The LINNEAN SOCIETY of London BOTANICAL Journal of the Linnean Society

Botanical Journal of the Linnean Society, 2013, **171**, 667–686. With 4 figures

Complex evolutionary relationships in *Origanum* section *Majorana* (Lamiaceae)

BRIGITTE LUKAS[1,2]*, ROSABELLE SAMUEL[2], EDUARD MADER[1], KEMAL HÜSNÜ CAN BAŞER[3], HAYRI DUMAN[4] and JOHANNES NOVAK[1]

[1]*Institute for Animal Nutrition and Functional Plant Compounds, University of Veterinary Medicine, Veterinärplatz 1, 1210 Vienna, Austria*
[2]*Department of Systematic and Evolutionary Botany, University of Vienna, Rennweg 14, 1030 Vienna, Austria*
[3]*Department of Pharmacognosy, Faculty of Pharmacy, Anadolu University, Eskisehir 26470, Turkey*
[4]*Department of Biology, Faculty of Science and Letters, Gazi University, Ankara 06330, Turkey*

Received 27 April 2012; revised 14 November 2012; accepted for publication 19 December 2012

Origanum (Lamiaceae) comprises a number of essential oil-rich species that have been used by humans 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 amongst its four taxa are unclear. In the present investigation, we used DNA sequence data from two nuclear regions [internal transcribed spacer (ITS) and 1-deoxy-D-xylulose 5-phosphate synthase (DXS)] 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 the older species in the section. *Origanum majorana* descends directly from *O. syriacum*. *Origanum dubium* was found to be of hybridogenous origin showing attributes of *O. onites*, *O. syriacum* and a third, unknown, *Origanum* species. Both sequence and microsatellite analyses provided evidence for recent hybridization between *O. onites* and *O. dubium* in Turkey. *Botanical Journal of the Linnean Society*, 2013, **171**, 667–686.

ADDITIONAL KEYWORDS: DXS – hybridization – ITS – marjoram – oregano.

INTRODUCTION

The genus *Origanum* L. (Lamiaceae) comprises 43 species and 18 hybrids (Ietswaart, 1980; Carlström, 1984; Danin, 1990; Duman *et al*., 1995; Danin & Künne, 1996; Duman, Baser & Aytec, 1998), most of them distributed in the eastern Mediterranean. As a result of their essential oils, *Origanum* spp. have been collected locally for centuries to flavour traditional dishes and for numerous purposes in traditional medicine (e.g. Fleisher & Fleisher, 1988; Della, Paraskeva-Hadjichambi & Hadjichambis, 2006). Today, two sensorial types of *Origanum*, marjoram (from *O. majorana* L.) and oregano (mainly *O. onites* L. but also *O. vulgare* L.), are commercially traded and widely used all over the world. Apart from the traditional use as kitchen herbs and folk remedies, preparations of *Origanum* plant material are applied in the food, feed, pharmaceutical and cosmetic industries in a wide variety of ways (e.g. Baričevič & Bartol, 2002; Singletary, 2010; Kuorwel *et al*., 2012).

Origanum is in the tribe Mentheae, with *Thymus* L., *Thymbra* L. and *Micromeria* Benth. as its closest relatives (Bräuchler, Meimberg & Heubl, 2010). As for many other genera of Menthinae, *Origanum* has a complex taxonomy, complicated by a considerable amount of morphological variation. Species, subspecies and varieties can be discerned in the typical form, but nearly all gradually merge into at least one other form. Hybridization, even between distantly related species, is a frequent phenomenon (Ietswaart, 1980). Among the different taxonomic concepts for

*Corresponding author: Brigitte.Lukas@vetmeduni.ac.at

Table 1. Morphological characteristics for the differentiation of *Origanum onites*, *O. syriacum*, *O. dubium* and *O. majorana* (Ietswaart, 1980, 1982, 1985)

	O. onites	O. syriacum	O. dubium	O. majorana
Inflorescence	Corymbiform	Paniculate	Paniculate Compact	Paniculate Elongated
Leaves	Serrate	Entire Acute Raised veins (abaxial)	Entire Obtuse No raised veins (abaxial)	Entire Obtuse No raised veins (abaxial)
Indumentum	Hirsute	Hirsute, hirsute-tomentose or tomentose	Tomentellous	Tomentellous
Essential oil chemotype	'Cymyl' type (Linalool type)	'Cymyl' type	'Cymyl' type (Linalool-type)	'Sabinyl' type
Arbutin	Not present or trace	Not present	High amounts	High amounts

Origanum (e.g. Bentham, 1834, 1848; Boissier, 1879; Briquet, 1895), the taxonomic revision of Ietswaart (1980) is the most widely accepted. However, for the reasons mentioned above, details of the taxonomic concept of Ietswaart (1980) are still debated. This is particularly true for section *Majorana* Benth. in which four essential oil-rich taxa, *O. onites* L., *O. syriacum* L., *O. dubium* Boiss. and *O. majorana* L., are among the most widely used *Origanum* species.

Within *Origanum*, the four taxa of section *Majorana* are united by a unique morphological feature: a bract-like calyx with a highly reduced lower lip. In section *Majorana*, however, morphological characters failed to explain species boundaries and evolutionary relationships. *Origanum onites* appears to be the best defined entity which can be characterized by corymbiform inflorescences and serrate leaves. Morphologically, *O. syriacum*, *O. dubium* and *O. majorana* differ in having paniculate inflorescences and usually entire leaves. The species boundaries of the last three species are not entirely clear and their differentiation relies mainly on subtle differences in indumentum and leaves (summarized in Table 1), morphological characters that are variable in natural populations. High morphological variation observed in *O. syriacum* led to the recognition of three varieties (var. *syriacum*, var. *bevanii* (Holmes) Ietsw. and var. *sinaicum* (Boissier) Ietsw. (Ietswaart, 1985). The taxonomic status of *O. dubium* and *O. majorana* has long been discussed. They were treated as distinct species (e.g. Boissier, 1879) before Ietswaart united them under *O. majorana* in his taxonomic revision (Ietswaart, 1980) and in the *Flora of Turkey* (Ietswaart, 1982). In the *Flora of Cyprus* (Ietswaart, 1985), Ietswaart again treated them as separate species after observing many specimens and using the length and shape of the inflorescence to differentiate them. Nowadays, both the 'one-species concept' and the 'two-species concept' are applied.

Phytochemical aspects are often considered when discussing phylogenetic relationships (Grayer, Chase & Simmonds, 1999; Wink, 2003; Larsson, 2007). In the case of the four taxa of section *Majorana*, the composition of the essential oil compounds and the accumulation/absence of arbutin, a hydroquinone derivative, have been investigated sufficiently to allow chemosystematic considerations. With respect to the essential oils, three main chemotypes were found in natural populations of *O. onites*, *O. syriacum*, *O. dubium* and *O. majorana* (Table 1). The most abundant is the 'cymyl' chemotype (Skoula & Harborne, 2002), accumulating large amounts of γ-terpinene, *p*-cymene, carvacrol and/or thymol and other related compounds. Essential oils rich in these compounds possess the pungent oregano flavour and are usually accumulated by *O. syriacum* (e.g. Fleisher & Fleisher, 1991; Lukas *et al*., 2009), *O. dubium* (Arnold, Bellomaria & Valentini, 1993; Baser, Kirimer & Tumen, 1993a) and *O. onites* (e.g. Vokou, Kokkini & Bessiere, 1988; Skoula *et al*., 1999). The second chemotype, an almost pure linalool chemotype uncommon in *Origanum*, somehow associates *O. dubium* with *O. onites*. This rare linalool chemotype had, until now, only been detected in sympatric populations of both taxa (Baser *et al*., 1993b; Lukas, Samuel & Novak, 2010a). The third chemotype, the 'sabinyl' chemotype, is a special feature of *O. majorana*, the volatiles of which are rich in 'sabinyl' compounds (*cis*-/*trans*-sabinene hydrate and *cis*-sabinene hydrate acetate; e.g. Fischer, Nitz & Drawert, 1987; Novak, Lukas & Franz, 2008b), which are responsible for the specific marjoram flavour. The extraordinary chemotype of *O. majorana* would support a 'two-species concept'

(Ietswaart, 1985), treating *O. majorana* and *O. dubium* as separate species. Arbutin, however, combined *O. dubium* with *O. majorana*. Both taxa accumulate large amounts of this compound, but arbutin was not detected (or was present in trace amounts only) in natural populations of *O. syriacum* and *O. onites* (Lukas *et al*., 2010b).

DNA-based investigations of *Origanum* are limited, either containing few species (Kaufmann & Wink, 1994; Paton *et al*., 2004; Walker *et al*., 2004; Azizi *et al*., 2009; Katsiotis *et al*., 2009; Bräuchler *et al*., 2010) or focusing on technical aspects (Azizi *et al*., 2009). To learn more about the taxonomic status of *O. onites*, *O. syriacum*, *O. dubium* and *O. majorana* and their evolutionary relationships, we performed sequence analyses of the nuclear regions of nrDNA internal transcribed spacers (ITS) and low-copy 1-deoxy-D-xylulose 5-phosphate synthase (DXS) and analyses of five polymorphic microsatellite loci. ITS1 and ITS2 are among the most commonly used molecular markers for evolutionary studies at the species level (e.g. Baldwin *et al*., 1995; Alvarez & Wendel, 2003). Despite the high level of variation, ITS can be a powerful tool for recognizing ancient and recent hybridization in flowering plants. In recent years, high intraspecific and intra-individual ITS variability has been reported in a number of taxa, providing valuable insights into the evolutionary history of complex groups (e.g. Mayol & Rosello, 2001; Koch, Dobes & Mitchell-Olds, 2003; Denk, Grimm & Hemleben, 2005; 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, Barfuss & Zizka, 2009; Vaezi & Brouillet, 2009). However, they are rarely used because of the lack of universal primers and the efforts associated with the establishment of primers for a new nuclear region for a taxon of interest. With DXS, a putative single-copy gene region in *Origanum* is introduced that may also be of interest for the study of phylogenetic relationships in *Origanum* and closely related genera of Lamiaceae. Microsatellites have developed into one of the most powerful genetic markers for the analysis of interspecific hybridization (e.g. Schwarzbach & Rieseberg, 2002; Muir & Schlötterer, 2005; Edwards, Soltis & Soltis, 2008).

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

MATERIAL AND METHODS

Plant material

Individual plants of *O. dubium*, *O. majorana*, *O. onites* and *O. syriacum* were collected during excursions to Italy, Greece, Turkey, Cyprus and Syria, covering a wide range of their natural distribution areas (Fig. 1). Additional plant material was taken from herbarium specimens and from plants grown in the glasshouse of the University of Veterinary Medicine, Vienna, Austria. Details about the geographical origin and 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 the case of *Origanum majorana s.l*., the *Flora of Cyprus* (Ietswaart, 1985) was used as a second reference to distinguish *O. dubium* and *O. majorana s.s.* Voucher specimens are kept at the herbarium of the Institute for Animal Nutrition and Functional Plant Compounds, University of Veterinary Medicine, Vienna, Austria.

DNA extraction, amplification, cloning and sequencing

Genomic DNA was extracted from young, silica gel-dried leaves using a cetyltrimethylammonium bromide (CTAB) extraction protocol based on Doyle & Doyle (1990). The nuclear ITS region was amplified using universal primers (ITS5 and ITS4; White *et al*., 1990; modified by Downie & Katz-Downie, 1996). The amplification reactions were performed on a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) in 20-μL volumes with the following reaction components: 0.5 μL template DNA (1–50 ng), 18 μL 1.1 × ReddyMix PCR Master Mix (2.5 mM $MgCl_2$) (ABGene, Epsom, Surrey, UK), 0.5 μL dimethylsulfoxide (DMSO), 0.2 μL double-distilled H_2O and 0.4 μL (400 nM) of each primer (Invitrogen, Carlsbad, CA, USA). Thirty-five 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 step at 72 °C. All polymerase chain reaction (PCR) products were checked on 1.4% agarose gels before purification with exonuclease 1 (EXO1) and shrimp alkaline phosphatase (SAP) (Fermentas, Burlington, ON, Canada) according to the manufacturer's instructions. Sequencing of both strands was performed using BigDye Terminators (Applied Biosystems) and using primers from the original amplifications. The sequences were generated with an ABI 3130x automated sequencer (Applied Biosystems) and edited using Chromas Vers. 2.24 (Technelyseum, Tewantin, Qld, Australia). Nearly all the directly sequenced

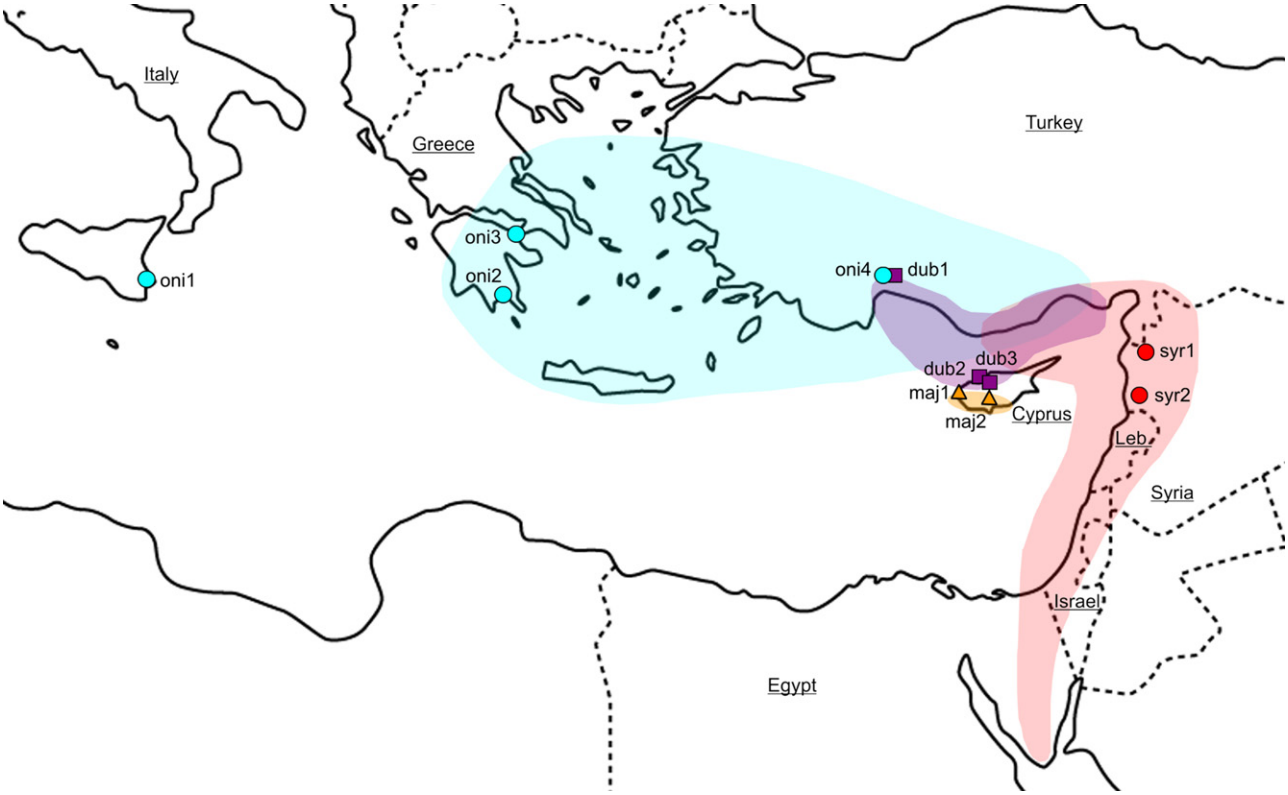

Figure 1. Natural distribution of *Origanum onites* (blue), *O. majorana s.s.* (yellow), *O. dubium* (violet) and *O. syriacum* (red) (Ietswaart, 1980; modified). The geographical location of the populations sampled for microsatellites is indicated. In Cyprus, the distribution of *O. majorana*, *O. dubium* and *O. syriacum* appears not to overlap; *O. majorana* is distributed in the south, whereas *O. dubium* seems to be restricted to the north (Troodos massif). *Origanum syriacum* occurs solely in the Turkish north-east of Cyprus. dub, *Origanum dubium*; maj, *O. majorana*; oni, *O. onites*; syr, *O. syriacum*.

accessions resulted in ambiguous sequence chromatograms characterized by the appearance of few to many polymorphic sites and/or noise or divergence of the sequences after/to a specific point. Therefore, PCR products of selected accessions were cloned into the pGEM-T easy-cloning vector system (Promega, Madison, WI, USA). Plasmid DNAs were re-amplified from 10–16 clones for each individual using the original ITS primers and the PCR conditions described above. After checking 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 gene was amplified using expressed sequence tag (EST)-derived primers (DXS24F1 and DXS390R1; Müller *et al.*, 2008) with the same amplification protocol 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 step at 72 °C. The PCR products were sequenced directly 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 in unambiguously separating different alleles from heterozygous plant individuals. PCR products corresponding to strongly divergent alleles of heterozygote individuals were cloned using the procedure described above.

Sequence analyses and phylogenetic reconstruction

ITS and DXS sequences were aligned using MEGA4 (Tamura *et al.*, 2007), with subsequent manual correction. Variable positions in the data matrices were checked against the original sequence chromatogram files.

In ITS, different degrees of intraspecific and intra-individual sequence divergence were detected. In order to gain information from ITS diversity, clones of

each accession and subsequently of each taxon were classified and grouped on the basis of alignment positions at which an overall variation of 5% or higher was observed (31 sites, Table 3). For all clones of each taxon, the presence/absence of mutations at these variable nucleotide positions was recorded and the frequencies of mutations were calculated (Table 3). To demonstrate the large amount of intra-individual ITS variability in *O. dubium*, *O. syriacum* and cultivated *O. majorana*, frequent ITS ribotypes (ITS variants that were isolated from more than one accession) were characterized (Table 3). The potential occurrence of pseudogenes among ITS repeats was assessed by checking for the presence of conserved angiosperm motifs in ITS1 (Liu & Schardl, 1994) and in the 5.8S rDNA (Jobes & Thien, 1997). Secondary structure predictions and minimum free energy (ΔG at 37 °C) estimates were conducted using mFold 3.1 (Zuker, 2003).

For the phylogenetic analyses of the ITS clones, the software programs G2CEF and EUKDIS (Göker & Grimm, 2008) were used, treating all ITS clones of a plant individual as 'associates' of a 'host'. In a first step, the sequence data of the 'associates' were transformed into a character matrix of the 'hosts' (program G2CEF, FRQ character transformation, gaps were treated as fifth character). The transformed characters represent the frequency of a nucleotide at a certain sequence position in all the clones of a 'host'. In a second step, from this character matrix, 'host'–'host' distances were computed (program EUKDIS; distance method, Euclidean distances). The distance matrix was visualized with SplitsTree 4.8 (Huson & Bryant, 2006) 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.

All sequences have been deposited in GenBank (accession numbers are provided in Table 2). For accessions with intra-individual ITS and/or DXS variability, multiple accession numbers were assigned.

Microsatellite analyses

The analysis of the five microsatellite loci (OR10, OR14, OR40, OR44, OR 64; Novak *et al*., 2008a) was performed via high-resolution-melt-analysis (HRM) according to the method of Mader, Lukas & Novak (2008). The 10-μL PCR contained 5.6 μL distilled H_2O 0.4 units Taq HOT FIREPol polymerase (Solis BioDyne, Tartu, Estonia), 1 μL Buffer B (Solis BioDyne), 1.4 μL $MgCl_2$ (25 mM), 0.2 μL DMSO, 0.1 μL deoxynucleoside triphosphate (dNTP)-mix (10 mM), 0.1 μL of each primer (10 pM) and 0.6 μL 36 μM BEBO (TATAA Biocenter, Gothenburg, Sweden); 1 μL of DNA solution was added to each reaction, containing between 0.25 and 0.8 ng μL^{-1} DNA. All reactions were performed in duplicate. The PCR cycling started with an initial phase of 15 min at 95 °C, followed by 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 to 90 °C in steps of 0.05 °C, each step with a 1-s hold. PCR and subsequent HRM were performed on a RotorGene 6500 (Corbett Research Pty Ltd, Sydney, Australia) equipped with an HRM module. The resulting melting curves were analysed using the RotorGene 6000 series software, Version 1.7.65. To obtain inter-run comparability, reference 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 & Smouse, 2006). To study population subdivision, pairwise F_{ST} values 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 criterion. Principal components analysis (PCA) (genetic distances were based on the 'codominant genotypic' distance option) was performed with GENALEX 6 and visualized with SPSS Version 16.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

ITS

A total of 277 ITS clones from 23 accessions of *O. dubium* (seven), wild *O. majorana* (three), cultivated *O. majorana* (four), *O. onites* (five) and *O. syriacum* (four) was generated for the ITS analyses. The number of clones per individual plant ranged from two to 16. Four accessions of *O. onites* (SR810, SR1387, SR1434 and SR1440) and one of *O. syriacum* (SP3) could be sequenced directly and were included in the alignment. The length of the aligned sequences varied from 642 to 644 base pairs (bp); the alignment of all ITS sequences resulted in a matrix of 647 characters, 275 of which were variable.

Diversity of cloned ITS sequences and intraspecific 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 clones. By comparing the aligned sequences, different degrees of intraspecific ITS variability became obvious.

Table 2. List of accessions examined in this study. Species, identification number (H, accessions sampled in herbaria; LC, living collection, University of Veterinary Medicine, Vienna, Austria; SR, accessions collected from wild populations), affiliation of wild accessions to populations sampled for microsatellite analysis, geographical origin (cv., cultivated plant; CY, Cyprus; ET, Egypt; GR, Greece; IL, Israel; IT, Italy; LB, Lebanon; SY, Syria; TR, Turkey; -, information not available) and GenBank accession numbers. 1-Deoxy-D-xylulose 5-phosphate synthase (DXS) allele identity is indicated. dub, *Origanum dubium*; maj, *O. majorana*; oni, *O. onites*; syr, *O. syriacum*

			Geographical origin			ITS	DXS	DXS
Species	ID	Pop.		N	E	NCBI accession no.	alleles	NCBI accession no.
O. dubium	H67^{ESSE}	–	TR	–	–	JX162774-JX162788	–	–
O. dubium	H137^{GAZI}	–	TR	–	–	JX162789-JX162803	–	–
O. dubium	SR535	dub1	TR	36°56′09.2″	31°28′34.1″	–	16/17	JX122650/JX122649
O. dubium	SR540	dub1	TR	36°56′09.2″	31°28′34.1″	–	16/17	JX122652/JX122651
O. dubium	SR558	dub1	TR	36°56′09.2″	31°28′34.1″	JX162842-JX162857	16/17	JX122654/JX122653
O. dubium	SR603	–	TR	36°29′47.2″	32°07′13.5″	–	4/4	JX122638
O. dubium	SR725	–	TR	36°06′14″	32°34′55.5″	JX162858-JX162869	17/17	JX122639
O. dubium	SR750	–	TR	36°05′30.5″	32°55′14.6″	–	4/4	JX122640
O. dubium	SR1028	–	CY	35°05′31.2″	32°32′31.8″	JX162915-JX162929	4/17	JX122656/JX122655
O. dubium	SR1054	–	CY	35°05′31.2″	32°32′31,8″	JX162930-JX162943	4/17	JX122658/JX122657
O. dubium	SR1056	dub2	CY	35°08′45.6″	32°31′58.5″	–	17/17	JX122642
O. dubium	SR1060	dub2	CY	35°08′45.6″	32°31′58.5″	–	4/17	JX122662/JX122661
O. dubium	SR1072	dub2	CY	35°08′45.6″	32°31′58.5″	–	17/17	JX122643
O. dubium	SR1076	dub2	CY	35°08′45.6″	32°31′58.5″	–	17/17	JX122644
O. dubium	SR1162	dub3	CY	35°01′19.1″	32°34′49.8″	–	17/17	JX122645
O. dubium	SR1165	dub3	CY	35°01′19.1″	32°34′49.8″	–	17/17	JX122646
O. dubium	SR1184	dub3	CY	35°01′19.1″	32°34′49.8″	–	17/17	JX122647
O. dubium	SR1187	dub3	CY	35°01′19.1″	32°34′49.8″	–	4/17	JX122664/JX122663
O. dubium	SR1189	dub3	CY	35°01′19.1″	32°34′49.8″	–	17/17	JX122648
O. dubium	SR1200	–	CY	35°01′42.9″	32°36′50.3″	JX162944-JX162957	17/17	JX122641
O. dubium	SR1204	–	CY	35°01′42.9″	32°36′50.3″	–	4/17	JX122660/JX122659
O. majorana	H44^{RNG}	–	CY	–	–	–	9/9	JX122665
O. majorana	SR837	maj1	CY	35°04′54.1″	32°17′36.9″	–	9/9	JX122666
O. majorana	SR839	maj1	CY	35°04′54.1″	32°17′36.9″	–	8/9	JX122671/JX122670
O. majorana	SR849	maj1	CY	35°04′54.1″	32°17′36.9″	JX162870-JX162885	–	–
O. majorana	SR942	maj2	CY	34°49′39.3″	32°49′43.6″	–	7/7	JX122667
O. majorana	SR951	maj2	CY	34°49′39.3″	32°49′43.6″	–	7/7	JX122668
O. majorana	SR961	maj2	CY	34°49′39.3″	32°49′43.6″	–	7/7	JX122669
O. majorana	SR998	–	CY	34°58′36.4″	32°28′23.0″	JX162886-JX162901	–	–

Table 2. *Continued*

Species	ID	Pop.	Geographical origin			ITS	DXS	DXS
				N	E	NCBI accession no.	alleles	NCBI accession no.
O. majorana	SR1087	–	CY	34°55′43.6″	32°26′32.5″	JX162902-JX162914	–	–
O. majorana	LC10	–	cv.	–	–	JX162804-JX162813	10/10	JX122675
O. majorana	Malta	–	cv.	–	–	JX162814-JX162822	5/5	JX122672
O. majorana	OBI	–	cv.	–	–	JX162832-JX162841	6/6	JX122673
O. majorana	Petronell	–	cv.	–	–	–	5/5	JX122674
O. majorana	Mira 2/3	–	cv.	–	–	JX162823-JX162831	–	–
O. onites	H38[RNG]	–	GR	–	–		3/3	JX122701
O. onites	H106[ESSE]	–	TR	–	–	JX162958-JX162959	–	–
O. onites	LC3	–	cv.	–	–	–	1/3	JX122713/JX122712
O. onites	SR498	oni4	TR	36°56′09.2″	31°28′34.1″	–	3/3	JX122702
O. onites	SR528	oni4	TR	36°56′09.2″	31°28′34.1″	–	3/17	JX122715/JX122714
O. onites	SR530	oni4	TR	36°56′09.2″	31°28′34.1″	JX162960-JX162970	16/17	JX122709/JX122708
O. onites	SR580	–	TR	36°35′38.5″	30°28′23.3″	–	3/3	JX122703
O. onites	SR656	–	TR	36°59′18.5″	30°28′05.1″	–	3/3	JX122704
O. onites	SR658	–	TR	36°59′18,5″	30°28′05,1″	–	17/17	JX122705
O. onites	SR777	–	TR	36°59′18.3″	31°12′25.4″	JX162971-JX162982	3/16	JX122717/JX122716
O. onites	SR779	–	TR	37°05′06.2″	31°13′51.0″	–	3/3	JX122706
O. onites	SR786	–	TR	37°15′55.8″	31°12′48.8″	JX162983-JX162997	3/17	JX122719/JX122718
O. onites	SR810	–	TR	37°16′00.3″	31°12′48.2″	JX163054	–	–
O. onites	SR1299	oni2	GR	36°51′05.5″	22°39′16.2″	–	2/3	JX122711/JX122710
O. onites	SR1372	oni3	GR	37°43′49.9″	22°45′22.8″	JX162998-JX163008	3/3	JX122707
O. onites	SR1387	oni3	GR	37°43′49.9″	22°45′22.8″	JX163051	–	–
O. onites	SR1434	oni1	IT	37°04′33.7″	15°16′30.4″	JX163052	3/3	JX122720
O. onites	SR1440	oni1	IT	37°04′33.7″	15°16′30.4″	JX163053	3/3	JX122721
O. onites	SR1460	oni1	IT	37°04′33.7″	15°16′30.4″	–	3/3	JX122722
O. syriacum	H50[RNG]	–	LB	34°11′	35°38′	JX163009-JX163024	11/14	JX122700/JX122699
O. syriacum var. *sinaicum*	H104[ESSE]	–	ET	–	–	JX163025-JX163031	5/5	JX122676
O. syriacum	LC7	–	cv.	–	–	JX163032-JX163038	–	–
O. syriacum	SP3	–	IL	–	–	JX163050	11/11	JX122677
O. syriacum var. *syriacum*	SR342	syr1	SY	35°49′53.7″	36°15′51″	–	12/15	JX122696/JX122695

Table 2. *Continued*

Species	ID	Pop.	Geographical origin			ITS	DXS	DXS
				N	E	NCBI accession no.	alleles	NCBI accession no.
O. syriacum var. *syriacum*	SR346	syr1	SY	35°49′53.7″	36°15′51″	–	12/12	JX122678
O. syriacum var. *syriacum*	SR349	syr1	SY	35°49′53.7″	36°15′51″	–	15/15	JX122679
O. syriacum var. *syriacum*	SR369	syr1	SY	35°42′40″	36°05′58.3″	–	12/12	JX122680
O. syriacum var. *syriacum*	SR371	syr1	SY	35°47′16.6″	36°02′12″	–	12/12	JX122681
O. syriacum var. *syriacum*	SR374	syr1	SY	35°47′16.6″	36°02′12″	–	12/12	JX122682
O. syriacum var. *syriacum*	SR378	syr1	SY	35°47′16.6″	36°02′12″	–	12/12	JX122683
O. syriacum var. *syriacum*	SR400	syr1	SY	35°20′57.2″	36°08′55.3″	–	12/13	JX122692/JX122691
O. syriacum var. *syriacum*	SR401	syr1	SY	35°20′57.2″	36°08′55.3″	–	12/12	JX122684
O. syriacum var. *syriacum*	SR402	syr1	SY	35°20′57.2″	36°08′55.3″	–	12/12	JX122685
O. syriacum var. *bevanii*	SR416	syr2	SY	35°05′49.7″	36°12′16″	–	12/12	JX122686
O. syriacum var. *bevanii*	SR418	syr2	SY	35°05′49.7″	36°12′16″	–	12/12	JX122687
O. syriacum var. *bevanii*	SR420	syr2	SY	35°05′49.7″	36°12′16″	–	12/13	JX122694/JX122693
O. syriacum	SR434	–	SY	34°59′46.7″	36°11′45.9″	–	12/12	JX122688
O. syriacum	SR437	–	SY	34°59′46.7″	36°11′45.9″	–	12/15	JX122698/JX122697
O. syriacum	SR438	–	SY	34°47′09.7″	36°09′28.9″	JX163039-JX163049	–	–
O. syriacum	SR442	–	SY	34°47′09.7″	36°09′28.9″	–	12/12	JX122689
O. syriacum	SR449	–	SY	34°47′09.7″	36°09′28.9″	–	12/12	JX122690

In *O. onites* and wild *O. majorana*, clones differed only by the presence or absence of point mutations at some of the 31 informative nucleotide positions defined (summarized in Table 3). ITS of *O. onites* was characterized by mutations at ten of these informative nucleotide positions (Table 3). Five mutations were present in all of the clones, five nucleotide positions were polymorphic and mutations occurred in 7–93% of the clones (Table 3). ITS of *O. majorana* was characterized by 14 informative nucleotide positions. Nine mutations were constantly present, five informative nucleotide positions were polymorphic and mutations occurred in 2–98% of the clones isolated from this species.

ITS of *O. syriacum*, cultivated *O. majorana* and *O. dubium* appeared to be much more complex, and exhibited variable nucleotide positions that were shared with more than one other taxon and putatively recombinant clones and a large number of clones with individual sequence composition. ITS of *O. syriacum* could be distinguished by 13 point mutations. Among all the different clones isolated, three ITS ribotypes (syr1 to syr3; Table 3; occurrence and frequencies of ITS ribotypes are summarized in Table 4) were present in more than one accession of *O. syriacum*. Ribotypes syr1 and syr2 differed only by the presence/absence of one mutation in ITS1, whereas ribotype syr3 was clearly distinct (Table 3). In ITS clones of cultivated *O. majorana*, 18 informative nucleotide positions were present and four frequent ITS ribotypes (cmaj1 to cmaj4, Table 3) were observed. Ribotype cmaj1 was identical to ribotype syr3 of *O. syriacum*. The sequence composition of ribotype cmaj2 matched that of ITS clones frequently present in accessions of wild *O. majorana*. Ribotypes cmaj3 and cmaj4 seemed to recombine features of ribotypes cmaj1 and cmaj2 in different ways. For ITS of *O. dubium*, 23 informative nucleotide positions were diagnostic. More than half were shared with either *O. syriacum*/O. *majorana* (in ITS1) or *O. onites* (mostly in ITS2). Five ITS ribotypes (dub1 to dub5, Table 3) were isolated from more than one accession of *O. dubium*. Ribotype dub1 was characterized by seven mutations, five of which were specific for *O. dubium.* Ribotype dub2 exhibited mutations that were shared with *O. syriacum* and/or *O. majorana* (ITS1) and *O. onites* (ITS2), whereas two further mutations were specific for *O. dubium.* Ribotype dub3 was characterized by four mutations that were constant in *O. onites* and two mutations specific for *O. dubium*. Ribotypes dub4 and dub5 combined features of dub1 and dub2 in different ways.

An ITS pattern similar to that of *O. dubium* was found in three Turkish accessions of *O. onites* (SR530, SR777, SR786). On closer examination of these accessions, complete ITS ribotypes of *O. dubium* (dub1 in SR777) and *O. onites* (in SR530, SR777, SR786) and putative recombinants (dub2 in SR786), and a high percentage of individual ribotypes of different sequence composition, were observed (Table 4). As point mutations specific for *O. dubium* were significantly involved in their intra-individual ITS polymorphisms, these accessions were treated as recent hybrids between *O. onites* and *O. dubium* (*oni* × *dub*, Table 3). In one accession of *O. syriacum* (H104), in addition to ribotype syr2, ITS clones identical to ribotypes of wild *O. majorana* and cmaj4 of cultivated *O. majorana* were observed, indicating recent hybridization between *O. syriacum* and (cultivated) *O. majorana* (*syi* × *maj*, Table 3).

All ITS sequences were checked for the presence of conserved angiosperm motifs in ITS1 (Liu & Schardl, 1994) and 5.8S (Jobes & Thien, 1997). These motifs were present in all of the clones, with the exception of one clone of cultivated *O. majorana* that lacked that in ITS1. In two clones of *O. dubium* (similar to the putative recombinants dub2 and dub4, respectively), a deletion (bp 493–510) in ITS2 was present. From these two clones, no valid secondary structures were obtained, indicating that the recombinant ITS ribotypes of *O. dubium* represent pseudogenes. Some of the clones of *O. dubium* with individual sequences resulted in chaotic secondary structures. In *O. dubium*, the overall highest free energies of the ITS2 region were observed for sequences of ribotype dub1. As this ribotype was also frequently isolated from all accessions analysed (Table 4), we assume that clones exhibiting ribotype dub1 represent functional ITS arrays of *O. dubium*.

Phylogenetic analyses of ITS

The high intra-individual and intraspecific ITS variability in *O. syriacum*, cultivated *O. majorana* and *O. dubium*, and the random combinations of shared and specific mutations in those ribotypes that exhibited an individual sequence composition, accounted for weakly resolved and confusing phylogenetic trees (data not shown). In order to use the high ITS variability as a tool, an alternative, distance-based approach (Göker & Grimm, 2008) was chosen to group the studied accessions according to their patterns of ITS variability. This alternative methodology has been shown to have a high potential to reflect evolutionary relationships (Göker & Grimm, 2008). In the resulting ITS network, three major groups could be clearly distinguished (Fig. 2). The first group is composed of *O. onites* from Italy (SR1434, SR1440), Greece (SR1372, SR1387) and Turkey (H106, SR810). These accessions exhibited solely ITS clones of the oni type (Table 4) and seem to represent the 'pure' *O. onites*. The second group comprises the accessions of *O. syriacum*, *O. majorana* and cultivated *O. majo-*

Table 3. Informative rDNA (internal transcribed spacer, ITS) nucleotide positions of the *Origanum* species under study. The numbers in the table head refer to the respective alignment position (ITS1: 13-223; 5.8S: 391; ITS2:410-582). Deviations from the consensus sequence (mutations) and the frequencies (%) of these mutations in the clones of a species/taxon are indicated. For dub, syr and cmaj, ITS variants (= ITS ribotypes) that were detected in more than one accession are listed. The order of these ribotypes corresponds to their frequencies in the respective taxon. cmaj, cultivated marjoram; dub, *Origanum dubium*; maj, *O. majorana*; oni, *O. onites*; syr, *O. syriacum*

	ITS1																					5.8S	ITS2										
	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	2	3	4	4	4	4	4	4	5	5	5	5	5	5
	1	1	1	1	1	2	2	3	4	5	7	8	9	0	1	6	7	7	8	2	9	1	2	2	2	4	7	0	0	4	4	7	8
	3	3	4	5	5	6	8	9	5	4	2	7	6	7	8	1	4	9	0	3	1	0	0	5	6	5	9	6	9	5	6	3	2
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 × 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	
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			
syr × 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.*	**C**	.	**C**	.	.	**T**	**T**	.	**C**	**G**	.	**T**	**A**	**T**	**C**	**A**	**C**	.	**T**	.	**A**	**T**	.	.	.	.	.	.	.	**G**	.	**G**	**G**
majorana	50		53			37	45		53	39		39	39	39	55	42	42		45		13	16								53		3	50
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	.
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	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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	.	.	.	.	.	.	.	.	.	.	.	.	.	.

The main results in this table are highlighted in bold type.

Table 4. Percentages of internal transcribed spacer (ITS) ribotypes (sequence characteristics of the ribotypes are indicated in Table 3) 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) (ind, number of clones with individual sequence characteristics that were isolated only once; cmaj, cultivated marjoram; dub, *Origanum dubium*; maj, *O. majorana*; oni, *O. onites*; syr, *O. syriacum*)

	ind.	oni	dub1	dub2	dub3	dub4	dub5	syr1 syr2	syr3 cmaj1	maj cmaj2	cmaj3	cmaj4
H106	–	100	–	–	–	–	–	–	–	–	–	–
SR1372	–	100	–	–	–	–	–	–	–	–	–	–
SR530	82	18	–	–	–	–	–	–	–	–	–	–
SR786	54	13	–	33	–	–	–	–	–	–	–	–
SR1054	50	–	14	21	7	7	–	–	–	–	–	–
SR777	75	17	8	–	–	–	–	–	–	–	–	–
H137	53	–	33	–	13	–	–	–	–	–	–	–
H67	20	–	73	–	–	–	7	–	–	–	–	–
SR558	56	–	38	–	–	6	–	–	–	–	–	–
SR1028	47	–	33	7	7	–	7	–	–	–	–	–
SR1200	54	–	31	–	–	8	–	–	–	–	–	–
SR725	25	–	42	25	–	8	–	–	–	–	–	–
H50	69	–	–	–	–	–	–	–	31	–	–	–
OBI	20	–	–	–	–	–	–	–	50	20	10	–
Malta	78	–	–	–	–	–	–	–	22	–	–	–
Mira2/3	44	–	–	–	–	–	–	–	11	22	–	22
LC10	60	–	–	–	–	–	–	–	–	20	–	20
SR1087	–	–	–	–	–	–	–	–	–	100	–	–
SR998	–	–	–	–	–	–	–	–	–	100	–	–
SR849	–	–	–	–	–	–	–	–	–	100	–	–
H104	43	–	–	–	–	–	–	29	–	14	–	14
SR438	–	–	–	–	–	–	–	100	–	–	–	–
LC7	–	–	–	–	–	–	–	86	14	–	–	–

rana. These three taxa are united by the presence of either the ITS ribotypes syr1, syr2 and syr3 or of ITS clones of the maj type (Table 4), which seem to be a descendant of syr1 and syr2. In *O. syriacum* from Israel (SP3, LC7) and Syria (SR438), syr1 and syr2 were the predominant ITS ribotypes. These accessions appear as sister to *O. majorana* from Cyprus (SR849, SR998, SR1087), which 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 an intermediate position. In Lebanese *O. syriacum* (H50) and cultivated *O. majorana* (Malta, OBI, Mira2/3, LC10), the ribotypes syr2, maj and syr3 were frequent. In addition, a large percentage of different recombinants was isolated, reflected by the formation of a conspicuous subnetwork. The third group comprises the accessions of Turkish and Cypriot *O. dubium* (H67, H137, SR558, SR725, SR1028, SR1054, SR1200) and putative hybrids between *O. onites* and *O. dubium* (SR530, SR777, SR786). The high intra- and inter-individual variability observed in *O. dubium* is reflected by the wide subnetwork. In clones of accession SR1054, a large proportion of oni-specific mutations was present, resulting in an ITS pattern related to that observed in the accessions of Turkish *O. onites* that are of putative hybrid origin.

DXS

With DXS, a new nuclear gene region was established to trace species relationships in *Origanum*. DXS is involved in the formation of isopentenylphosphate (IPP), a basic structure for molecules of primary and secondary metabolism (Cordoba, Salmi & Leon, 2009). The DXS gene seems to be part of a small gene family with two distinct gene subfamilies (DXS1 and DXS2). In *Medicago truncatulata* Gaertn., genes of the two

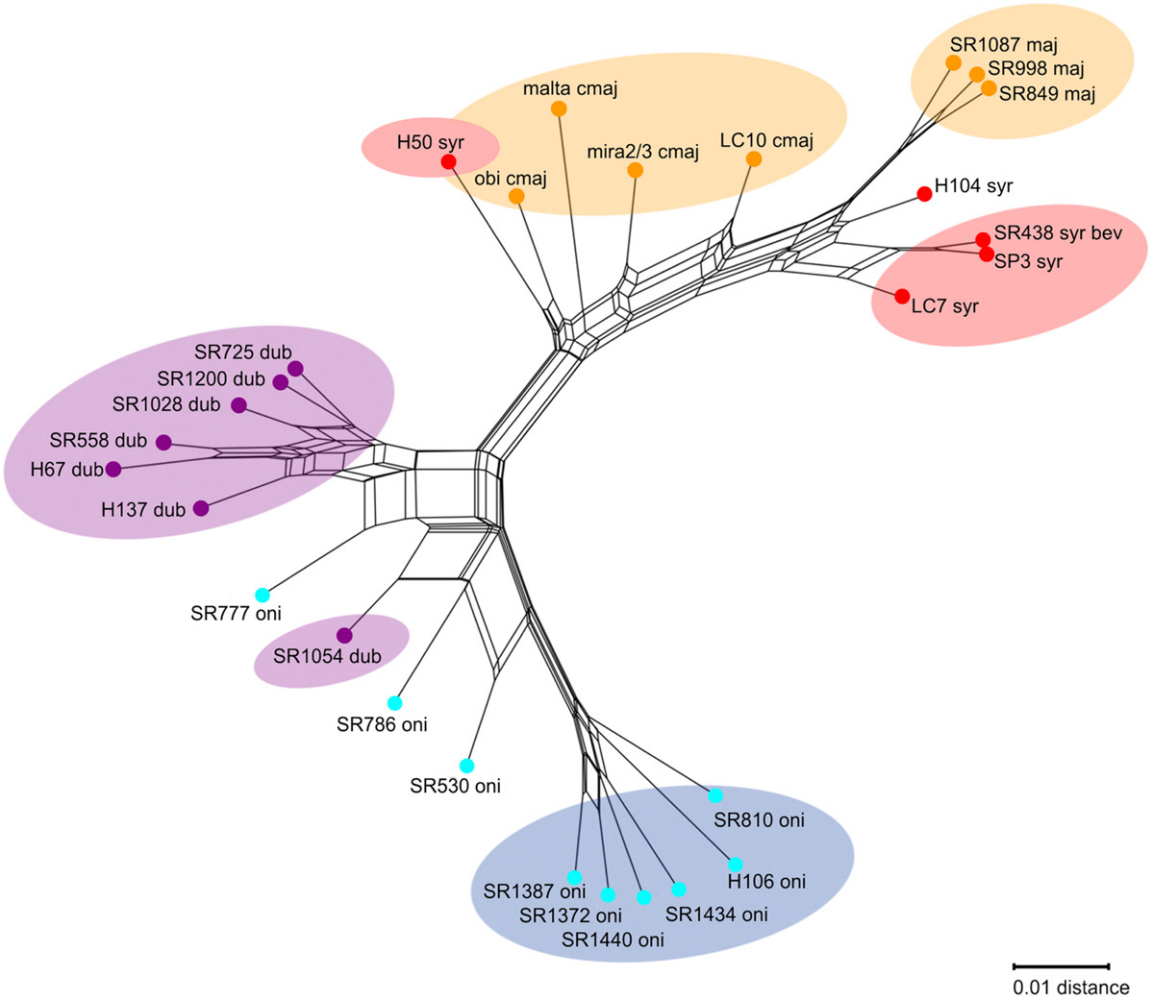

Figure 2. Neighbour-Net splits graph showing interindividual distances based on internal transcribed spacer (ITS) data. The sequence data of the ITS clones of each individual plant were transformed into a character matrix, with the transformed characters representing the frequency of a nucleotide at a certain sequence position (FRQ character transformation; Göker & Grimm, 2008). In a second step, from this character matrix, interindividual distances (Euclidean distances; Göker & Grimm, 2008) were computed (cmaj, cultivated marjoram; dub, *Origanum dubium*; maj, *O. majorana*; oni, *O. onites*; syr, *O. syriacum*).

subfamilies share about 70% identity in their amino acid sequences (Walter, Hans & Strack, 2002). In *Origanum*, sequencing and cloning of DXS did not reveal more than two different, not strongly diverging alleles from a single plant. Therefore, we are confident that all DXS fragments amplified correspond to a single locus (DXS2).

A total of 86 DXS sequences from 66 accessions of *O. dubium* (20), wild *O. majorana* (six), cultivated *O. majorana* (four), *O. onites* (16) and *O. syriacum* (20) were included in the analyses. Twenty of the 66 individuals examined showed heterozygosity at the DXS locus. The length of the aligned sequences varied from 413 to 421 bp. 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 109 bp was localized accounting for bp 216 to 324 of the alignment. Forty variable characters were observed.

Phylogenetic analyses of DXS

Seventeen DXS alleles were isolated from the four species investigated. In the DXS network, three distinct major groups can clearly be distinguished (Fig. 3, DXS allele identity of all accessions analysed is listed in Table 2). The first group comprises DXS alleles 1–3, which were only isolated from *O. onites*. Allele 3 was the most frequent and was present throughout the distribution area of *O. onites*. The second group is the most diverse, comprising 12

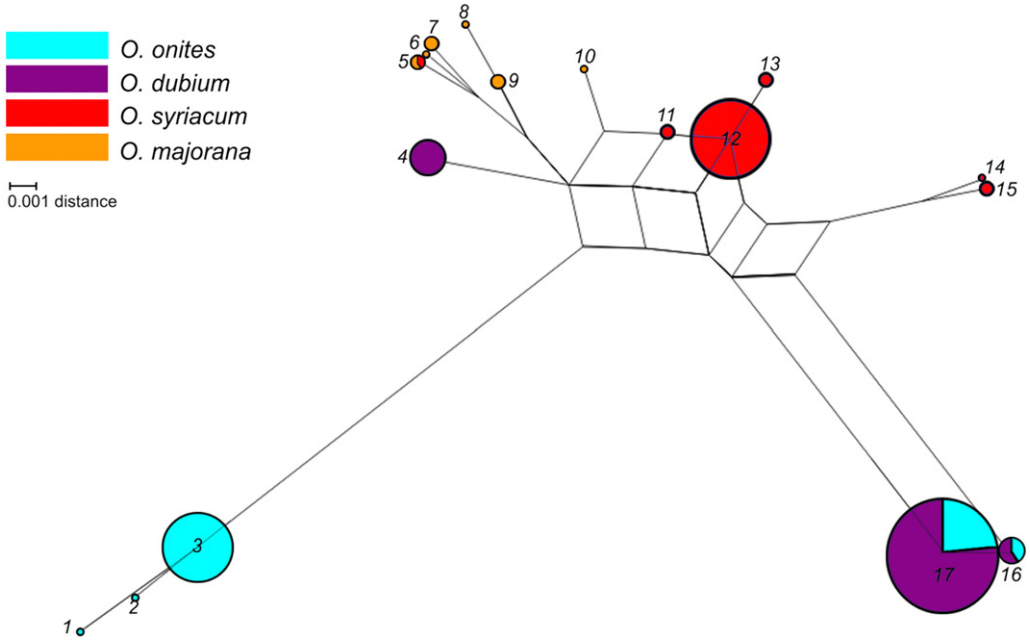

Figure 3. Neighbour-Net splits graph based on uncorrected *p* distances of 1-deoxy-D-xylulose 5-phosphate synthase (DXS) data. Numerals refer to the different DXS alleles (alleles 1–17) isolated. The sizes of the circles roughly correspond to the frequencies of the DXS alleles across the plant individuals analysed. The colours of the circles refer to *O. onites*, *O. syriacum*, *O. dubium* and *O. majorana*. In *O. majorana*, alleles 7, 8 and 9 were isolated from wild accessions and alleles 5, 6 and 10 were derived from different cultivars. The identity of DXS alleles of all plants analysed is listed in Table 2.

closely related DXS alleles (alleles 4–15), which were isolated from *O. dubium*, wild and cultivated *O. majorana* and *O. syriacum*. With the exception of allele 10, which seemed to be more closely related to DXS of *O. syriacum*, the DXS alleles of wild and cultivated *O. majorana* clustered closely together. In accessions of *O. syriacum*, allele 12 was the most frequent. Those two accessions of *O. syriacum* that did not originate from Syria (H50 and SP3; Table 2) exhibited two of the rare DXS alleles. Accession H104, which was classified as *O. syriacum* var. *sinaicum*, possessed a DXS allele (allele 5) similar or closely related to that isolated from cultivated *O. majorana*, supporting the hypothesis of a hybridogenous origin. *Origanum dubium* was exceptional as it exhibited rather distinct alleles (4 and 16/17). The rare allele 4 shares a common ancestor with the DXS alleles isolated from *O. majorana*, whereas the most frequent DXS allele 17 and the rarer allele 16 appeared to be clearly separated in the network. Alleles 16 and 17 were also isolated from some Turkish accessions of *O. onites* (Table 2), which exhibited DXS allele 3 in combination with allele 16 or 17, respectively. The co-occurrence of an *O. onites*-specific and an *O. dubium*-specific DXS allele in these accessions directly indicated hybridization. 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*.

MICROSATELLITES

Five polymorphic microsatellites were screened for three populations of 'pure' *O. onites* (onites1, onites2 and onites3), sympatrically occurring *O. onites* and *O. dubium* (onites4, 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) (Fig. 1). The mean expected heterozygosity of the populations analysed ranged from 0 to 0.44, the mean observed heterozygositiy ranged from 0 to 0.47 and the mean number of alleles per locus ranged from 1.0 to 3.6 (Table 5). Cultivated *O. majorana* was monomorphic at each locus. The population onites4 possessed the highest number of alleles per locus (Table 5).

Pairwise F_{ST} values varied widely between population pairs (Table 6). Significant F_{ST} values yielding from interspecific comparisons (0.23–0.74) were commonly higher than those resulting from intraspecific comparisons (0.17–0.44). In *O. onites*, high intraspecific pairwise F_{ST} values (0.30–0.44) were observed between the Sicilian and Greek populations (onites1,

Table 5. Mean diversity indices for the five investigated microsatellite loci (*n*, number of plants analysed; *a*, mean number of alleles/locus; H_e, expected heterozygosity; H_o, observed heterozygosity; cmaj, cultivated marjoram; dub, *Origanum dubium*; maj, *O. majorana*; oni, *O. onites*; syr, *O. syriacum*

Species	Population	*n*	*a*	H_e	H_o
O. onites	oni1	10	2.0	0.22	0.16
	oni2	24	2.4	0.31	0.23
	oni3	15	2.0	0.30	0.28
	oni4	33	3.6	0.35	0.30
O. dubium	dub1	27	2.8	0.42	0.47
	dub2	12	3.4	0.44	0.40
	dub3	12	3.0	0.42	0.42
O. majorana	maj1	12	2.2	0.20	0.10
	maj2	12	1.6	0.13	0.08
	cmaj	10	1.0	0.00	0.00
O. syriacum	syr1	15	2.4	0.26	0.20
	syr2	8	2.2	0.27	0.20

onites2, onites3), on the one hand, and the Turkish 'mixed' population (onites4) on the other, indicating high population differentiation. Although there seems 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 also be partly the result of 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 those between Sicilian and Greek populations of *O. onites* and *O. dubium* (0.47–0.56). In *O. dubium*, no significant pairwise F_{ST} value was observed between the two Cypriot populations, whereas significant genetic divergence existed 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 whether the genetic divergence is a result of 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* were among the lowest interspecific pairwise F_{ST} values (0.23–0.35). All F_{ST} values correlated to cultivated *O. majorana* were noticeably high (0.52–0.82), indicating high genetic differentiation between the marjoram cultivars and wild *Origanum* populations. However, these high values may reflect the strikingly different levels of genetic variation when comparing completely monomorphic cultivated *O. majorana* with heterozygous wild populations. In the PCA, cultivated *O. majorana* appeared to be close to the cluster composed of intermixed individuals of *O. syriacum* and *O. majorana* (Fig. 4). *Origanum onites* was clearly separating into two groups. Individuals of onites1, onites2 and onites3 clustered closely together, whereas plant individuals of population onites4 clustered in a second group that tended clearly towards *O. dubium*. No such apparent differentiation was visible between dubium1 and individuals of dubium2 or dubium3.

DISCUSSION

Evaluation of species and species boundaries

Origanum section *Majorana* has been regarded as a difficult group, and species boundaries and the taxonomic status of some taxa have remained unresolved (Ietswaart, 1980, 1982, 1985). Morphologically, *O. onites* appears to be the best defined and most homogeneous species, which can clearly be distinguished by its corymbiform inflorescences and usually 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*), our results confirmed the stable and independent character of this species. However, Turkish *O. onites*, originating from an area in which the distributions of *O. onites* and *O. dubium* overlap (Fig. 1), was clearly genetically distinct (Fig. 4). The sequence characteristics that were observed in some Turkish accessions of *O. onites* strongly suggest that gene flow from *O. dubium* to *O. onites* is significant and contributes to the genetic differentiation. The presence of *O. dubium*-specific DXS alleles in several Turkish accessions of *O. onites* directly indicated recent hybridization (Fig. 3). Some of these putative hybrids exhibited a complex mixture of the ITS patterns typical for both *O. onites* and *O. dubium* (Table 3). This local hybridization is probably also reflected in the secondary metabolites of *O. onites* and *O. dubium*. Earlier studies have shown that hybrids may express all or only some of the secondary compounds of the parental taxa, and novel compounds may arise. Concentrations of parental compounds may vary (Orians, 2000; Pichersky & Gang, 2000; Schwab, 2003). The essential oils of *O. onites* and *O. dubium* usually contain large amounts of 'cymyl' compounds but, in Turkish populations of both species, in addition, an almost pure linalool chemotype was detected (Baser, Kirimer & Tumen, 1993a; Baser *et al.*, 1993b; Lukas *et al.*, 2010a). The occurrence of such an exceptional chemotype in a putative hybrid zone could indicate that this chemotype is a result of the genetic consequences of hybridization.

Table 6. 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). Numerals in bold indicate nonsignificant F_{ST} values

	oni1	oni2	oni3	oni4	dub1	dub2	dub3	maj1	maj2	cmaj	syr1	syr2
oni1												
oni2	**0.091**											
oni3	**0.146**	**–0.023**										
oni4	0.439	0.306	0.300									
dub1	0.563	0.522	0.513	0.295								
dub2	0.513	0.479	0.473	0.283	0.169							
dub3	0.549	0.509	0.499	0.329	0.181	**–0.008**						
maj1	0.683	0.553	0.537	0.451	0.357	0.419	0.366					
maj2	0.740	0.604	0.602	0.450	0.389	0.454	0.383	**–0.002**				
cmaj	0.806	0.658	0.700	0.582	0.521	0.613	0.616	0.740	0.819			
syr1	0.595	0.510	0.510	0.383	0.289	0.350	0.305	0.336	0.347	0.503		
syr2	0.605	0.505	0.497	0.376	0.259	0.309	0.248	0.233	0.246	0.615	**–0.016**	

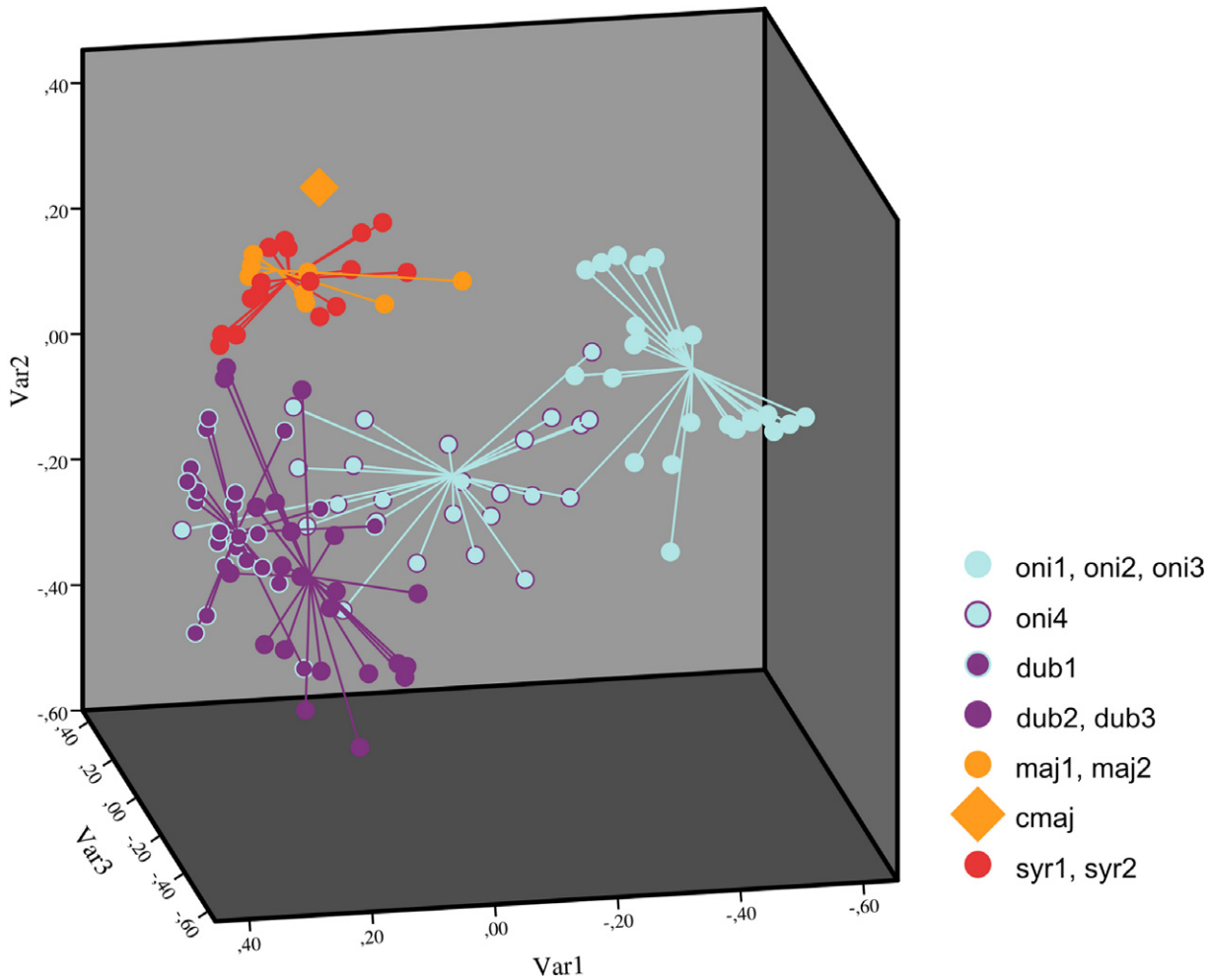

Figure 4. Principal components analysis (PCA) of the microsatellite data. Centroids of groups are indicated ['pure' *Origanum onites* (oni1, oni2 and oni3), sympatric occurring *O. onites* and *O. dubium* (oni4 and dub1), 'pure' *O. dubium* (dub2 and dub3), *O. majorana* (maj1 and maj2), cultivated *O. majorana* (cmaj) and *O. syriacum* (syr1 and syr2)]. Coordinates of the centroids are the weighted means for each axis.

Considering arbutin, Turkish populations of *O. onites* were also exceptional, containing small amounts of this compound, whereas populations of 'pure' *O. onites* were free of arbutin (Lukas *et al*., 2010b). The occurrence of arbutin in Turkish *O. onites* could therefore be a result of gene flow from arbutin-rich *O. dubium* into arbutin-free *O. onites*.

In contrast to *O. onites*, *O. syriacum*, *O. majorana* and *O. dubium* possess paniculate inflorescences and usually entire leaves. Morphologically, these three species are a complex of closely related taxa among which boundaries appear to be partly blurred. Characters that are used for their differentiation (Table 1) were found to be variable in natural populations. In *O. syriacum*, the striking morphological variability led to the description of three varieties (Ietswaart, 1980). This morphological heterogeneity parallels the high genetic diversity observed in *O. syriacum*. Three different ITS ribotypes were frequent, two of which were closely related and one was clearly distinct (Table 3). The simultaneous occurrence of such divergent ITS types could indicate ancient hybridization of *O. syriacum* with a sympatric (so far unknown) *Origanum* species. Local variations of morphological characters in *O. syriacum* could therefore be a consequence of introgression of dissimilar genetic material. With respect to DXS, five different alleles were isolated from 20 accessions (Fig. 3), again demonstrating the high genetic diversity. However, as plant individuals of *O. syriacum* from outside Syria were underrepresented in this investigation, no clear correlation between the geographical distribution of ITS ribotypes, DXS alleles and the distribution of the three morphological variants could be established. To achieve a clearer concept of the genetic identity and diversity of *O. syriacum*, a more comprehensive analysis of correctly designated plant material from the whole distribution area would be needed.

The largely unclear degree of genetic heterogeneity in *O. syriacum* also makes the taxonomic treatment of *O. majorana* difficult. From the simple sequence repeat (SSR) analysis, a close genetic relationship between populations of Syrian *O. syriacum* and *O. majorana s.s.* was observed. The close relationship was also obvious in ITS and DXS, but the position of *O. majorana s.s.* as a separate species seems to be better supported than with SSR data. When considering phytochemical characters, *O. majorana* is clearly distinct from *O. syriacum*. *Origanum majorana* possesses an exceptional essential oil chemotype accumulating large amounts of 'sabinyl' compounds (Fischer *et al*., 1987; Novak *et al*., 2008b), but lacking the oregano-typical 'cymyl' compounds that characterize the essential oils of *O. syriacum* (Fleisher & Fleisher, 1991; Lukas *et al*., 2009). Such exceptional 'sabinyl' chemotypes have not been described for wild populations of *O. syriacum* to date. Similar patterns were found for arbutin, a compound that is present in large amounts in wild and cultivated *O. majorana* and *O. dubium*, but not in *O. syriacum* (Lukas *et al*., 2010b).

The relationship between *O. syriacum* and *O. majorana* appears to be even more complex when considering the genetic characteristics of cultivated marjoram. Cultivated and wild marjoram showed closely related DXS alleles, but ITS revealed significant differences, relating the marjoram cultivars to accessions of *O. syriacum* from Lebanon and Israel. The sharing of ITS ribotype syr3 between cultivated *O. majorana* and *O. syriacum* (Table 4) is difficult to interpret, as the current knowledge about genetic diversity in *O. syriacum* and the historical origin of cultivated marjoram is limited. Probably, crossings with *O. syriacum* took place in its early cultivation history that could, to some degree, explain the morphological tendencies of cultivated *O. majorana* (e.g. larger and more greenish leaves; Novak *et al*., 2008b) towards *O. syriacum*. Accession H104 appears to be such a connecting link between *O. majorana* and *O. syriacum*. This Egyptian accession of *O. syriacum* was intermediate in ITS (Table 4) and possessed a DXS allele also present in cultivated *O. majorana* (Fig. 3). The hybrid origin of this specimen was also suggested by its intermediate essential oil composition, exhibiting both oregano-typical 'cymyl' compounds and marjoram-typical 'sabinyl compounds' (Baser *et al*., 2003).

Origanum dubium, which is morphologically close to *O. majorana*, was found to be a complex species. Sequence characteristics of the ITS ribotypes dub2, dub3, dub4 and dub5 (Table 3) indicated a hybridogenous origin from *O. onites* and *O. syriacum*. As the putative donor of the distinct ITS ribotype dub1, a third, unidentified *Origanum* species is assumed to have been involved in the speciation history of *O. dubium*. An interesting phenomenon is also the occurrence of distinct DXS alleles (Fig. 3). One is closely related to DXS of wild and cultivated *O. majorana*, indicating a common ancestor, probably an ancient *O. syriacum*. Two other alleles were clearly distinct and could be remnants of the third, unidentified ancestor. From the results of this investigation, it became clear that, although *O. majorana* and *O. dubium* are morphologically similar, they have distinct speciation histories. The close morphological relationship that has complicated their taxonomic classification (Ietswaart, 1980, 1982, 1985) most probably originates from their common ancestor *O. syriacum*.

As demonstrated previously, *O. dubium* hybridizes in the westernmost range of its distribution with its supposed ancestor *O. onites*. Although our data

provided evidence for gene flow from *O. dubium* to *O. onites*, gene flow from *O. onites* to *O. dubium* could not be detected. It remains unclear whether pre- or postzygotic mechanisms impede introgression of genetic material from *O. onites* into the genetic background of *O. dubium*.

Evolutionary relationships in section *Majorana*

Origanum onites and *O. syriacum* have been shown to be putative ancestors of *O. dubium*, and *O. majorana* is assumed to have its origin in *O. syriacum*. Therefore, we consider *O. onites* and *O. syriacum* to be ancient species in section *Majorana*. Probably, both derived from the same ancestor, *O. onites*, spread across Turkey and Greece, whereas *O. syriacum* developed in the eastern Mediterranean. In southern populations of *O. syriacum*, gene flow between *O. syriacum* and an unknown species may have occurred. At the north-western border of its distribution, hybridization with sympatric *O. onites* took place, presumably laying the basis for the formation of a 'proto-*O. dubium*'. Recurrent, independent hybridization and backcrossing with the two parental species may have occurred. *Origanum syriacum* var. *bevanii*, distributed in Turkey and the northern parts of Syria, is described as somewhat intermediate between *O. onites* and *O. syriacum* var. *syriacum*, differing from the latter by, for example, hirsute stems and larger, green leaves (Ietswaart, 1982). The morphological tendencies characteristic for *O. syriacum* var. *bevanii* could be a result of introgression of genetic material from *O. onites* 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 therefore, for some reason, genetic exchange with the parental species must have stopped after some time. The 'proto-*O. dubium*' was possibly able to undergo undisturbed speciation before a second hybridization event, involving a third, unknown species, led to the final formation of *O. dubium*. *Origanum onites* is not present in Cyprus and therefore *O. dubium* is assumed to have its origin in the Turkish Taurus Mountains, the diversity hot spot of *Origanum*. Fourteen species of different sections are exclusively distributed along the Turkish coast. Their formation is likely to be associated with range alternations and secondary contact of previously isolated species induced by Quaternary climatic oscillations (Ietswaart, 1982). Probably, hybridization between the 'proto-*O. dubium*' and the third parental species took place during this geo-historical period. *Origanum majorana* seems to have its origin in Syrian *O. syriacum* and seems to have developed by isolation and differentiation on the island of Cyprus.

Species distribution and recent hybridization

Recent hybridization has often been correlated with migration and range expansions following the last cold period of the Pleistocene. In the case of *Origanum* spp., the human factor must also be considered because species of the genus have been used extensively since ancient times. The popularity of oregano and marjoram in the ancient world is well documented (Bostock & Riley, 1855; Berendes, 1902; Edmondson & Bierkowski, 1993). Recently, *Origanum* DNA was amplified from clay material of a 2400-year-old Greek amphora (Hansson & Foley, 2008; Foley *et al.*, 2012). As oregano and marjoram played a role in the daily life for centuries, it can be assumed that the current distribution of *Origanum* spp. is not only a result of natural migration, but also of distribution by humans along trade routes. This could be an explanation of the nonsignificant or low intraspecific F_{ST} values, as they were especially obvious between the distant populations of the 'pure' *O. onites* (onites1 to onites3). The small population of *O. onites* in Sicily (onites1) is located in and around the ancient city of Syracuse which was founded by the Greeks in 733 BC. The isolated occurrence of this population, together with its narrow distribution on historical grounds, and its close genetic relationship to the two Greek populations, leads to the conclusion that *O. onites* was actively brought to Sicily by the Greeks.

Concluding remarks

The four species of section *Majorana* are widely used and are traded and processed in large quantities (e.g. Fleisher & Fleisher, 1988; Baser, 2002). Whenever *Origanum* plant material is commercially used, taxonomic ambiguities constitute not only a scientific challenge and also concern industry and consumers, as the unambiguous identification and classification of the original plant material is a prerequisite for high-quality products. Confusion arises especially from the unclear taxonomic status of *O. dubium* and *O. majorana*, when carvacrol-rich, oregano-flavoured plant material of *O. dubium* is designated as *O. majorana*, the species that has traditionally been linked to sweet marjoram. One of the primary goals of this investigation was to evaluate the species complex comprising *O. dubium*, *O. majorana s.s.* and *O. syriacum* to provide a broader basis for the ongoing taxonomic discussions. The results presented here revealed an astonishingly complex species history of *O. dubium* that is reflected in a unique pattern of ITS and DXS sequence characteristics allowing its reliable identification. As the genotypic discontinuity between *O. dubium* and the other two taxa of the complex is obvious, a classification system treating *O. dubium* as a separate species seems to be appro-

priate. This would be in accordance with the latest taxonomic treatment of *O. dubium* in the *Flora of Cyprus* (Ietswaart, 1985), where inflorescence characteristics (length and shape of the inflorescence) were used for its characterization and differentiation from the morphologically similar *O. majorana*.

In the case of *O. majorana* and *O. syriacum*, the close morphological relationship is in conformity with the low interspecific genetic differentiation. This does not necessarily mean that *O. majorana* would be better treated as a subspecies or a fourth variety of *O. syriacum*, because phenotypic (Ietswaart, 1980, 1982) and chemotypic (e.g. Fischer *et al*., 1987; Fleisher & Fleisher, 1991; Novak *et al*., 2008b; Lukas *et al*., 2009) incoherencies between *O. syriacum* and *O. majorana* are present. Therefore, we favour the taxonomic concept established by Ietswaart (1980, 1985), treating them as separate species.

ACKNOWLEDGEMENTS

The authors thank Professor Tod F. Stuessy, Head of the Department of Systematic and Evolutionary Botany, University of Vienna, for providing access to laboratory facilities, C. Schmiderer for her support in collecting plant material in Turkey, Cyprus, Greece and Italy, and the Universities of Reading, Eskisehir and Ankara for their permission to sample herbarium collections. The authors wish to extend their gratitude to V. Klejna and C. Schmiderer for their help with laboratory work and phytochemical analyses. Two anonymous reviewers are acknowledged for their suggestions that have helped to improve the paper. Financial support for this study was provided by the Austrian Science Foundation (FWF) (grant no. P18027-B06).

REFERENCES

Alvarez I, Wendel JF. 2003. Ribosomal ITS sequences and plant phylogenetic inference. *Molecular Phylogenetics and Evolution* **29:** 417–434.

Arnold N, Bellomaria B, Valentini G. 1993. Comparative study of the essential oils from three species of *Origanum* growing wild in the eastern Mediterranean region. *Journal of Essential Oil Research* **5:** 71–77.

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

Baldwin BG, Sanderson MJ, Porter JM, Wojciechowski MF, Campbell CS, Donoghue MJ. 1995. The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. *Annals of the Missouri Botanical Garden* **82:** 247–277.

Baričevič D, Bartol T. 2002. The biological/pharmacological activity of the *Origanum* genus. In: Kintzios SE, ed. *Oregano: the genera* Origanum *and* Lippia. London, New York: Taylor and Francis, 177–213.

Baser KHC. 2002. The Turkish *Origanum* species. In: Kintzios SE, ed. *Oregano: the genera* Origanum *and* Lippia. London, New York: Taylor and Francis, 109–126.

Baser KHC, Kirimer N, Tumen G. 1993a. Composition of the essential oil of *Origanum majorana* L. from Turkey. *Journal of Essential Oil Research* **5:** 577–579.

Baser KHC, Kurkcuoglu M, Demirci B, Ozek T. 2003. The essential oil of *Origanum syriacum* L. var. *sinaicum* (Boiss.) Ietswaart. *Flavour and Fragrance Journal* **18:** 98–99.

Baser KHC, Ozek T, Tumen G, Sezik E. 1993b. Composition of the essential oils of Turkish *Origanum* species with commercial importance. *Journal of Essential Oil Research* **5:** 619–623.

Bentham G. 1834. *Labiatarum genera et species*. London: Ridgway and Sons.

Bentham G. 1848. Labiatae. In: de Cantolle A, ed. *Prodromus systematis naturalis regni vegetalis Vol. 12*. Paris: Treuttel and Wurtz, 191–197.

Berendes J. 1902. *Des Pedanios Dioskurides aus Anazarbos Arzneimittellehre in fünf Büchern*. Stuttgart: Ferdinand Enke.

Boissier E. 1879. *Flora Orientalis 4*. Geneva, Basle: George.

Bostock J, Riley HT. 1855. *The Natural History of Pliny Book 13*. London: Henry G. Bohn.

Bräuchler C, Meimberg H, Heubl G. 2010. Molecular phylogeny of Menthinae (Lamiaceae, Nepetoideae, Mentheae) – taxonomy, biogeography and conflicts. *Molecular Phylogenetics and Evolution* **55:** 501–523.

Briquet J. 1895. Labiatae. In: Engler A, Prantl K, eds. *Die naturrlichen Pflanzenfamilien*. Leipzig: Engelmann, 183–375.

Carlström A. 1984. New species of *Alyssum*, *Consolida*, *Origanum* and *Umbilicus* from SE Aegean sea. *Willdenowia* **14:** 15–26.

Clark AG. 1990. Inference of haplotypes from PCR-amplified samples of diploid populations. *Molecular Biology and Evolution* **7:** 111–122.

Cordoba E, Salmi M, Leon P. 2009. Unravelling the regulatory mechanisms that modulate the MEP pathway in higher plants. *Journal of Experimental Botany* **60:** 2933–2943.

Danin A. 1990. Two new species of *Origanum* (Labiatae) from Jordan. *Willdenowia* **19:** 15–26.

Danin A, Künne I. 1996. *Origanum jordanicum* (Labiatae), a new species from Jordan, and notes on other species of sect. *Campanulaticalyx*. *Willdenowia* **25:** 601–611.

Della A, Paraskeva-Hadjichambi D, Hadjichambis AC. 2006. An ethnobotanical survey of wild edible plants of Paphos and Larnaca countryside of Cyprus. *Journal of Ethnobiology and Ethnomedicine* **2:** 34.

Denk T, Grimm GW, Hemleben V. 2005. Patterns of molecular and morphological differentiation in *Fagus* (Fagaceae): phylogenetic implications. *American Journal of Botany* **92:** 1006–1016.

Downie SR, Katz-Downie DS. 1996. A molecular phylogeny of Apiaceae subfamily Apioideae: evidence from nuclear ribosomal DNA internal transcribed spacer sequences. *American Journal of Botany* **83:** 234–251.

Doyle JJ, Doyle JL. 1990. Isolation of plant DNA from fresh tissue. *Focus* **12:** 13–15.

Duman H, Aytec Z, Ekici M, Karaveliogullari EA, Donmez A, Duran A. 1995. Three new species (Labiatae) form Turkey. *Flora Mediterranea* **5:** 221–228.

Duman H, Baser KHC, Aytec Z. 1998. Two new species and a new hybrid from Anatolia. *Turkish Journal of Botany* **22:** 51–55.

Edmondson J, Bierkowski P. 1993. Analysis of essential oils in funerary wreaths from Hawara. In: Davies WV, Walker R, eds. *Biological anthropology and the study of Ancient Egypt*. London: British Museum Press, 169–174.

Edwards CE, Soltis DE, Soltis PS. 2008. Using patterns of genetic structure based on microsatellite loci to test hypotheses of current hybridization, ancient hybridization and incomplete lineage sorting in *Conradina* (Lamiaceae). *Molecular Ecology* **17:** 5157–5174.

Fischer N, Nitz S, Drawert F. 1987. Original flavour compounds and the essential oil composition of marjoram (*Majorana hortensis* Moench). *Flavour and Fragrance Journal* **2:** 55–61.

Fleisher A, Fleisher Z. 1988. Identification of biblical hyssop and origin of the traditional use of oregano-group herbs in the Mediterranean region. *Economic Botany* **42:** 232–241.

Fleisher A, Fleisher Z. 1991. Chemical composition of *Origanum syriacum* L. essential oil. Aromatic plants of the Holy Land and the Sinai, part V. *Journal of Essential Oil Research* **3:** 121–123.

Foley BP, Hansson MC, Kourkoumelis DP, Theodoulou TA. 2012. Aspects of ancient Greek trade re-evaluated with amphora DNA evidence. *Journal of Archaeological Science* **39:** 389–398.

Göker M, Grimm GW. 2008. General functions to transform associate data to host data, and their use in phylogenetic inference from sequences with intra-individual variability. *Evolutionary Biology* **8:** 86. Available at: http://www.goeker.org/mg/distance

Goudet J. 1995. Fstat version 1.2: a computer program to calculate Fstatistics. *Journal of Heredity* **86:** 485–486.

Grayer RJ, Chase MW, Simmonds MSJ. 1999. A comparison between chemical and molecular characters for the determination of phylogenetic relationships among plant families: an appreciation of Hegnauer's 'Chemotaxonomie der Pflanzen'. *Biochemical Systematics and Ecology* **27:** 369–393.

Hansson MC, Foley BP. 2008. Ancient DNA fragments inside Classical Greek amphoras reveal cargo of 2400-year-old shipwreck. *Journal of Archaeological Science* **35:** 1169–1176.

Huson DH, Bryant D. 2006. Application of phylogenetic networks in evolutionary studies. *Molecular Biology and Evolution* **23:** 254–267.

Ietswaart JH. 1980. *A taxonomic revision of the genus* Origanum. Leiden: Leiden University Press.

Ietswaart JH. 1982. *Origanum*. In: Davis PH, ed. *Flora of Turkey and the East Aegean Islands, Volume 7*. Edinburgh: Edinburgh University Press, 297–313.

Ietswaart JH. 1985. *Origanum*. In: Meikle RD, ed. *Flora of Cyprus, Volume 2*. Kew: The Bentham-Moxon Trust, Royal Botanic Gardens Kew, 1262–1270.

Jobes DV, Thien LB. 1997. A conserved motif in the 5.8S ribosomal RNA (rRNA) gene is a useful diagnostic marker for plant internal transcribed spacer (ITS) sequences. *Plant Molecular Biology Reporter* **15:** 326–334.

Katsiotis A, Nikoloudakis N, Linos A, Drossou A, Constantinidis T. 2009. Phylogenetic relationships in *Origanum* spp. based on rDNA sequences and intra-genetic variation of Greek *O. vulgare* subsp. *hirtum* revealed by RAPD. *Scientia Horticulturae* **121:** 103–108.

Kaufmann M, Wink M. 1994. Molecular systematics of the Nepetoideae (Family Labiatae): phylogenetic implications from *rbcL* gene sequences. *Zeitschrift für Naturforschung* **49c:** 635–645.

Koch MA, Dobes C, Mitchell-Olds T. 2003. Multiple hybrid formation in natural populations: concerted evolution of the internal transcribed spacer of nuclear ribosomal DNA (ITS) in North American *Arabis divaricarpa* (Brassicaceae). *Molecular Biology and Evolution* **20:** 338–350.

Kuorwel KK, Cran MJ, Sonneveld K, Miltz J, Bigger SW. 2012. Essential oils and their principal constituents as antimicrobial agents for synthetic packaging films. *Journal of Food Science* **76:** 164–177.

Larsson S. 2007. The 'new' chemosystematics: phylogeny and phytochemistry. *Phytochemistry* **68:** 2904–2908.

Liu JS, Schardl CL. 1994. A conserved sequence in internal transcribed spacer 1 of plant nuclear rRNA genes. *Plant Molecular Biology* **26:** 775–778.

Lukas B, Samuel R, Novak J. 2010a. Oregano or marjoram? The γ-terpinene synthase affects chemotype formation in the genus *Origanum*. *Israel Journal of Plant Sciences* **58:** 211–220.

Lukas B, Schmiderer C, Franz C, Novak J. 2009. Composition of essential oil compounds from different Syrian populations of *Origanum syriacum* L. (Lamiaceae). *Journal of Agricultural and Food Chemistry* **57:** 1362–1365.

Lukas B, Schmiderer C, Mitteregger U, Novak J. 2010b. Arbutin in marjoram and oregano. *Food Chemistry* **121:** 185–190.

Mader E, Lukas B, Novak J. 2008. A strategy to setup codominant microsatellite analysis for high-resolution-melting-curve-analysis (HRM). *Genetics* **9:** 69.

Mayol M, Rosello JA. 2001. Why nuclear ribosomal DNA spacers (ITS) tell different stories in *Quercus*. *Molecular Phylogenetics and Evolution* **19:** 167–176.

Muir G, Schlötterer C. 2005. Evidence for shared ancestral polymorphism rather than recurrent gene flow at microsatellite loci differentiating two hybridizing oaks (*Quercus* spp.). *Molecular Ecology* **14:** 549–561.

Müller M, Lukas B, Novak J, Simoncini T, Genazzani AR, Jungbauer A. 2008. Oregano: a source for peroxisome proliferator-activated receptor gamma antagonists. *Journal of Agricultural and Food Chemistry* **56:** 11 621–11 630.

Novak J, Lukas B, Bolzer K, Grausgruber-Gröger S, Degenhardt J. 2008a. Identification and characterization of simple sequence repeat markers from a glandular *Origanum vulgare* expressed sequence tag. *Molecular Ecology Resources* **8:** 599–601.

Novak J, Lukas B, Franz CM. 2008b. The essential oil composition of wild growing sweet marjoram (*Origanum majorana* L., Lamiaceae) from Cyprus – three chemotypes. *Journal of Essential Oil Research* **20:** 339–341.

Orians CM. 2000. The effects of hybridization in plants on secondary chemistry: implications for the ecology and evolution of plant–herbivore interactions. *American Journal of Botany* **87:** 1749–1756.

Paton AJ, Springate D, Suddee S, Otieno D, Grayer RJ, Harley MM, Willis F, Simmonds MSJ, Powell MP, Savolainen V. 2004. Phylogeny and evolution of basils and allies (Ocimeae, Labiatae) based on three plastid DNA regions. *Molecular Phylogenetics and Evolution* **31:** 277–299.

Peakall R, Smouse PE. 2006. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes* **6:** 288–295.

Pichersky E, Gang DR. 2000. Genetics and biochemistry of secondary metabolites in plants: an evolutionary perspective. *Trends in Plant Science* **5:** 439–445.

Schulte K, Barfuss MHJ, Zizka G. 2009. Phylogeny of Bromelioideae (Bromeliaceae) inferred from nuclear and plastid DNA loci reveals the evolution of the tank habit within the subfamily. *Molecular Phylogenetics and Evolution* **51:** 327–339.

Schwab W. 2003. Metabolome diversity: too few genes, too many metabolites? *Phytochemistry* **62:** 837–849.

Schwarzbach AE, Rieseberg LH. 2002. Likely multiple origins of a diploid hybrid sunflower species. *Molecular Ecology* **11:** 1703–1715.

Singletary K. 2010. Oregano: overview of the literature on health benefits. *Nutrition Today* **45:** 129–138.

Skoula M, Gotsiou P, Naxakis G, Johnson CB. 1999. A chemosystematic investigation on the mono- and sesquiterpenoids in the genus *Origanum* (Labiatae). *Phytochemistry* **52:** 649–657.

Skoula M, Harborne JB. 2002. The taxonomy and chemistry of *Origanum*. In: Kintzios SE, ed. *Oregano: the genera* Origanum *and* Lippia. London, New York: Taylor and Francis, 67–108.

Tamura K, Dudley J, Nei M, Kumar S. 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* **24:** 1596–1599.

Vaezi J, Brouillet L. 2009. Phylogenetic relationships among diploid species of *Symphyotrichum* (Asteraceae: Astereae) based on two nuclear markers, ITS and GAPDH. *Molecular Phylogenetics and Evolution* **51:** 540–553.

Vokou D, Kokkini S, Bessiere JM. 1988. *Origanum onites* (Lamiaceae) in Greece: distribution, volatile oil yield, and composition. *Economic Botany* **42:** 407–412.

Walker JB, Kenneth JS, Treutlein J, Wink M. 2004. *Salvia* (Lamiaceae) is not monophyletic: implications for the systematics, radiation and ecological specializations of *Salvia* and tribe Mentheae. *American Journal of Botany* **91:** 1115–1125.

Walter MH, Hans J, Strack D. 2002. Two distantly related genes encoding 1-deoxy-D-xylulose 5-phosphate synthases: differential regulation in shoots and apocarotenoid-accumulating mycorrhizal roots. *Plant Journal* **31:** 243–254.

White TJ, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds. *PCR protocols: a guide to methods and applications*. San Diego, CA: Academic Press, 315–322.

Wink M. 2003. Evolution of secondary metabolites from an ecological and molecular phylogenetic perspective. *Phytochemistry* **64:** 3–19.

Zheng X, Cai D, Yao L, Teng Y. 2008. Non-concerted ITS evolution, early origin and phylogenetic utility of ITS pseudogenes in *Pyrus*. *Molecular Phylogenetics and Evolution* **48:** 892–903.

Zuker M. 2003. Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Research* **31:** 3406–3415. Available at: http://frontend.bioinfo.rpi.edu