2	271.1	224.9, 148.9, 200.1	apigenin

[a] With full MS, molecule ions $[M - H]^-$ are derived; with MS/MS, fragment patterns are derived, which serves for identification of a specific substance.

peak at a retention time of 34.5 min and the main fragment of 160.85 g. The dimer of rosmarinic acid, salvianolic acid B, was the second most abundant peak. All other compounds occurred only in small amounts.

DISCUSSION

Metabolic syndrome is a multifactorial, severe health problem that affects a large number of people worldwide. This syndrome comprises metabolic risk factors such as abdominal obesity, insulin resistance, hyper/dyslipidemia, and hypertension, a combination that increases the risk for developing cardiovascular disease or type 2 diabetes (*1*). Primary treatment of this syndrome should be a change in lifestyle, including increased physical activity, calorie restriction, and changes in diet. If the cardiovascular risk is too high or the lifestyle changes are not sufficient, medical treatment of the metabolic syndrome is recommended. Treatment of several perturbations of the metabolic syndrome is recommended for reducing cardiovascular risk because the risk factors have a multiplicative impact on cardiovascular risk (*1*).

PPARs have been shown to have a positive effect on the disorders related to metabolic syndrome (*2*). Oregano extract is a putative candidate for treating several aspects of metabolic syndrome, and we found that different oregano extracts are moderate PPARγ ligands. Oregano extract contains mainly PPARγ antagonists such as quercetin, luteolin, rosmarinic acid, and diosmetin, SPPARγMs like naringenin and apigenin, and PPARγ agonists like biochanin A.

Extracts of oregano and almost all associated substances moderately antagonized rosiglitazone-mediated DRIP205/TRAP220 recruitment. This cofactor was recently classified among the so-called "adipogenic factors", indicating that it promotes adipogenesis and adipocyte differentiation in cell culture and thus weight gain in humans. PPARγ antagonists have been suggested to have potential as antiobesity drugs (*3, 4*). Recently, it was shown that insulin resistance is also improved by PPARγ antagonists; because of a lower adipocyte mass, lower levels of adipokines that promote insulin resistance are secreted, thus resulting in a reduced risk of developing diabetes (*4*). Hypoglycemic activity of oregano extract also has been reported, but the proposed mechanism differs. It was proposed that oregano extracts exert hypoglycemic activity through inhibition of pancreatic amylase and α-glucosidase (*7*−*10*).

As mentioned, oregano extract contains the PPARα agonists rosmarinic acid and biochanin A, which might be expected to contribute to an improved lipid profile. However, because the extract did not transactivate the receptor, the agonistic activity of those substances may be too low or oregano may also contain PPARα antagonists that counteract the agonistic activity.

Oregano extracts also have the potential to treat secondary complications of diabetes. Human endothelial cells are central for the function of human vessels in physiological and pathological conditions, and the synthesis of the vasodilatory and anti-inflammatory molecule NO by endothelial cells is of paramount importance for vascular function (*28*). As an activator of eNOS, oregano extract has the potential to improve endothelial dysfunction and thus atherosclerosis (*6*). The oregano compounds quercetin and kaempferol showed inhibitory effects on glucose-induced low-density lipoprotein lipid peroxidation, which helps to prevent arteriosclerosis (*29*).

Currently, diabetes is treated with glitazones like rosiglitazone and pioglitazone, and fibrates are used for treatment of dyslipidemia. Limitations and side effects of these drugs, such as weight gain and edema, have reinforced the need for drugs that ameliorate perturbations of metabolic syndrome without severe side effects (*30*). Oregano extracts represent an interesting alternative for treatment of obesity and metabolic syndrome because of its wide spectrum of health-ameliorating effects, starting with the prevention of obesity and thus of metabolic syndrome, and ameliorating metabolic syndrome and diabetic complications. We showed that it is a candidate for investigation in the treatment of obesity and diabetes via PPARγ antagonism, treatment of hyperlipidemia because it contains PPARα agonists, and the prevention and amelioration of atherosclerosis by eNOS activation. Literature reports showed the hypotensive effect of oregano extracts or its compounds (*9, 12*).

Further studies are required for standardizing oregano extracts for treatment of obesity and related disorders. Species, climate, and growing area seem to have important influences on the synthesis of PPARγ antagonists.

Here, we present only putative effects of oregano extract based on in vitro data. In vivo assays and clinical trials would be necessary to confirm any health-ameliorating effects of oregano extract in humans and to decide which biologic properties contribute to amelioration of metabolic syndrome. It would also be of interest to evaluate whether different mechanisms act together in producing beneficial effects.

In conclusion, oregano extract contains PPARγ antagonists, like quercetin, luteolin, rosmarinic acid, and diosmetin, SPPARγMs like naringenin and apigenin, PPARγ agonists, like biochanin A, and PPARα agonists rosmarinic acid and biochanin A. Rosiglitazone-mediated DRIP205/TRAP220 recruitment is antagonized by oregano extract and nearly all components, which could contribute to weight reduction. We also demonstrated activation of eNOS, which has the potential to improve diabetes-related vascular complications. Further studies are necessary to determine whether administration of oregano extract could aid in preventing the development of metabolic syndrome or in its treatment and amelioration of its complications.

ABBREVIATIONS USED

DMEM, Dulbecco's minimum essential medium; DMSO, dimethylsulfoxide; eNOS, endothelial nitric oxide synthase; DXS, 1-deoxy-D-xylulose 5-phosphate synthase; FCS, fetal calf serum; GST, glutathione-*S*-transferase; HUVECs, human um-

bilical vein endothelial cells; ITS, internal transcribed spacer; LBA, ligand binding assay; LBD, ligand binding domain; NO, nitric oxide; PPAR, peroxisome proliferator-activated receptor; SPPARγMs, selective PPARγ modulators; TR-FRET, time-resolved fluorescence resonance energy transfer.

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ANNEX TO PART 2

The Essential Oil Composition of Wild Growing Sweet Marjoram (*Origanum majorana* L. Lamiaceae) from Cyprus – Three Chemotypes

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The Essential Oil Composition of Wild Growing Sweet Marjoram (*Origanum majorana* L., Lamiaceae) from Cyprus –Three Chemotypes

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Abstract

Individual plants grown in a greenhouse from seeds of a natural population of sweet marjoram (*Origanum majorana* L.) from Cyprus were analyzed for their essential oil composition. Three chemotypes were detected in the population. Besides the standard 'marjoramy' composition ('sabinyl chemotype') with 74% of oil compounds belonging to the bicyclic compounds sabinene, *trans*- and *cis*-sabinene hydrate and *cis*-sabinene hydrate acetate ('sabinyl compounds'), two further chemotypes were present in the population, namely a pure α–terpineol chemotype (73% α–terpineol) and a mixed sabinyl/α–terpineol chemotype (41% sabinyl compounds, 40% α–terpineol). The chemotype frequencies found in this population were 56% of the plants belonging to the sabinyl chemotype, 4% to the pure α–terpineol chemotype and 40% to the mixed sabinyl/α–terpineol chemotype.

Key Word Index

Origanum majorana L., Lamiaceae, essential oil composition, biosynthesis, *cis*-sabinene hydrate, *trans*-sabinene hydrate, *cis*-sabinene hydrate acetate, α–terpineol.

Introduction

'Sweet marjoram' (*Origanum majorana* L. syn. *Majorana hortensis* Moench, Lamiaceae) is an herbaceous, perennial plant native to the Eastern Mediterranean. It grows on dry and rocky limestone hillsides 100 m to 1500 m above sea level, and flowers from May to September (1).

In the food industry marjoram is mainly used as an aromatic herb in sausages and meat dishes. The essential oil is added to baked goods, processed vegetables, condiments, soups, snack foods and gravies (2). The main compounds of the oil of cultivated marjoram are the bicyclic monoterpene alcohols *trans*- and *cis*-sabinene hydrate and the acetate of *cis*-sabinene hydrate (3). *Cis*-sabinene hydrate is a compound of intensive spicy, 'marjoramy' aroma, whereas *trans*-sabinene hydrate has no typical 'marjoramy' properties (4–6). The often reported high contents of terpinen-4-ol, α–terpinene and γ–terpinene in the essential oil (examples cited by Lawrence (7,8)) are due to component rearrangements occurring during the process of distillation (9–10).

Skoula et al. (11) grouped *trans*-sabinene hydrate, *cis*-sabinene hydrate and their acetates together with sabinene as 'sabinyl-compounds', whereas they summarized the oregano typical compounds (γ–terpinene, p-cymene, thymol and carvacrol) as the 'p-cymyl group'. They also reported the existence of hybrids of *Origanum* species containing both groups of compounds in which the p-cymyl group was present in far greater abundance than the sabinyl group. The sabinyl group, however, was never completely absent.

The wild growing *O. majorana* from the south-western part of Turkey (12) was found to be rich in carvacrol. In *Origanum majorana* var. *tenuifolium* (13) from Cyprus, however, the typical 'marjoram' compounds (sabinyl group) were predominant, whereas the p-cymyl group was almost absent. The only difference to cultivated marjoram was the elevated content of α–terpineol. Novak et al. (3) found in an old sample of marjoram (age estimated to more than 60 years) both groups to be present (the sabinyl-group as well as the p-cymyl group), a fact also found by Pino et al. (14) from marjoram plants grown at an experimental station in Cuba.

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Table I. Essential oil compounds of marjoram of the first and second sampling with their retention indices (RI). Values are mean ± standard error of mean.

Compound	RI	Chemotype (no. of plants)		
		sabinyl chemotype (n=28)	*mixed chemotype* (n=18)	α*–terpineol chemotype* (n=2)
α–thujene	930	0.5 ± 0.01	0.3 ± 0.01	0.1 ± 0.01
α–pinene	936	1.1 ± 0.04	0.7 ± 0.03	0.5 ± 0.05
camphene	951	0.5 ± 0.04	0.6 ± 0.04	0.7 ± 0.08
sabinene	976	9.4 ± 0.23	5.2 ± 0.17	1.1 ± 0.02
β–pinene	978	0.6 ± 0.03	0.3 ± 0.04	0.2 ± 0.01
myrcene	993	2.0 ± 0.03	1.5 ± 0.03	1.0 ± 0.01
limonene/β–phellandrene	1031	3.3 ± 0.05	2.3 ± 0.04	1.3 ± 0.01
1,8-cineole	1034	0.6 ± 0.04	0.6 ± 0.06	0.4 ± 0.16
(Z)-β–ocimene	1043	0.1 ± 0.03	0.0 ± 0.02	0.0 ± 0.00
γ–terpinene	1063	0.2 ± 0.01	0.0 ± 0.01	0.0 ± 0.00
trans-sabinene hydrate	1072	4.1 ± 0.10	2.4 ± 0.11	0.7 ± 0.22
cis-sabinene hydrate/linalool	1102	60.6 ± 0.90	32.6 ± 0.47	4.2 ± 1.20*)
borneol	1172	1.8 ± 0.10	1.9 ± 0.10	2.2 ± 0.03
terpinen-4-ol	1183	0.3 ± 0.02	0.2 ± 0.01	0.2 ± 0.02
α–terpineol	1196	3.7 ± 0.13	40.0 ± 0.74	72.5 ± 2.59
cis-sabinene hydrate acetate	1258	1.0 ± 0.13	0.7 ± 0.09	0.0 ± 0.00
linalyl acetate	1260	3.4 ± 0.30	3.7 ± 0.37	6.4 ± 1.91
bornyl acetate	1290	0.2 ± 0.02	0.2 ± 0.02	0.2 ± 0.07
unknown	1341	0.2 ± 0.01	0.3 ± 0.02	0.4 ± 0.02
β–caryophyllene	1425	2.3 ± 0.10	2.3 ± 0.10	3.5 ± 0.50
α–humulene	1459	0.1 ± 0.01	0.1 ± 0.01	0.2 ± 0.02
bicyclogermacrene	1501	2.6 ± 0.10	3.1 ± 0.15	3.7 ± 0.20
germacrene-D-4-ol	1584	0.3 ± 0.07	0.7 ± 0.08	0.2 ± 0.01

*) linalool only, no *cis*-sabinene hydrate present

It is interesting to note that in wild growing populations of *O. majorana cis*-sabinene hydrate acetate was never detected, while it is one of the main compounds in cultivated marjoram (3). Even in cultivated marjoram, the concentration of this acetate is subject to chemotype variation with a complete absence in individual plants of many accessions (15).

The aim of this study is to characterise the essential oil composition of *Origanum majorana* from a population from Cyprus based on the analysis of individual plants.

Experimental

Samples of* Origanum majorana*: Seeds *of Origanum majorana* L. were collected from a wild population near the village of Vouni, Limassol district, Cyprus, in April 2000 (remaining seeds from 1999). Voucher specimens were deposited at the Herbarium of the Institute for Applied Botany, University of Veterinary Medicine. The seeds were germinated and cultivated in the greenhouse under conditions of 24°C day and 15°C night temperature. Artificial light was supplied to complement daylight to a constant 14 h day length with 'full sunshine' (optimized assimilation programme). Plants of cultivated marjoram (*Origanum majorana* cv. 'Erfo', N.L.Chrestensen, Erfurt, Germany) were grown in parallel for comparison. The plants from the wild population were sampled at the stage of flower bud development in October, 2000. The plants of cv. 'Erfo' were not sampled and were not analyzed since they started to flower much earlier and, hence, could not be directly compared to the wild population.

Extraction and GC/MS-analyses: Two tenth of a gram of the dried tissue from each plant was extracted with 1.5 mL dichloromethane in an ultrasonic bath for 15 min. GC/MS-analyses were performed on a HP 6890 coupled with a HP 5972 MSD and fitted with Rtx-5MS 30 m x 0.25 mm capillary column coated with a film thickness of 0.25 µm. The analytical conditions were: Helium as carrier gas, injector temperature at 250°C, split ratio 20:1, temperature program 40–240°C with 3°C/min. Components were identified by comparing their retention indices and spectra to published retention times and spectral data (16,17).

The sabinene hydrate ratio (*trans*-sabinene hydrate / (*cis*-sabinene hydrate + *cis*-sabinene hydrate acetate)) (18) was calculated where a baseline separation between *cis*-sabinene hydrate and linalool allowed a correct integration and separation of these compounds. Due to the extremely high contents of *cis*-sabinene hydrate in some plants, a correct separation between *cis*-sabinene hydrate and linalool was not always possible.

Results and Discussion

Although the plants from the wild population were undoubtedly *Origanum majorana* L., the differences to *O. majorana* cv. 'Erfo' (a standard cultivar in large scale marjoram cultivation) regarding plant development were very high. Plants of cultivar 'Erfo' germinated much faster and started their generative cycle (bud formation) weeks before the plants from Cyprus. 'Erfo' was much greener in its leaf color compared to a more greyish/silvery look in the plants from the wild population where

also the leaves were in general smaller and more numerous (data not shown). The plants from the wild population were more sensitive to stress that was imposed on them by standard greenhouse conditions leading once even to a complete loss of their leaves. However, the leaves regenerated rather quickly from the nodes at the deciduous plants.

Chemotype formations: In this study, the p-cymyl compounds (γ–terpinene, p-cymene, carvacrol and thymol) were present in all plants but at very low levels (< 0.5%). Therefore there is no similarity to the populations rich in p-cymyl compounds from south-eastern Turkey (12) and to the 'mixed' p-cymyl/sabinyl types from Cuba (14) or from Austria (3). The population studied here resembles the oil composition found in cultivated marjoram (3) or other populations from Cyprus (13) consisting mainly of sabinyl-compounds and α–terpineol.

Within the population three distinct chemotypes could be identified (Table 1), a 'marjoram typical' sabinyl-chemotype (74% sabinyl compounds, i.e. sabinene, *trans*- and *cis*-sabinene hydrate and *cis*-sabinene hydrate acetate), a pure α–terpineol chemotype (73% α–terpineol) and a mixed chemotype (41% sabinyl compounds and 40% α–terpineol). 56% of all plants analyzed were consistent with the pattern of the sabinyl chemotype, 4% with the pure α–terpineol chemotype and 40% with the mixed chemotype.

A chemotype with a high content of α–terpineol, as previously described in *O. ramonense* (19), is rather rare in the genus *Origanum.* The reason why Arnold et al. (13) found an elevated content of α–terpineol in a population of *O. majorana* var. *tenuifolia* from Cyprus in comparison to cultivated marjoram might be that only one sample had been analysed, probably consisting of plants of different chemotypes. A high number of individuals of the mixed or α–terpineol chemotype in the sample might have resulted in an atypically high content of α–terpineol.

Although the high content of α–terpineol leads to a pleasant floral odor, it radically changes the sensorial impression that is typical for marjoram.

Two plants of the α–terpineol chemotype were especially interesting because they lacked the ability to form any sabinyl compound. Not only the formation of *cis*-sabinene hydrate was completely suppressed, but also the formation of sabinene and *trans*-sabinene hydrate. Of course, no acetates of *trans*- or *cis*-sabinene hydrate could be found in these two plants. The simultaneous absence of *trans*- and *cis*-sabinene hydrate was expected because it has been demonstrated, that in cultivated marjoram (*O. majorana*) the same enzyme ('sabinene hydrate synthase', EC 4.6.1.9) produces both, *trans*- and *cis*-sabinene hydrate (20,21). It could not be expected, however, that sabinene would be missing too, because sabinene was not an end-product of the purified enzyme preparations which resulted in the formation of *cis*- and *trans*-sabinene hydrate. It was suggested, that sabinene in marjoram would be formed by a different way. This result indicates a mechanism of suppression of a whole group of most probably similar enzymes.

Ratio of sabinene hydrates: In cultivated marjoram (O. majorana) the ratio of the sabinene hydrates was found to be stable and characteristic, while it was very variable in wild populations of *O. microphyllum* (18,20). This ratio was found to be quite constant but different to above mentioned studies in our population from Cyprus with values of 0.079 ± 0.003 (mean ± standard error of mean). The ratio was found to be in it's variability as narrow as in different accessions of cultivated marjoram (18), but the level was closer to the ratio of 1:10 observed by Hallahan and Croteau (20) than the 1:20 ratio found by Novak et al. (18).

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Validation of a Quantitative Assay of Arbutin using Gas Chromatography in *Origanum majorana* and *Arctostaphylos uva-ursi* Extracts

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Validation of a Quantitative Assay of Arbutin using Gas Chromatography in *Origanum majorana* and *Arctostaphylos uva-ursi* Extracts

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ABSTRACT:

Introduction – Arbutin is a skin-whitening agent that occurs naturally in the bark and leaves of various plants. It is commonly quantified in plant extracts and skin-whitening products by HPLC.

Objective – To develop an alternative gas chromatographic method for the separation and quantification of arbutin in *Origanum majorana* and *Arctostaphylos uva-ursi* extracts.

Methodology – *N,O*-Bis(trimethylsilyl)acetamide and trimethylchlorosilane were used as silylation reagents, and the gas chromatographic separation of silylated extracts and standards was performed using a DB-5 narrow bore column. GC-MS was used for the compound identification, and the quantification was carried out by GC-FID. The quantitative results were compared with those of HPLC analysis.

Results – The developed method gave a good sensitivity with linearity in the range 0.33–500 mg/mL and recovery >98%, allowing the quantification of arbutin in *O. majorana* and A. *uva-ursi* extracts. The relative standard deviations (RSD) relating to intra-day and inter-day precision were <0.002% and <4.8%, respectively. The GC results correlated well with those obtained by HPLC analysis.

Conclusion – The analysis of marjoram and bearberry samples showed that the established GC method was rapid, selective, and demonstrated that arbutin could be screened alternatively by gas chromatography.

Keywords: GC-MS; GC-FID; quantitative assay; HPLC; fast-GC; arbutin; *Origanum majorana*; *Arctostaphylos uva-ursi*

Introduction

Arbutin (4-hydroxyphenyl-β-D-glucopyranoside) is a hydroquinone derivative present in marjoram (*Origanum majorana*, Lamiaceae), pears (*Pyrus communis*, Rosaceae) and species of Ericaceae. It is used as a skin whitening agent in cosmetics owing to its inhibitory effect on tyrosinase activity. Arbutin is also known as a sunscreen agent and as an antioxidant (Couteau and Coiffard, 2000; Petkou *et al*., 2002). Leaves of bearberry (*Arctostaphylos uva-ursi*, Ericaceae) may be used internally as a urinary antiseptic by virtue of their high content of arbutin. Extracts of the leaves of bearberry have been widely used in cosmetic preparations to lighten the skin. Hydroquinones, originating from arbutin after hydrolysis, however, exhibit some adverse effects. They have been shown to be hepatotoxic and nephrotoxic, mutagenic and carcinogenic in animal studies (Nowak *et al*., 1995; Peters *et al*., 1997).

A number of methods have been reported for the quantitative and qualitative analysis of arbutin in plant extracts, whitening agents and cosmetic products. Reversed-phase HPLC was found to be the more suitable chromatographic method for arbutin separation (Assaf *et al*., 1987; Lutterbach *et al*., 1993; Masse *et al*., 2001; Parejo *et al*., 2001; Lin *et al*., 2005; Quintus *et al*., 2005). HPLC methods have been improved for the isolation and purification of arbutin and formulation into cosmetic products (Chang and Chang, 2003), and recently an isocratic HPLC method for the separation and quantitative analysis of arbutin in plant tissue cultures has been developed (Kittipongpatana *et al*., 2007). The most sensitive HPLC method was developed by Thongchai *et al*. (2007) for arbutin quantification in skin-whitening creams and medicinal plant extracts with limits of detection and quantification of 0.02 and 0.2 μg/mL per injection volume of 100 μL, respectively. Rapid HPLC methods can resolve arbutin at a retention time of 3.7 min (Parejo *et al*., 2001) and 3.9–4 min (Kittipongpatana *et al*., 2007).

Several other methods for arbutin quantification have been reported, including spectrophotometry (Barsoom *et al*., 2006; Thongchai *et al*., 2009), thin-layer chromatography (Masse *et al*., 2001), capillary zone electrophoresis (Glöckl *et al*., 2001; Lin *et al*., 2007) and chromato-spectrophotometry (Assaf *et al*., 1987). To our knowledge, no validated gas chromatography method has been developed for arbutin quantification in plant extracts.

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In this study, an alternative gas chromatographic method was developed for the separation and quantification of arbutin in *O. majorana* and *A. uva-ursi* extracts. The method allowed the rapid and precise analysis of arbutin in plant extracts, and was applied to the detection and quantification of arbutin in methanol extracts of *A. uva-ursi* and *O. majorana*. The quantification results were compared with those produced by HPLC analysis.

Materials and Methods

Chemicals and solvents. Arbutin, salicin, *N,O*-Bis(trimethylsilyl)-acetamide (BSA) and trimethyl chlorosilane (TMCS) were from Carl Roth (Karlsruhe, Germany). Tetrahydrofuran (THF) was HPLC grade from Scharlau (Barcelona, Spain), and methanol for extraction was of analytical grade (Merck, Darmstadt, Germany). Methanol, HPLC grade, from Carl Roth was used for the HPLC analysis.

Sample preparation. Five leaf samples of marjoram (*O. majorana*) from Cyprus and one leaf sample of bearberry (*A. uva-ursi*) from a local pharmacy (Vienna, Austria) were used in this study. The samples were ground to a fine powder in a mill (Culatti, Zürich, Switzerland) and 50 mg extracted with 8 mL of 50% methanol for 1 h in a cooled ultrasonicator (Bandelin Sonorex, Berlin, Germany). The filtered extracts (Whatman paper filter) were aliquoted into 2 mL Eppendorf tubes and kept at –20°C until analysis.

GC-FID calibration. The standard arbutin and the internal standard (salicin) were dissolved in THF to yield concentrations of 1 mg/mL. Seven calibration levels of arbutin were prepared using THF. A quantity of 170 µL of each arbutin concentration was trimethylsilylated by adding 100 µL of BSA, 10 µL of TMCS and 20 µL of salicin solution (1 mg/mL in THF). The stoppered tubes with the mixtures were placed in an ultrasonic bath for 5 min and kept at 60°C for 30 min.

Derivatisation and GC analytical method. An aliquot of 300 µL of each plant extract was evaporated to dryness using a rotary evaporator at 40°C. The dried extract was dissolved in 170 µL of THF and trimethylsilylated by adding 100 µL of BSA, 10 µL of TMCS and 20 µL of salicin solution (1 mg/mL in THF) as internal standard. The stoppered tubes with the mixtures were placed in an ultrasonic bath for 5 min and kept at 60°C for 30 min. The tubes were cooled to room temperature before GC analysis.

The analysis was carried out using a GC-FID (6890N Network GC system Agilent Technologies, Palo Alto, CA, USA) equipped with a flame ionisation detector and a DB-5 narrow bore column (10 m × 0.1 mm id.; 0.17 µm film thickness; Agilent). Helium (average velocity 45 cm/s) was used as carrier gas and the oven temperature was increased from 240 to 280°C at 20°C/min and held for 3 min at 280°C, with a post run time of 2 min. Samples (0.2 µL) were injected at 260°C front inlet temperature and the split ratio was 50:1.

The GC-MS (HP 6890 coupled to HP 5972 MSD; Hewlett Packard, Palo Alto, USA) was equipped with a HP-5MS column (30 m × 0.25 mm i.d.; 0.25 µm film thickness; Agilent), and data were analysed on a computer equipped with ChemStation software. Helium (average velocity 39 cm/s) was used as carrier gas and the oven temperature was increased from 200 to 275°C at 3°C/min. Samples (1 µL) were injected at 250°C and the split ratio was 1:1. Identification of arbutin and salicin was performed by GC-MS using computer matching with the library, and the retention indices (RI) relative to *n*-alkanes was calculated.

HPLC analysis of arbutin in the extracts. The HPLC analyses were carried out on a Waters (Milford, MA, USA) chromatograph equipped with a quaternary pump, a photodiode array detector and an auto-injector, and Empower software Build 1150 from Waters Corporation was used for data collection and processing. A reversed-phase Luna (Torrance, CA, USA) C18 column (150 × 4.6 mm i.d.; 5 µm) was used and the mobile phase was 95% water and 5% methanol. The elution programme was 15 min isocratic at a flow rate of 2 mL/min. The injection volume was 10 µL and the spectral data were analysed at 280 nm. The concentration of arbutin was determined using a calibration curve established in the concentration range from 50 to 500 µg/mL of standard arbutin.

Statistical validation. The validation of the method was performed according to Bliesner (2006). Limits of detection and quantification were taken as 3 and 10 times the signal-to-noise ratio, respectively. For the precision and accuracy investigation, three replicates of *O. majorana* samples were prepared by spiking arbutin and analysed to evaluate the intra-day variation. The inter-day variation was estimated from the mean of five replicates of *O. majorana* samples on five different days within a 14 day period. Pearson correlation coefficient was used to compare the GC and the HPLC results.

Results and Discussion

The chromatograms of arbutin and salicin (internal standard) after derivatisation are presented in Fig. 1(A, B) and their mass spectra and molecular structure are shown in Fig. 1(C, D). The silylated products were pentakis(trimethylsilyl)hydroquinone-β-D-glucopyranoside for arbutin, and pentakis(trimethylsilyl)2-(hydroxylmethyl)phenyl-β-D-glucopyranoside for salicin. The internal standard salicin with a molecular weight of 286 g/mol (close to that of arbutin at 272 g/mol) was separated at a retention time (RT) of 3.2 min. The retention indices (RI) of the salicin and arbutin derivatives relative to *n*-alkanes on a non-polar column were 2578 and 2648, respectively. The differences in the RT and RI of salicin (3.2 min, RI 2578) and arbutin (3.5 min, RI 2648) were 18.6 s and 70 RI units, respectively. The characteristic ions common to derivatives of both arbutin and salicin were *m/z* 73, 147, 217 and 361. The purity of the arbutin peak in extract chromatograms was investigated by comparing the mass spectra of the compound in the samples with that of the reference.

The chromatographic conditions allowed the separation and determination of arbutin in plant extracts of bearberry and marjoram. The regression equation of the calibration curve was $y = 14.411\ (\pm 0.497)x + 0.037\ (\pm 0.025)$ with a correlation coefficient $r^2 = 0.998$. The detection limit estimated as 3 times the signal-to-noise ratio was 1.6 µg/mL per injection volume of 0.2 µL, and 0.13 µg/mL per injection volume of 1 µL. The quantification limit estimated as 10 times the signal-to-noise ratio was 8 and 0.33 µg/mL per injection volume of 0.2 and 1 µL, respectively. The calibration curve for arbutin was constructed using five replicate analyses and the linearity of the curve was demonstrated in the concentration range 0.33–500 µg/mL. The calibration curves of the five replicates were linear over the concentration ranges tested with a coefficient of determination $R^2 > 0.995$. The recoveries of the method were investigated at concentrations of 33, 67 and 133 µg/mL, and were >98% (Table 2).

The intra-day precision (RSD) evaluated by performing three replicates of three spiked samples was between 0.002 and 0.004%. The inter-day precision was evaluated by performing five replicate analyses of three spiked *O. majorana* samples. The inter-day

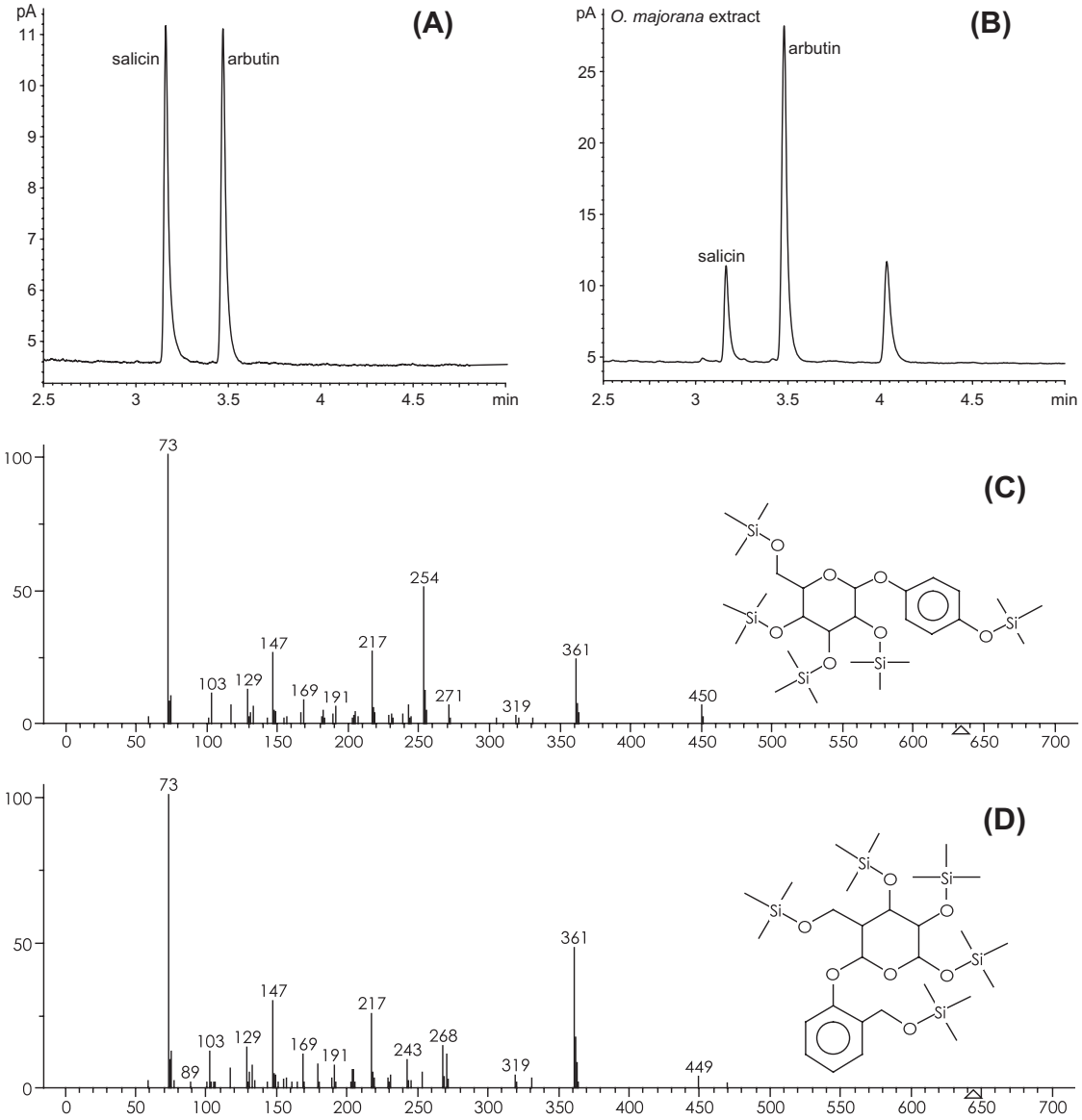

Figure 1. GC-FID and GC-MS analysis of arbutin using salicin as internal standard: (A) GC/FID of a mixture of pure substances; (B) leaf extract of *O. majorana* + salicin as internal standard; (C) mass spectrum of silylated arbutin with characteristic ions at *m/z* 73, 254, 361, 217, 147; (D) mass spectrum of silylated salicin with characteristic ions at *m/z* 73, 361, 147, 217, 268.

precision (RSD) on the basic of content of arbutin was between 2.5 and 4.8% (Table 2).

In comparison with reported methods, the described gas chromatographic protocol (Tables 1 and 2) showed higher sensitivity and precision than the HPLC method of Kittipongpatana *et al*. (2007), previously developed for arbutin quantification in plant tissue cultures, where the limit of detection was 5 µg/mL with 0.4% intra-day RSD, and that of Glöckl *et al*. (2001) using capillary zone electrophoresis with a limit of quantification of 20 µg/mL with 1.7–5.1% inter-day RSD. The HPLC and spectrophotometric methods developed by Thongchai *et al*. (2007, 2009) for arbutin quantification in skin whitening creams and medicinal plant extracts seem to be more sensitive (Table 1), but when considering the injection volumes, the present GC offers a higher sensitivity with a detection limit of 0.02 ng per injection volume of 1 µL. The spectrophotometric method of Barsoom *et al*. (2006) for arbutin determination in whitening agent had a limit of quantification of 25 µg/mL with 6.2% RSD.

Arbutin was identified and quantified in methanol extracts of five samples of *O. majorana* and one sample of *A. uva-ursi* by GC and HPLC. Each sample was analysed in triplicate and the results are shown in Fig. 2. The GC analysis results were 45.7 mg/g dried weight in bearberry and 33.5–59.8 mg/g dried weight in marjoram samples. The HPLC analysis ($y = 1.925x + 1.6379$; $r^2 = 0.999$) of arbutin content in the same samples gave 46.9 mg/g dried weight in the bearberry and 33.5–61.3 mg/g dried weight in marjoram samples. The arbutin retention time of the HPLC analysis was 2.4 min for an elution programme of 15 min. A highly significant correlation of 0.99 ($p = 0.001$) was obtained between the GC and the HPLC data. These values are also comparable with those of the HPLC methods for both bearberry (Parejo *et al*., 2002) and marjoram (Assaf *et al*., 1987) samples.

Table 1. Sensitivity and linearity of the developed gas chromatographic method compared with other methods

Methods of quantification of arbutin	Injection volume	Limit of detection		Limit of quantification		Linear range
	(μL)	(μg/mL)	(ng)	(μg/mL)	(ng)	(μg/mL)
Developed GC method	0.2	1.6	0.33	8	1.6	8–500
	1	0.13	0.02	0.33	0.06	0.33–500
HPLC (Masse *et al*., 2001)	20	1.85	37	—	—	—
HPLC (Chang *et al*., 2005)	25	—	—	—	—	10–300
HPLC (Kittipongpatana *et al*., 2007)	10	—	50	—	160	16–1024
HPLC (Thongchai *et al*., 2007)	100	0.02	2	0.2	20	0.5–30
Capillary electrophoresis (Lin *et al*., 2007)	—	5.4	—	—	—	20–200
Spectrophotometry (Barsoom *et al*., 2006)	—	25	—	—	—	25–125
Spectrophotometry (Thongchai *et al*., 2009)	300	0.04	12	0.13	39	1–30

Table 2. Recovery and precision for the determination of arbutin in *O. majorana*

	Amount spiked (μg/mL)	Amount found mean ± SD (μg/mL)[a,b]	Recovery mean (%)[b]	Precision (RSD%)	
				Intra-day[b]	Inter-day[c]
Arbutin	33	32.37 ± 0.001	98.08	0.004	3.24
	67	66.35 ± 0.002	99.03	0.002	2.48
	133	132.91 ± 0.002	99.94	0.002	4.76

[a]Determination on one day.
[b]$n = 3$.
[c]$n = 3$ replicates × 5 days within 14 day period.

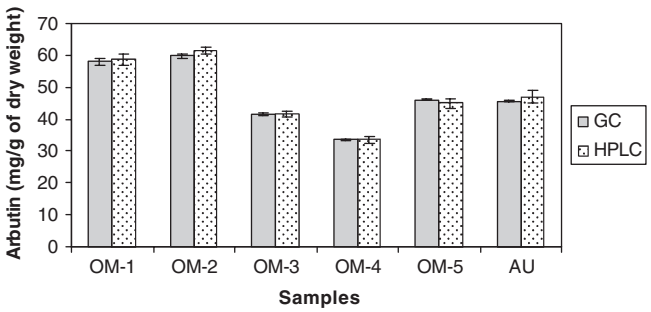

Figure 2. Content (mg/g) of arbutin in samples of *O. majorana* (OM) and *A. uva-ursi* (AU) obtained by GC and HPLC analysis.

The developed gas chromatographic method is simple, sensitive, rapid and reproducible for the detection and quantification of arbutin in plant extracts. A good separation of arbutin from the other components in the plant was obtained within 5 min and the GC results correlated well with those of the HPLC analysis. The established fast-GC method demonstrated that arbutin could be detected at a wide range of amount, and screened alternatively by gas chromatography.

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ANNEX TO PART 3

Temperature Influences Thymol and Carvacrol Differentially in *Origanum* spp. (Lamiaceae)

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ABSTRACT

The composition of secondary metabolites is often modified by environment and ontogenesis. To explicitly study the influence of temperature on essential oil compositions, an experiments on a hybrid of *Origanum vulgare* x *Origanum majorana* (containing both major monoterpene pathways of the genus *Origanum*, 'sabinyl' and 'cymyl'-pathway) and on *Origanum syriacum* ssp. *syriacum* (only 'cymyl'-pathway) were conducted in growth chambers. Clonally propagated plants were grown at three different temperature levels and the solvent extracts analyzed by GC/MS for their composition of essential oil compounds. The major 'sabinyl'-compound *cis*-sabinene hydrate was not influenced by temperature, while temperature significantly influenced thymol and carvacrol and other essential oil compounds. Although thymol and carvacrol are closely related monoterpene phenols, they reacted to varying levels of temperature in an opposite way. Thymol increased with decreasing temperatures while carvacrol increased with increasing temperatures.

KEYWORDS. *Origanum vulgare* x *Origanum majorana*, *Origanum syriacum* ssp. *syriacum*, Lamiaceae, temperature, essential oil compounds, carvacrol, thymol, *cis*-sabinene hydrate

Introduction

The genus *Origanum* contains two important aromatic plants with different sensorial qualities, marjoram (*Origanum majorana* L.) and oregano (*Origanum* sp., but also species from other genera with similar sensorial qualities are called 'oregano' *(1)*. The bicyclic monoterpene *cis*-sabinene hydrate and its acetate derived from the 'sabinyl' pathway are responsible for the quality of marjoram, while the phenolic monoterpene carvacrol, arising from the 'cymyl' pathway *(2)*, is the character compound of oregano (Scheme 1). The monoterpene phenols carvacrol and thymol are well known for their high antimicrobial *(3-6)* and antioxidant activities (7,8).

The composition of such complex mixtures like essential oils is principally genetically determined but may vary under different environmental conditions *(9)*. The term 'environmental conditions' is a complex term sub summarising many different factors, like e.g. water supply and light intensity *(10)*, daylength *(11)* or temperature *(12-15)*.

The temperature sometimes has and sometimes has not influence on the essential oil compositions. Clark and Menary *(15)* found that low temperatures lead to higher concentrations of 1,8-cineole and menthone and lower concentrations of pulegone in peppermint (*Mentha* x *piperita*). *Cymbopogon nardus* raised at 27°C/21°C showed elevated citronellol content compared to 32°C/27°C *(14)*. Duriyaprapan et al. *(16)*, however, could not find any influence of temperature on menthol content in Japanese mint (*Mentha canadensis*).

Dudai *et al. (17)* used a thymol chemotype of *O. syriacum* to analyse the effect of day length as well as temperature on composition. Under long-day conditions, the temperature had no influence on the composition. However, when kept under short-day conditions, the composition changed significantly with γ-terpinene and thymol decreasing and p-cymene increasing with increasing temperatures.

In this paper we present results of the oil compositions from two different *Origanum* species of two different chemotypes (a 'mixed' type with both pathways (sabinyl/p-cymyl) and a pure p-cymyl chemotype (Scheme 1)) raised at different temperatures.

Material and Methods

Plant Material

Cuttings were prepared from two single plants of *Origanum* sp. cv. 'Kaliteri' (Richters, Goodwood, Canada) and *Origanum syriacum* ssp. *syriacum* and potted in 17x17cm pots. Voucher specimens were deposited at the Herbarium of our institute.

The plants were grown in triplicate in growth chambers at three different temperature levels (18°C, 22°C and 26°C) kept constant at day and night under long-day conditions with 16h day length.

Extraction and GC/MS Analyses

0.2 g of the dried herb was extracted with 1.5 mL of dichloromethane for 15 minutes in an ultrasonic bath. The extract was filtered and put into the sample vial for GC/MS analyses.

GC/MS analyses were performed on an HP 6890 coupled with a HP 5972 MSD and fitted with an Rtx-5MS 30m x 0.25mm capillary column (0.25 µm film thickness). The analytical conditions were: carrier gas helium, injector temperature 250°C, split ratio 50:1, temperature programme 60-280°C with 3°C/min.

Retention Indices (RI) of the sample components were determined on the basis of homologous n-alkane hydrocarbons under the same conditions. The quantitative composition was obtained by peak area normalization, and the response factor for each component was considered to equal 1. The compounds were identified by comparing their retention indices and mass spectra with published data.

Results and Discussion

The two genotypes investigated in this study represent two different chemotypes. *O.* sp. cv. 'Kaliteri' is a mixed type with both main pathways of the genus *Origanum* showing *cis*-sabinene hydrate (sabinyl pathway) and carvacrol (p-cymyl pathway) as main compounds.

The variety 'Kaliteri', offered on the market as *Origanum* sp., proved to be a hybrid between *Origanum vulgare* and *Origanum majorana* (identification based on the shape of the calyces *(18)* and DNA-sequencing (data not shown)). *Origanum syriacum*, however, was a p-cymyl pathway chemotype with carvacrol as the dominating major compound.

cis-Sabinene hydrate in *O. vulgare* x *O. majorana* cv. 'Kaliteri' was not affected at all by increasing temperature, while sabinene (another compound of the sabinyl pathway) (Scheme 1), increased linearly from 3.9% to 5.3% (Table I). The difference seemed to be rather small, but proved to be highly significant. Myrcene significantly increased in both species from 18°C to 22°C and remained equal between 22°C and 26°C. *p*-Cymene, however, showed an opposite trend in both species, an increase in *O. vulgare* x *O. majorana* and a decrease in *O. syriacum* which was a pronounced effect in this species from 2.9% at 18°C to 0.5% at 26°C. In *O. vulgare* x *O. majorana* γ-terpinene followed the trend of p-cymene, while this compound just slightly increased in *O. syriacum*. *trans*-Sabinene hydrate slightly increased in both species from 18°C to 22°C while the main compound of *O. vulgare* x *O. majorana*, *cis*-sabinene hydrate, remained constant.

Interestingly, thymol significantly decreased while carvacrol, the main compound in *O. syriacum,* increased in both species with increasing temperatures. Both very similar compounds are synthesised from the precursor p-cymene by two still hypothetical hydroxylases (Scheme 1). This differential reaction of the two compounds to environmental influences is a further indication for the existence of two independent hydroxylases for thymol and carvacrol, respectively.

The differences in the oil composition under short day conditions found by Dudai *et al. (17)* seem to be on the first glance contradictory to our results. But considering the different chemotypes used and the biosynthesis of the monoterpene phenols thymol and carvacrol the results are plausible. Duday *et al. (17)* used a thymol chemotype with very low amounts of carvacrol (below 2%). Higher temperatures may hinder the activity of the thymol hydroxylase in the thymol chemotype. This could

lead in absence of the carvacrol hydroxylase to an increase of the precursor p-cymene, while in a carvacrol chemotype higher temperatures favour the carvacrol hydroxylase leading to an increase of carvacrol and a decrease in the precursor p-cymene.

Kokkini *et al. (19)* observed in *Origanum vulgare* ssp. *hirtum* an increase of p-cymene in autumn oil (collected in November in Greece) compared to the summer oil. The explanation of this increase could be the lower activity of both hydroxylases at cooler temperatures in late autumn which would then result in an accumulation of p-cymene due to a bottleneck-situation for the next step in biosynthesis. Carvacrol content was always diminished in their study. Thymol, however, reacted controversially with a decrease in the (cooler) Northern population and an increase in the (warmer) Southern population. The reaction of the Southern population would be in accordance with our hypothesis, the November temperature at the Northern population may be already below the optimal range for the thymol hydroxylase.

Whether the differential reaction of thymol and carvacrol to temperature is regulated on the level of gene expression or rather simply an effect of temperature on the kinetic properties of the enzymes remains to be clarified, since the gene sequences for the enzymes as well as the enzymatic properties are not described yet.

Although thymol and carvacrol are structurally quite similar, two different enzymes are necessary for their formation from the precursor p-cymene. Our results demonstrate that the synthesis of both similar monoterpene phenols is influenced by temperature in an opposite way.

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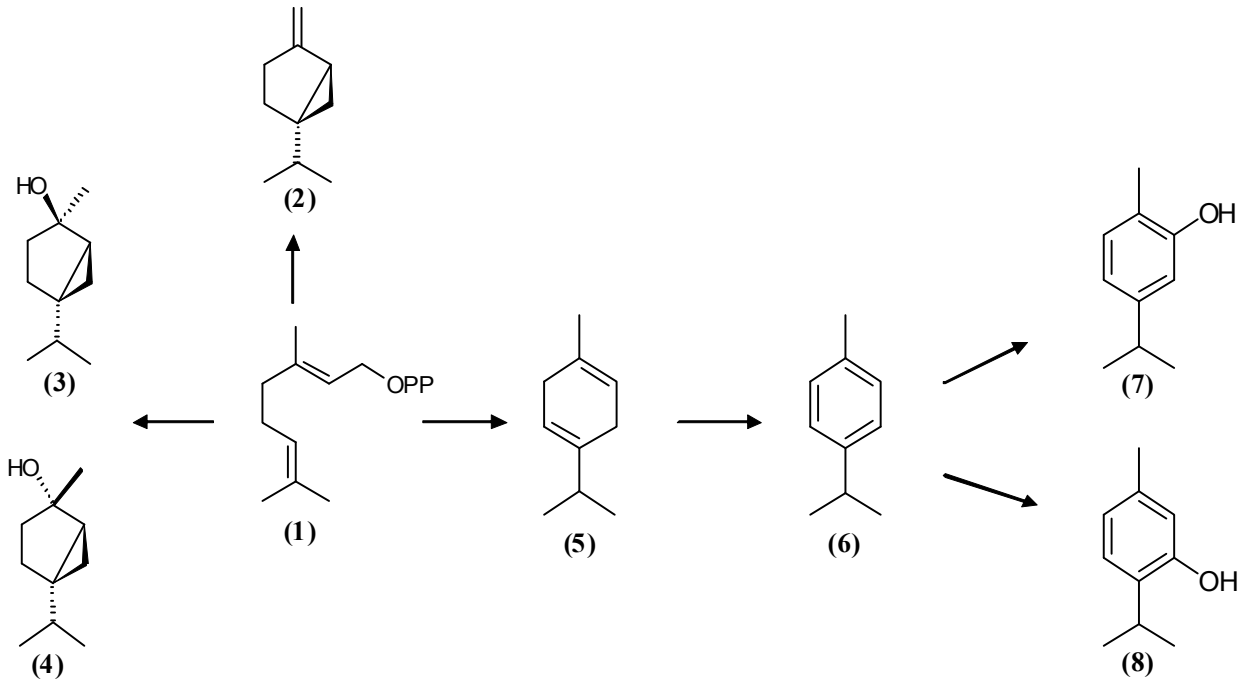

Scheme 1. Biosynthesis of 'sabinyl' and 'cymyl'-type monoterpenes in *Origanum*. ((1)…geranylpyrophosphate; 'sabinyl'-types: (2)…sabinene, (3)…*cis*-sabinene hydrate, (4)…*trans*-sabinene hydrate; 'cymyl'-types: (5)…γ-terpinene, (6)…*p*-cymene, (7)…carvacrol, (8)…thymol)

Table I. Composition of essential oil compounds [%] of two *Origanum* species at different levels of constant temperature.

compound	***Origanum vulgare* x *O. majorana***			***Origanum syriacum***		
	18°C	**22°C**	**26°C**	**18°C**	**22°C**	**26°C**
α-thujene	0.6 b	tr. a	tr. a	0.9 b	tr. a	tr. a
α-pinene	0.7 b	tr. a	tr. a	0.4 b	tr. a	tr. a
sabinene	3.9 a	4.8 b	5.3 c	0.0 a	0.4 b	0.3 b
myrcene	1.5 a	1.8 b	1.8 b	1.6 a	1.9 a	2.0 a
α-terpinene	0.8 b	0.1 a	0.1 a	0.1 a	0.1 a	0.1 a
p-cymene	0.1 a	0.7 b	0.8 c	2.9 b	2.7 b	0.5 a
γ-terpinene	tr. a	1.0 b	1.7 c	3.2 a	3.7 b	3.6 b
trans-sabinene hydrate	1.3 a	2.2 b	2.5 b	0.1 a	0.8 a	0.9 a
cis-sabinene hydrate	48.0 a	49.8 a	48.2 a	0.4 a	0.4 a	1.3 a
linalool	0.1 a	tr. a	tr. a	tr. a	tr. a	tr. a
terpinen-4-ol	1.0 b	0.2 a	0.3 a	0.2 a	0.3 a	0.2 a
α-terpineol	1.8 a	1.9 a	1.9 a	0.1 a	0.1 a	0.1 a
thymol	4.4 c	3.0 b	1.5 a	1.4 a	1.0 a	0.5 a
carvacrol	23.8 a	27.9 b	30.6 b	82.8 a	84.6 b	86.8 b
β-caryophyllene	2.4 ab	2.7 a	2.2 b	2.8 b	2.1 b	1.8 b

tr. ... traces; temperature levels within a species do not differ significantly

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CONCLUSIONS AND PERSPECTIVES

Conclusions and perspectives

PART 1: Tracing evolutionary relationships in section *Majorana*

The results summarized in the first chapter of my thesis are primary contributions to the field of molecular phylogenetics in a plant group only scarcely investigated. To gain detailed insights into the evolutionary history of the four taxa of section *Majorana* a broad approach was chosen combining information from nuclear and chloroplast DNA sequences with evidence based on microsatellite and phytochemical analyses (Lukas et al., in preparation). The data obtained told a fascinating story about reticulate evolution and hybrid speciation and allowed us to draw hypotheses on species boundaries, species histories and evolutionary relationships of *O. onites*, *O. dubium*, *O. majorana* and *O. syriacum*. As already concluded from morphological analyses (Ietswaart, 1980), hybridisation was confirmed to be an important speciation mechanism in *Origanum*.

The main conclusions from my work on section *Majorana* were based on sequence information from two nuclear gene regions, ITS (Internal transcribed spacers) and DXS (1-deoxy-D-xylulose 5-phosphate synthase). In ITS high intraindividual sequence variability was observed in all of the four taxa examined and cloning revealed (sometimes strongly) different ITS haplotypes from single accessions. A comprehensive analysis of informative nucleotide positions of the clones revealed *O. onites* and *O. syriacum* as ancient species within section *Majorana* and provided evidence for a complex hybrid origin of *O. dubium* (from *O. onites* and *O. syriacum*), an ancient hybridisation of *O. syriacum* with an unknown *Origanum* species and a more recent hybridisation between *O. onites* and *O. dubium*. *Origanum majorana* seems to be a specialised chemovariant of *O. syriacum*. With DXS a new (in *Origanum* putative single-copy) nuclear gene region was introduced that complemented conclusions drawn from ITS and might be useful for future phylogenetic studies in *Origanum* and closely related Lamiaceae genera. ITS and DXS, both, provided evidence for a distantly related *Origanum* species being involved in the speciation history of the hybrid species *O. dubium*. Considerable efforts were made to find chloroplast DNA regions suitable for phylogenetic investigations within *Origanum*. A number of non-coding cpDNA regions were amplified and sequenced but only little or even no sequence variation was observed. In the case of the most variable cpDNA region, the *psbA-trnH* ingergenic spacer, five haplotypes out of six were shared between two or more of the four *Majorana* taxa emphasizing a close interlocking of species histories. Via microsatellite analyses gene-flow between Turkish *O. onites* and *O. dubium* became impressively visible. The genetic consequences following hybridisations are probably the origin of an exceptional linalool-chemotype. The ‘sabinyl’-chemotype seems to be a result from the specialisation of *O. majorana*.

The data presented allows particular insights into processes of intrageneric evolution in *Origanum*. The experiences obtained from the comprehensive research on section *Majorana* will be helpful when analysing and interpreting the molecular data resulting from sequencing ITS, DXS and *psbA-trnH* of

the whole *Origanum* genus (Lukas et al., in preparation). Preliminary analyses of data sets comprising sequence information from species of all nine *Origanum* sections showed that the 'morphological' groups and some of the sections as defined by Ietswaart (1980) are not reflected when the species are grouped on the basis of comparative sequence analyses (Figure 8). From ITS sequence chromatogram characteristics it can be concluded that some more taxa within the genus might have complex histories as observed for section *Majorana*.

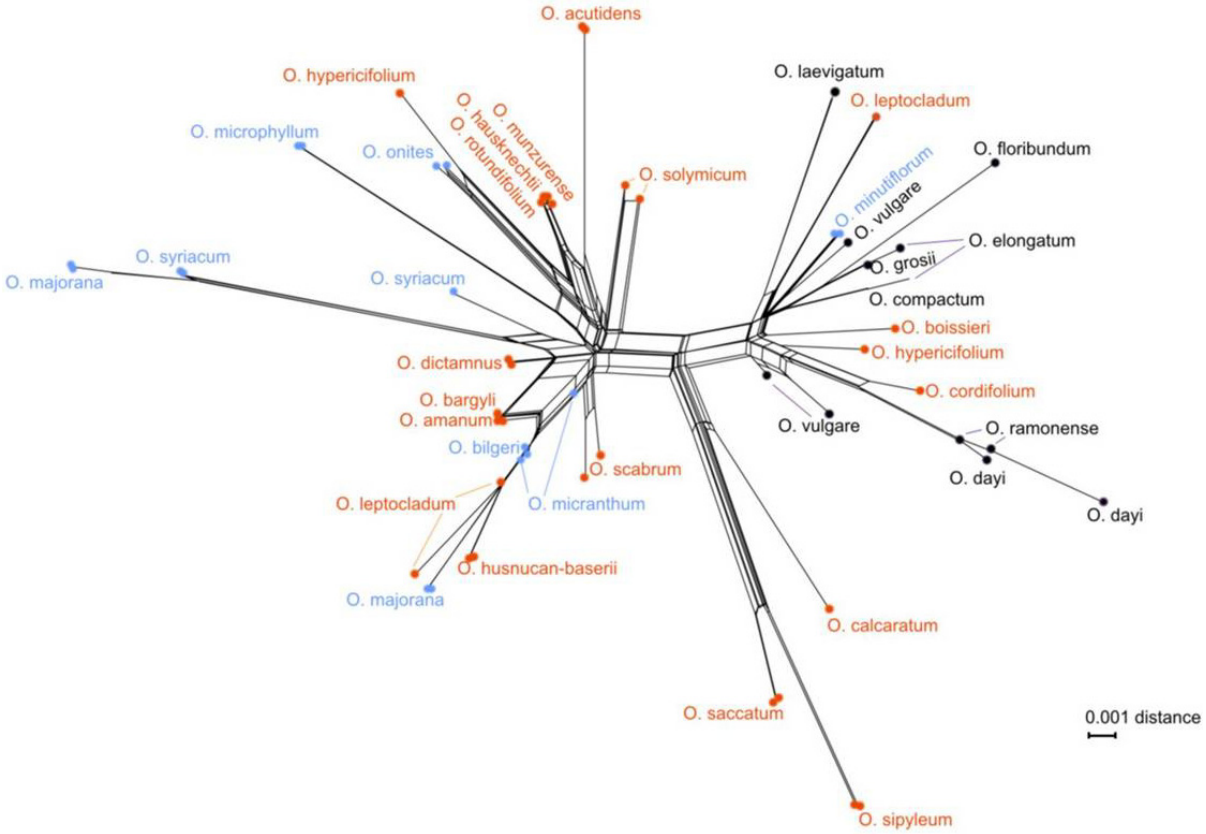

Figure 8: Neighbour Net splits graph of ITS sequences based on uncorrected p-distances. Species in red are of group A, species in blue are of group B, species in black are of group C. The *Origanum* species cluster within three groups. The grouping based on ITS is not in accordance with the grouping on the basis of morphological characteristics (Ietswaart, 1980).

DNA sequence analyses or sequence based approaches can be used as an alternative tool for the botanical classification of plant material ("DNA-barcoding") even when the questioned plant material is (highly) processed. The DNA sequence data obtained in the course of my studies will find practical application when serving as reference data for quality control or molecular identification of herbs or medicinal plant formulations. Due to the difficulties described above cpDNA sequences can not be recommended as a basis for DNA-barcoding in *Origanum*. With the knowledge of the existing ITS and DXS variability in mind, an identification method based on these two nuclear regions seem to be useful for a DNA based identity proof of *Origanum* plant material and its preparations (Müller et al., 2008).

PART 2: Phytochemical aspects of the genus *Origanum*

The investigations presented in chapter 2 contributed to a more complete knowledge of secondary compound biodiversity in *Origanum* and give an idea about the enormous inter- and intraspecific chemical polymorphisms present.

In case of *O. vulgare* (Lukas et al., 2008) the work presented is the first on the composition of essential oil compounds from populations in Corsica and one of only few studies available that research essential oils of *O. vulgare* from the north-western Mediterranean. The essential-oil-poor populations of Corsican *O. vulgare* are of interest from a chemotaxonomical point of view because two subspecies, ssp. *viride* and ssp. *vulgare*, are reported to co-occur. The Corsican populations were found to be rather heterogeneous. Usually morphological characteristics of both subspecies were present together with individuals exhibiting intermediate characteristics. The three essential oil chemotypes observed resembled the qualitative essential oil composition of Greek and Italian *O. vulgare* ssp. *viride* ('cymyl'-chemotype), of Italian *O. vulgare* ssp. *vulgare* ('sabinyl'-chemotype) and of *O. vulgare* ssp. *virens* ('mixed'-chemotype) from France. Although poor in essential oils Corsican *O. vulgare* showed high antioxidative properties (Lukas et al., data in preparation). Plant material of oregano biotypes low in essential oil (and therewith poorly oregano flavoured) but with high antioxidative capacity would especially be suitable for food-protective applications.

In case of *O. syriacum* (Lukas et al., 2009) the work presented is the first comprehensive analysis of essential oil characteristics of Syrian populations. All of the plant individuals analysed were rich in the oregano typical 'cymyl-'compounds and exhibited high percentages of carvacrol and/or thymol. A special characteristic of Syrian *O. syriacum* was the presence of thymoquinone, a bioactive compound with, amongst others, antioxidative and antiinflammatory properties. Moreover thymoquinone was reported to be a promising anticancer candidate. In the samples analysed thymoquinone was present in a broad range between 0.04 and 24 %. Selected biotypes producing high percentages of this compound could serve as a competitive natural source for thymoquinone.

Concerning arbutin, for the first time information about inter- and intraspecific arbutin variability in *Origanum* is provided (Lukas et al., 2010). The therapeutical properties of arbutin have been used for short-term medicinal and cosmetical treatments. The substance is non-desired in the regular human diet because a metabolite of arbutin, hydroquinone, exhibits a number of adverse effects. Eight *Origanum* species were analysed for the presence and variability of arbutin. Two of them, *O. dubium* and *O. majorana*, were shown to accumulate high concentrations of the compound (up to 118 mg/g dry mass). In commercial marjoram samples the arbutin value ranged between 2 and 24 mg/g dry mass, commercial oregano was in most cases free of arbutin. Crossing experiments between *O. majorana* and arbutin free *O. vulgare* indicated that only one gene is responsible for the arbutin polymorphism in *Origanum*. Classical breeding techniques could open a way to breed arbutin-free marjoram cultivars to be used in food-industry. Selected biotypes producing high amounts of arbutin could serve as a natural source for this compound.

Specific applications of *Origanum* preparations in food industry and medicine require homogeneous crops with certain chemical properties. A controlled production of reliable *Origanum* genotypes in the field might be the future method to produce plant material adequate to specific requirements. The natural chemical variability of *Origanum* species would offer a wide range of valuable biotypes for cultivation or targeted breeding programs. Comprehensive population analyses as presented here provide an overview about the range of chemical variability present and can support a more efficient selection.

PART 3: Tracing factors that influence qualitative and quantitative essential oil composition within *Origanum*:

Sequence analyses of the γ-terpinene synthase gene were performed to gain information about its genomic organisation in *Origanum* and to verify whether the γ-terpinene synthase gene is involved in the formation of essential oil chemotypes present in section *Majorana* (Lukas et al., accepted). On the basis of its gene structure (six introns were identified) the γ-terpinene synthase gene can be classified as a typical class III-type TPS gene. According to sequence characteristics present in intron 3 six different gene variants (among them one putatively pseudogeneous variant) were identified. The formation of 'cymyl'-compounds was found to be associated with the presence of certain variants of the γ-terpinene synthase gene. In plants lacking these 'active' gene variants (the pure 'sabinyl'-chemotypes of *O. majorana* and the pure linalool-chemotypes of *O. onites* and *O. dubium*) 'cymyl'-compounds were completely absent or were not exceeding traces. The background for this genotype/chemotype correlation remains unclear for the moment. The 'non-active' variants could represent pseudogeneous variants or gene variants that are not expressed in the aerial parts of the plant. To learn more about the complex mechanisms regulating terpene variation within *Majorana* further research, including expression studies and biochemical studies, is needed.

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Summary

The genus *Origanum* (Lamiaceae) comprises 43 species and 18 hybrids. Marjoram (*O. majorana*) and oregano (several *Origanum* species) have worldwide been used as kitchen herbs and folk remedies. The high antimicrobial and antioxidant activity of some *Origanum* species opens a wide range of further application possibilities of *Origanum* plant material in food-, feed-, pharmaceutical- and cosmetics-industry.

The taxonomy of the genus *Origanum* is complex and nearly all of the nine sections are afflicted with taxonomic uncertainties. DNA sequence information of the two nuclear gene regions ITS (Internal transcribed spacers) and DXS (1-deoxy-D-xylulose 5-phosphate synthase) as well as of the chloroplast *psbA-trnH* intergenic spacer was used to test the currently accepted genus concept for its applicability. The investigation focused on the evolutionary relationships of the four essential oil rich taxa of section *Majorana* (*O. majorana, O. onites, O. syriacum and O. dubium*), which are amongst the most widely used *Origanum* species (Lukas et al., in preparation). The combined DNA data revealed *O. onites* and *O. syriacum* as ancient species in the section. *Origanum dubium* was found to be of hybridogeneous origin combining attributes of *O. onites*, *O. syriacum* and a third *Origanum* species that remained unknown. The results show that *O. majorana* directly derived from *O. syriacum*. Sequence analysis as well as the analysis of five microsatellite loci provided evidence for recent hybridisation between Turkish *O. onites* and *O. dubium*. The SSR markers used were developed from expressed sequence tags (ESTs) of essential oil glands of *O. vulgare* (Novak et al., 2007). In total, thirteen EST-SSR loci were evaluated using one population of *O. vulgare* and one of *O. majorana*. All primer developed for *O. vulgare* cross-amplified in the distantly related *O. majorana* and may therefore be useful for evolutionary analyses of the genus and of closely related Lamiaceae like *Thymus* or *Satureja*. To analyse microsatellites a novel strategy was introduced (Mader et al., 2008). HRM (high resolution melting curve analyses) is a technique that measures the decreasing fluorescence of an intercalating dye in the process of dissociation of double stranded DNA. The shape of the resulting melting curve depends on GC content, length and sequence composition of the amplicon. HRM can be used for microsatellite analysis but also for other codominant marker systems by implementing a protocol of comparative melting curve assignment. To aid the correct assignment of heterozygous samples artificial mixtures of homozygous samples have to be included. The method was shown to be fast, cheap and more sensitive than standard protocols for microsatellite analysis.

In the course of an investigation dealing with PPAR (peroxisome proliferator-activated receptors) activation by oregano sequence analysis of ITS and DXS was performed to identify the botanical origin of the plant material investigated (Müller et al., 2008). In case of commercial plant material sequence analysis was useful to complement evidence from morphological characteristics. The two different DNA markers used allowed an unambigous determination of the respective plant material. In

case of dried plant extracts sequence analysis was complicated by the tiny amounts of extractable DNA. When dealing with such traces of DNA the amplification of DXS was not possible and the amplification of ITS was successful for some of the dried extract samples only.

Different groups of active compounds are responsible for the use of *Origanum* species as medicinal and aromatic plants. The most important are the mono- and sesquiterpenes, responsible for flavour and antimicrobial activity. Selected *Origanum* species were analysed for presence and quantity of these active compounds. In populations of Corsican *O. vulgare* three different chemotypes were present: a 'cymyl'-type with either carvacrol or thymol as main compound, a 'sabinyl'-type with large amounts of sabinene and *cis*-sabinene hydrate and a 'mixed' chemotype combining compounds of the 'cymyl'- and the 'sabinyl'-pathway (Lukas et al., 2008). In populations of Syrian *O. syriacum* the essential oil was dominated by carvacrol and/or thymol (Lukas et al., 2009). In Syrian *O. syriacum* thymoquinone, a promising substance for cancer therapy, was present in a wide range. Selected accessions of *O. syriacum* could be the basis for an oregano cultivar rich in thymoquinone. In populations of Cypriot *O. majorana* three different chemotypes were detected (Novak et al., 2008). Besides the standard 'marjoram' composition (a pure 'sabinyl'-chemotype), a pure α-terpineol chemotype and a mixed sabinyl/α-terpineol chemotype were also present.

Arbutin, a hydroquinone derivate, is a substance non desired in the human diet. Within the genus *Origanum* the formation of arbutin is polymorphic, with arbutin present in considerable amounts (*O. majorana* and *O. dubium*), minor amounts (*O. microphyllum*, *O. onites*, *O. saccatum*, *O. solymicum*) or completely absent (*O. husnucan-baseri*, *O. syriacum*, *O. vulgare*) (Lukas et al., 2010). Whereas the most important commercial oregano species (*O. onites* and *O. vulgare*) contain no or only minor amounts of arbutin, marjoram (*O. majorana*) has considerably high amounts. From the results of an inheritance study it was concluded that only one gene is responsible for the arbutin polymorphism in *Origanum*. The absence of arbutin in *O. vulgare* and *O. syriacum* would enable the breeding of arbutin-free marjoram. For the analysis of arbutin in marjoram and bearberry a rapid and selective GC method was established (Lamien-Meda et al., 2009).

Polymorphic essential oil variation can be observed among and within *Origanum* species. To learn more about the formation of essential oil chemotypes sequence analyses of the γ-terpinene synthase gene, a key enzyme in the biosynthesis of 'cymyl'-compounds, were performed (Lukas et al., accepted). The analyses revealed six different variants of the gene. The formation of 'cymyl'-compounds was associated with the presence of certain variants in a plant. In plants lacking these variants 'cymyl'-compounds were absent or present in traces only. The composition of secondary metabolites is also modified by environmental conditions. To study the influence of temperature clones of *O. vulgare* x *O. majorana* (containing 'cymyl'- and 'sabinyl'-compounds) and *O. syriacum* ssp. *syriacum* (containing 'cymyl'-compounds only) were cultivated at different temperature levels (Novak et al., accepted). The biosynthesis of the major 'sabinyl'-compound was not effected by temperature while the biosynthesis of the major 'cymyl'-compounds was significantly influenced.

Zusammenfassung

Die Gattung *Origanum* (Lamiaceae) umfaßt 43 Arten. Majoran (*O. majorana*) und Oregano (verschiedenen *Origanum* Arten) werden als Arznei- und Gewürzpflanzen genutzt. Die antimikrobielle und antioxidative Aktivität einiger *Origanum* Arten eröffnet auch eine Reihe von neuen Anwendungsfeldern in der Pharma-, Kosmetik- sowie Lebens- und Futtermittelindustrie.

Origanum ist eine schwierige Gattung und nahezu alle seiner 9 Sektionen sind mit taxonomischen Unsicherheiten behaftet. Um das bestehende Gattungskonzept auf seine Anwendbarkeit zu prüfen wurden anhand von DNA-Sequenzinformation (ITS – Internal transcribes spacers; DXS – 1-deoxy-D-xylulose 5-phosphate synthase; *psbA-trnH*) die Verwandschaftsverhältnisse in der Gattung analysiert. Spezielle Aufmerksamkeit wurde hierbei Sektion Majorana gewidmet deren Mitglieder (*O. majorana*, *O. syriacum*, *O. onites* und *O. dubium*) zu den am stärksten genutzten *Origanum* Arten zählen (Lukas et al., in Vorbereitung). Die erarbeiteten Daten weisen *O. onites* und *O. syriacum* als alte Arten der Sektion *Majorana* aus. *Origanum dubium* ist eine Hybridart die Merkmale von *O. onites*, *O. syriacum* und einer dritten, unbekannten Art aufweist, *O. majorana* stammt direkt von *O. syriacum* ab. Die Analyse von fünf Mikrosatelliten-Loci zeigte, daß *O. onites* und *O. dubium* in ihrem Überschneidungsgebiet in der Türkei rezent miteinander hybridisieren. Die verwendeten SSR-Marker wurden anhand von „expressed sequence tags“ (ESTs) von *O. vulgare* entwickelt (Novak et al., 2007). Insgesamt dreizehn EST-SSR Loci wurden evaluiert. Alle der dreizehn Loci konnten auch in in der entfernt verwandten Art *O. majorana* amplifiziert werden und scheinen somit für evolutionäre Studien in der Gattung und eventuell auch in nah verwandten Gattungen wie *Thymus* oder *Satureja* geeignet. Für die Analyse der Mikrosatelliten wurde eine neue Methode entwickelt die auf High-resolution melting curve analyses (HRM) basiert (Mader et al., 2008). Bei der HRM wird im Anschluß and die PCR die Floureszenz eines interkalierenden Farbstoffes gemessen, der entsprechend eines Temperaturgradienten während der Dissoziation der doppelsträngigen DNA frei wird. Die Form der resultierenden Schmelzkurve ist spezifisch für das PCR-Produkt und hängt von GC-Gehalt, Länge und Sequenzkomposition des Amplikons ab. Zur Analyse von Mikrosatelliten werden die Schmelzkurven vergleichend analysiert und gruppiert. Zur korrekten Zuordnung von heterozygoten Proben werden die entsprechenden Schmelzkurven mit jenen von künstlichen DNA Mischungen homozygoter Proben verglichen. Die Methode hat sich als schnell, kostengünstig und sensitiver als herkömmliche Analyse-Methoden erwiesen.

Im Zuge einer Untersuchung über die PPAR (peroxisome proliferator-activated receptors) Aktivierung durch Oregano oder Oregano-Komponenten wurden DNA-Seqenzanalysen (ITS und DXS) durchgeführt um den botanischen Ursprung des für die Studie verwendeten Pflanzenmaterials zu bestimmen (Müller et al., 2008). Im Falle handelsüblicher Oregano-Proben erlaubte die DNA-Analyse eine eindeutige Identifizierung des Pflanzenmaterials. Die Analyse von Oregano-Trockenextrakten erwies sich aufgrund der geringen Mengen an extrahierbarer DNA hingegen als

schwierig. Bei der Arbeit mit DNA aus Trockenextrakten war eine Amplifikation von DXS nicht möglich, eine Amplifikation von ITS gelang lediglich bei einem Teil dieser Proben.

Verschiedene Gruppen von Pflanzeninhaltsstoffen sind für den Gebrauch von *Origanum* Arten als Arznei- und Gewürzpflanzen verantwortlich. Zu den wichtigsten zählen hier die Mono- und Sesquiterpene (Bestandteile des ätherischen Öls) die für das Aroma und die antimikrobielle Aktivität verantwortlich sind. Um bestehende Wissenslücken über die intra- und interspezifische Variabilität dieser Inhaltsstoffe zu schließen, wurden Populationen ausgewählter *Origanum* Arten hinsichtlich dieser Inhaltsstoffe untersucht. In Populationen von korsischem *O. vulgare* waren drei verschiedene Chemotypen präsent: ein ‚cymyl'-Chemotyp mit Carvacrol oder Thymol als Hauptkomponente, ein ‚sabinyl'-Chemotyp mit hohem Gehalt an Sabinen und *cis*-Sabinenhydrat sowie ein ‚gemischter'Chemotyp (Lukas et al., 2008). Das Ätherische Öl von syrischem *O. syriacum* wurde von Thymol und/oder Carvacrol dominiert und enthielt außerdem hohe Mengen an Thymoquinon, einer für die Krebstherapie vielversprechende Substanz (Lukas et al., 2009). In Populationen von zypriotischem *O. majorana* wurden drei verschiedene Chemotypen detektiert: ein reiner sabinyl'-Chemotyp (mit typischem Majoran-Aroma), ein reiner α-terpineol-Chemotyp sowie ein ‚gemischter' Chemotyp (Novak et al., 2008).

Arbutin ist ein Inhaltsstoff der wegen seiner hohen biologischen Aktivität in Lebensmitteln unerwünscht ist. Eine umfassende Analyse ergab, daß *O. dubium* und *O. majorana* hohe Mengen an Arbutin anreichern und daß auch kommerziell gehandelter Majoran und Oregano hohe Arbutinmengen aufweisen. *O. microphyllum*, *O. onites*, *O. saccatum*, *O. solymicum*, *O. husnucan-baseri*, *O. syriacum* und *O. vulgare* enthalten nur geringe Mengen bzw. kein Arbutin (Lukas et al., 2010). Es wurde außerdem gezeigt, daß nur ein Gen für den Arbutin-Polymorphismus in *Origanum* verantwortlich ist. Durch gezielte Züchtungsarbeit wäre somit die Produktion von arbutinfreiem Majoran und Oregano möglich. Zur Analyse von Arbutin in Majoran und Beerenträubel-Blättern wurde eine neue, schnelle und selektive GC-Methode etabliert (Lamien-Meda et al., 2009).

In der Gattung *Origanum* variiert die Komposition des Ätherischen Öls sowohl zwischen als auch innerhalb einzelner Arten. Um mehr über die genetischen Grundlagen der Chemotypen zu erfahren wurde ein Teil des γ-Terpinen Synthase Gens, ein Schlüssel-Gen für die Biosynthese der ‚cymyl'-Komponenten, sequenziert (Lukas et al., akzeptiert). Sechs verschiedene Varianten des Gens wurden identifiziert wobei die Synthese der ‚cymyl'-Komponenten an das Vorhandensein bestimmter Genvarianten gebunden war. Die Komposition des Ätherischen Öls wird aber auch von Umweltfaktoren beeinflußt. Um den Einfluß der Temperatur zu untersuchen wurden Klone von *O. vulgare* x *O. majorana* (enthält 'cymyl'- und 'sabinyl'-Komponenten) und *O. syriacum* ssp. *syriacum* (enthält nur 'cymyl'-Komponenten) unter unterschiedlichen Temperaturbedingungen kultiviert (Novak et al., akzeptiert). Die Biosynthese der wichtigsten ‚sabinyl'-Komponenten wurde durch die Temperatur nicht beeinflußt. Die Biosynthese der wichtigsten ‚cymyl'-Komponenten zeigte sich jedoch von der Temperatur beeinflußt.

Curriculum vitae

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School Education

1981 – 1985: Primary School; Petronell-Carnuntum

1985 – 1989: Secondary General School; Hainburg an der Donau

1989 – 1994: Business College; Bruck an der Leitha

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University Studies

1994 – 2002: Studies of Biology/Ecology at the Institute of Ecology and Conservation Biology, University of Vienna
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27. 6. 2002: Academic degree Mag.rer.nat. (equivalent to MSc), obtaining first-class honours

2006 – 2010 PhD studies in Systematic Botany and Phytochemistry
Topic of Thesis: "Molecular and phytochemical analyses of the genus *Origanum* L. (Lamiaceae)" at the Faculty of Life Sciences, Department for Systematic and Evolutionary Botany, University of Vienna (supervision: Univ.-Prof. Dr. Mary Rosabelle Samuel, in collaboration with the Institute for Applied Botany and Pharmacognosy, University of Veterinary Medicine Vienna, supervision: Ao. Univ.-Prof. Dipl.-Ing. Dr. Johannes Novak)
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Professional/Research experience

2000 – 2002 undergraduate student, Institute for Applied Botany and Pharmacognosy, University of Veterinary medicine, Vienna, with Ao.Univ.-Prof. Dr. Remigius Chizzola

2003 – 2010 research assistant/graduate student, Institute for Applied Botany and Pharmacognosy, University of Veterinary medicine, Vienna, with Ao. Univ.-Prof. Mag. Dr. Karin Zitterl-Eglseer and Ao. Univ.-Prof. Dipl.-Ing. Dr. Johannes Novak

Grant: 2005/09 – 2009/05 "Molecular Phylogeny and Phytochemistry of the genus *Origanum*" (FWF project Nr. P18027-B06 – Project leader: Ao. Univ.-Prof. Dipl.-Ing. Dr. Johannes Novak).

Meetings, presentations and lectures

2005/07 XVII International Botanical Congress (IBC); 12. – 16. 7. 2005, Vienna, Austria

2006/09 37th International Symposium on Essential Oils (ISEO); 7. 9. – 10. 9. 2006, Grasse, France (poster presentation)

2006/09 12. Österreichisches Botanikertreffen; 21. – 24. 9. 2006, Kremsmünster, Österreich (oral presentation)

2006/11 57. Tagung der Vereinigung der Pflanzenzüchter und Saatgutkaufleute Österreichs; 21. – 23. 11. 2006, Gumpenstein, Austria

2007/04 1st International medicinal and aromatic plants conference on culinary herbs; 29. 4. - 4. 5. 2007, Antalya, Turkey (poster presentation)

2007/08 Systematics. The Sixth Biennial Conference of the Systematics Association; 28. 8. - 31. 8. 2007, Edinburgh, Scotland (poster presentation)

2007/09 38th International Symposium on Essential Oils (ISEO); 9. 9. - 12. 9. 2007, Graz, Austria (poster presentation)

2008/02 Bernburger Winterseminar und 5. Fachtagung Arznei- und Gewürzpflanzen; 18. 2. – 21. 2. 2008, Bernburg, Deutschland (poster presentation)

2008/05 Invitation of the IPK Gatersleben, Germany (oral presentation)

2009/06 4th International Symposium Breeding Research on Medicinal and Aromatic Plants; 17. 6. -21. 6. 2009; Ljubljana, Slovenia (poster presentation)

Teaching experience

2009 Tutor at the Faculty of Life Sciences, Department for Systematic and Evolutionary Botany

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2010 Assistant at the Faculty of Life Sciences, Department for Systematic and Evolutionary Botany

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Thesis, publications and abstracts:

Thesis:

Lukas, B. (2002) Cadmium- und Spurenelementgehalt in Johanniskrautarten an Wildstandorten in Ostösterreich. Diplomarbeit, Universität Wien.

Publications:

Chizzola, R., **Lukas**, B. (2005) Variability of the cadmium content in *Hypericum* species collected in the wild in eastern Austria. *Water, Air and Soil Pollution* 170: 331-343.

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