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ORIGINAL ARTICLE

Molecular and chemical characterization of the most widespread *Ocimum* species

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Abstract DNA fingerprinting (AFLP) and chemical analyses of essential oils were utilized to define the extent of variation existing in the genus *Ocimum*. Research was carried out on 22 *Ocimum* accessions representing seven species. Concerning the essential oil composition of all investigated accessions, 115 compounds were identified. UPGMA cluster analysis, based on Euclidian distances of essential oil constituents between all pairs of accessions, showed four well-supported clusters (*O. tenuiflorum, O. basilicum/O. africanum, O. basilicum*, and *O. americanum/O. africanum*). Relating to the essential oil composition of all of ?A3B2 tlsb?> the investigated accessions, 17 compounds were identified as the main ones, and according to them 13 chemotypes were determined. AFLP relationships were determined by neighbor-joining (NJ) cluster analysis based on Dice's distance matrix and by maximum parsimony (MP) analysis. *O. basilicum*, *O. americanum/O. africanum*, *O. tenuiflorum*, and *O. gratissimum* represented four clusters supported with high bootstrap values. A neighbor-net diagram allowed the visualization of apparently conflicting data by revealing relationships between genotypes and chemotypes. Concerning the *O. africanum* species, two distinct chemotypes, geranial/neral (accession 11) and estragol (accession 10), have been established, while all the studied *O. americanum* accessions belong to the geranial/neral chemotype. This could be additional evidence that *O. americanum* is one of the parents of *O. africanum*. Furthermore, the fact that the *O. africanum* accession (10) as well as *O. basilicum* 'Purpurascens' and *O. basilicum* 'Erevanskii' accessions belong to the estragol chemotype supports the theory that *O. africanum* is one of the parents of these two *O. basilicum* accessions.

Keywords AFLP · Basil · Chemotypes · Essential oil · GC-MS · Genotypes

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Introduction

Ocimum L. is a member of the Lamiaceae family, and estimates of the species number in the genus vary from 30 (Paton 1992) to 160 (Pushpangadan and Bradu 1995). Plants of the genus are collectively called basil and are used as a source of essential oils, spices, ornamentals, and medicines (Simon et al. 1990). The most used species, for essential oil production and as a pot herb, are *O. basilicum* L., *O. americanum* L., and their putative hybrid *O. africanum* Lour. (syn. *O.* x *citriodorum* Vis.). The species *O. kilimandscharicum* Baker ex Gürke is extensively grown in the tropics for camphor production, while the major economic importance of *O. gratissimum* L. is production of essential oil. *O. tenuiflorum* L. is widely used as a pot herb, medicinally, and in essential oil production,

while *O. campechianum* Mill. is used as a pot herb and medicinally (Paton et al. 1999).

Species classification within the genus is still uncertain because species identification relies on morphological characters whose expression is known to be affected by developmental and environmental factors (Labra et al. 2004). On the other hand, the study of the taxonomy of the genus is difficult because of the abundant cross pollination and interference of humans with selection, cultivation, and hybridization. Basil is known to occur as several chemotypes that differ in their essential oil compositions (Simon et al. 1990). Thanks to this diversity and since chemotaxonomy can also be used to assess inter- or intra-species variability, the chemical composition of essential oils of plants in the genus *Ocimum*, especially *O. basilicum*, have been the object of many studies (Hiltunen and Holm 1999). Many species of the genus *Ocimum* contain essential oils based primarily on monoterpene derivatives such as camphor, limonene, thymol, citral, geraniol, and linalool. Other members of the genus, including common basil (*O. basilicum*), contain an essential oil based primarily on high proportions of phenolic derivatives, such as eugenol, methyl chavicol (estragole), and methyl cinnamate, often combined with various proportions of linalool (Labra et al. 2004). Because the aroma of each species, variety, or cultivar is predominantly determined by its genotype and depends on the main chemical components of the essential oils, genetic characterization represents an extremely important task (De Masi et al. 2006). AFLP (Vos et al. 1995) is one of DNA markers that have become extremely important for the genetic analysis of plants because of their ability to reveal genetic diversity among individuals, populations, and species. AFLP has already been used to investigate genetic variation in *Ocimum* (Carović-Stanko et al. 2010a; Labra et al. 2004).

The objectives of this research were to study the genetic relationships among the basil taxa, to determine their essential oil compositions and chemotypes, to verify the occurrence of any correlation among genetic distance and essential oil profiles, and to gain better insight into the origin and genetic relationships among the studied basil taxa.

Materials and methods

Plant material

Research was carried out on 22 accessions, representing the seven most widespread and economically most important *Ocimum* species (*O. africanum* Lour., *O. americanum* L., *O. basilicum* L., *O. campechianum* Mill., *O. gratissimum* L., *O. kilimandscharicum* Baker ex Gürke, and *O. tenuiflorum* L.). All investigated accessions are listed in Table 1. *O. basilicum* accessions were selected to represent morphotypes (true basils, small-leaf basils, lettuce-leaf basils, purple basil A, and purple basil B) as previously reported by Carović-Stanko et al. (2011). Seeds were obtained from the collection of medicinal and aromatic plants of the Department of Seed Science and Technology, Faculty of Agriculture, University of Zagreb, Croatia (http://cpgrd.zsr.hr). This site also housed the voucher specimens.

Essential oil extraction and gas chromatography-mass spectrometry

One hundred grams of plant material and 500 ml of water were placed in a Clevenger type apparatus. The essential oil was isolated by hydrodistillation for 3 h. The obtained essential oil was separated, dried over anhydrous sodium sulfate, and stored under argon in a sealed vial at −20°C before usage. The voucher specimens of the basil essential oils are deposited in the Laboratory of Biochemistry, Faculty of Chemistry and Technology, Split, Croatia.

The analyses of the volatile compounds were run on a Hewlett-Packard gas chromatography-mass spectrometry (GC-MS) system (GC 5890 series II; MSD 5971A, Hewlett-Packard, Vienna, Austria). Two columns of different polarity were used: a HP-101 column (methyl silicone fluid, Hewlett-Packard; 25 m × 0.2 mm i.d., film thickness 0.2 μm) and a HP-20 M column (Carbowax, Hewlett-Packard; 50 m × 0.2 mm i.d., film thickness 0.2 μm). Oven temperature was programmed as follows: held isothermally at 70°C for 4 min, then increased to 180°C at a rate of 4°C/min, and subsequently held isothermally for 15 min (for the HP-20 M column); held isothermally at 70°C for 2 min, then increased to 200°C at a rate of 3°C/min, and held isothermally for 15 min (for HP-101 column). The carrier gas was helium (1 ml/min). The injection port temperature was 250°C, and the detector temperature was 280°C. Ionization of the sample components was performed in the EI mode (70 eV). The injected volume was 1 μl. The linear retention indices for all the compounds were determined by co-injection of the samples with a solution containing the homologous series of C8–C22 *n*-alkanes (Van Den Dool and Kratz 1963). The individual constituents were identified by their identical retention indices referring to the compounds known from the literature data (Adams 1995) and also by comparing their mass spectra with spectra of either the known compounds or with those stored in the Wiley mass spectral database (Hewlett-Packard, Vienna, Austria).

AFLP markers

Total genomic DNAs were extracted from fresh leaves of a single plant per accession using the DNeasy Plant Mini Kit

Table 1 Accession number, species, cultivar name, and morphotype of 22 *Ocimum* accessions included in the analysis

No.	Accession no.[a]	Species	Cultivar	Morphotype[b]
1	MAP00294	*O. basilicum*	Genovese	True basil
2	MAP00232	*O. basilicum*	Sweet basil	True basil
3	MAP01648	*O. basilicum*	Green globe	Small-leaf basil
4	MAP00559	*O. basilicum*	Blistered lettuce-leaf	Lettuce-leaf basil
5	MAP00334	*O. basilicum*	Purpurascens	Purple basil (A)
6	MAP00335	*O. basilicum*	Erevanskii	Purple basil (A)
7	MAP00586	*O. basilicum*	Thai-basilikum	Purple basil (A)
8	MAP01657	*O. basilicum*	Thai basil 'Queenette'	Purple basil (B)
9	MAP00284	*O. basilicum*	Dark opal	Purple basil (B)
10	MAP01635	*O. africanum*		
11	MAP00156	*O. africanum*		
12	MAP00594	*O. americanum*		
13	MAP01647	*O. americanum*		
14	MAP01651	*O. americanum*		
15	MAP01625	*O. gratissimum*		
16	MAP01631	*O. gratissimum*		
17	MAP01636	*O. kilimandscharicum*		
18	MAP01627	*O. tenuiflorum*		
19	MAP00160	*O. tenuiflorum*		
20	MAP01656	*O. tenuiflorum*		
21	MAP01628	*O. tenuiflorum*		
22	MAP01624	*O. campechianum*		

[a] Accession number from the Collection of Medicinal and Aromatic Plants, Zagreb, Croatia, as available at the Croatian Plant Genetic Resources Database (http://cpgr.zsr.hr)

[b] Morphotypes taken over from a previous report by Carović-Stanko et al. (2011)

(Qiagen). The quality and quantity of the extracted DNAs were checked on 0.8% agarose gels with λ DNA as a standard (Boehringer Mannheim). The DNA concentrations were measured using a QubitTM fluorometer (Invitrogen).

The AFLP protocol followed that of Vos et al. (1995) with several modifications. Restriction digestion and adapter ligation were performed simultaneously on 200 ng of genomic DNA in a total volume of 33 µl. Fifteen units of high concentration restriction enzyme *Eco*RI (50 U/µl, Fermentas) and 3 units of high concentration restriction enzyme *Tru*1I (=*Mse*I) (50 U/µl, Fermentas) were used for DNA digestion. For ligation of 25 pmol *Eco*RI and 25 pmol *Tru*1I double-stranded nucleotide adapters, three units of high-concentration T4 DNA ligase (30 U/µl, Fermentas) were applied. Digestion and ligation were performed for 2 h at 37°C and 14 h at 23°C. Pre-amplification and amplification were performed with the GeneAmp PCR System 9600. For pre-amplification, one-nucleotide selective primers were used, and the program was: 2 min at 70°C followed by 20 cycles: at 94°C for 20 s, 56°C for 30 s, 72°C for 2 min, and 60°C for 30 min. Four-primer combinations were selected for amplification (FAM-*Eco*RI-ACT + *Mse*-CAG, NED-*Eco*RI-AGA + *Mse*-CAG, VIC-*Eco*RI-ACG + *Mse*-CGA, and PET-*Eco*RI-ACC + *Mse*-CGA). The amplification program was: 2 min at 94°C; 10 cycles at 94°C for 20 s, 66°C for 30 s, 72°C for 2 min (the annealing temperature was 1°C lower in each cycle during that 10 cycles); 20 cycles at 94°C for 20 s, 56°C for 30 s, 72°C for 2 min, and the final cycle was 60°C for 30 min. Samples were detected using an ABI3130 DNA sequencer (Applied Biosystems). The presence or absence of fragments was scored on the chromatograms with GeneMapper 4.0 Software (Applied Biosystems). All fragments between 50 and 500 bp were scored. The peaks obtained were automatically transposed into a binary matrix. When the peak height exceeded an absolute value of 50, adjusted with the Peak Amplitude Threshold Settings of GeneMapper 4.0 Software, a peak was scored as present (1); otherwise it was scored as absent (0).

Data analysis

Euclidean distances were calculated between all pairs of *Ocimum* accessions based on 115 essential oil constituents. Percentages were normalized by arcsine transformation. Thus, obtained Euclidean distances were hereby referred to as biochemical dissimilarities between pairs of accessions. A hierarchical cluster procedure was performed using the UPGMA algorithm. Statistical support of the branches was tested with bootstrap analysis using 1,000 replicates (Felsenstein 1985). The calculations were made using PAST version 2.01 (Hammer et al. 2001). Scores between 50 and 74 bootstrap percentages (BS) were defined as weak support, scores between 75 and 89% BS as moderate

support, and scores greater than 90% BS as strong support. The cophenetic correlation coefficient was calculated, and Mantel's test (Mantel 1967) was performed to check the goodness of fit of a cluster analysis to the matrix, which was based on using 1,000 random permutations with NTSys-pc version 2.1 (Rohlf 2000).

AFLP amplified fragments were scored for the presence (1) or absence (0) of homologous bands to create binary matrices. Pairwise genetic distances were calculated using Dice's coefficient (Dice 1945), and the cluster analysis was performed by the neighbor-joining method (Saitou and Nei 1987) as implemented in TREECON for Windows version 1.3 b (Van de Peer and De Wachter 1994). Bootstrap analysis was performed on 1,000 bootstrap samples. A neighbor-net diagram (Bryant and Moulton 2004) was constructed from Dice's distances using SplitsTree 4 (Huson and Bryant 2006).

Pearson's correlation coefficient and Mantel's test were used to compute and test the linear correlation between the matrix of genetic distances and biochemical dissimilarity matrix. The significance level was assessed after 1,000 random permutations in NTSYS-pc.

Determination of the phylogenetic signal in the molecular data set and maximum parsimony analysis were performed using PAUP version 4.0b10 (Swofford 2003). The phylogenetic signal was determined using the RANDOM TREES option from the tree-length distribution of 1,000,000 trees and comparing the g1 value for the distribution to the critical value of -0.15 (at $\alpha = 0.01$ for 500 variable characters and 15 taxa; Hillis and Huelsenbeck 1992). Data sets that produce g1 values less than -0.15 are significantly more structured than the random data. Maximum parsimony (MP) analyses were conducted using heuristic searches of the AFLP data, with tree bisection reconnection (TBR) branch swapping and 1,000 random addition sequence replicates. In the first round, all characters were specified as unweighted. Other searches were run using successive weighting (Farris 1969). Characters were reweighted by the maximum value of the rescaled consistency index (RC) calculated in the previous search, i.e., characters that showed high homoplasy were given lower weight. The procedure was repeated until reaching identical weights, lengths, and topologies in two successive rounds. Bootstrap support values were calculated from 1,000 heuristic search replicates with TBR branch swapping and 100 random addition sequence replicates.

Results

Essential oil composition

Using GC/MS analyses 115 volatiles were identified as constituents of investigated essential oils. In each accession identified compounds represented more than 90% of the oil. Seventeen main compounds (>10% of the total oil content) of essential oils were identified in 22 analyzed accessions (Table 2). Chemotypes were designated by the main compound if found in concentrations higher than 50% or by the most abundant components. In addition, 57 more constituents of essential oils were identified in concentrations higher than 1%, but lower than 10%, and 41 compounds more found in concentrations less than 1%. The number of compounds per accession ranged from 10, as in *O. basilicum* 'Erevanskii' (06), to 33 as in *O. gratissimum* (16). A total of 56 compounds were identified as "private" (i.e., compounds found in a single accession); additionally 16 compounds were found in more than one accession of a species, but not in other species. Minimal biochemical dissimilarity between pairs of accessions (0.279) was observed between *O. basilicum* 'Purpurascens' (05) and *O. basilicum* 'Erevanskii' (06), while maximal (1.737) was observed between *O. basilicum* 'Genovese' (01) and *O. basilicum* 'Erevanskii' (06). Concerning the average biochemical dissimilarity among all pairs of accessions belonging to the same species, the highest dissimilarity was noticed between *O. africanum* accessions (1.313) followed by *O gratissimum* (1.302), *O. basilicum* (1.066), *O. americanum* (0.630), and *O. tenuiflorum* (0.493) accessions.

The cophenetic correlation coefficient between the matrix of cophenetic values and the original distance matrix was high ($r = 0.877$; $P < 0.001$). The dendrogram obtained by cluster analysis (Fig. 1) of the composition of essential oils of all accessions showed four well-supported clusters. The first cluster was composed of *O. tenuiflorum* accessions representing three chemotypes: (1) β-bisabolene chemotype (accession 18 with 56.97% β-bisabolene), (2) β-bisabolene/1,8-cineole chemotype (accessions 19 and 20 with 25.89–26.68% β-bisabolene and 21.77–24.74% 1,8-cineole), and (3) 1,8-cineole/β-bisabolene chemotype (accession 21 with 24.90% 1,8-cineole and 24.60% β-bisabolene). Investigated *O. basilicum* accessions were separated in two clusters; the first one (cluster two) comprised accessions rich in estragol representing two chemotypes: (1) estragol chemotype (accessions 05–07 with 78.20–94.57% estragol) and (2) estragol/linalool chemotype (accession 04 with 47.52% estragol and 20.82% linalool). In this cluster also an *O. africanum* accession (10) with an estragol chemotype was included. The second one (cluster three) comprised linalool-rich *O. basilicum* accessions representing three chemotypes: (1) linalool chemotype (accessions 01, 02, and 09 with 58.79–66.40% linalool), (2) linalool/(Z)-methyl cinnamate chemotype [accession 08 with 46.16% of linalool and 18.75% (Z)-methyl cinnamate], and (3) linalool/eugenol chemotype (accession 03 with 30.49 linalool and 19.57% eugenol).

Table 2 Main compounds of the essential oils of 22 *Ocimum* accessions

No.	Species	Cultivar	Linalool	Eugenol	Estragol	(Z)-methyl cinnamate	Geranial	Neral	α-bergamotene	Thymol	p-cimene	Camphor	β-bisabolene	1,8-cineole	Caryophyllene	α-farnesene	Nerol	Caryophyllene oxide	β-elemene
1	*O. basilicum*	Genovese	66.40	8.26	0.00	0.00	0.00	0.00	7.96	0.00	0.00	0.49	0.00	7.23	0.00	0.00	0.00	0.00	1.00
2	*O. basilicum*	Sweet basil	60.04	12.26	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.61	0.00	7.36	0.00	2.82	0.00	0.00	0.68
3	*O. basilicum*	Green globe	30.49	19.57	0.59	0.00	0.00	0.00	0.00	0.30	0.59	1.34	0.00	2.73	0.00	16.40	0.00	0.00	0.00
4	*O. basilicum*	Blistered lettuce-leaf	20.82	0.00	47.52	1.21	0.00	0.00	6.84	0.00	0.17	0.60	0.00	6.17	0.00	0.33	0.00	0.00	0.00
5	*O. basilicum*	Purpurascens	0.00	0.00	87.75	0.00	0.00	0.00	0.68	0.00	0.00	0.00	0.00	0.43	0.00	0.00	0.00	0.00	0.00
6	*O. basilicum*	Erevanskii	0.00	0.43	94.57	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.50	0.00	0.00	0.00	0.00	0.00	0.00
7	*O. basilicum*	Thai-basilikum	1.30	0.00	78.20	0.00	0.00	0.00	4.60	0.00	0.00	3.47	0.00	2.51	0.00	0.00	0.00	0.46	0.00
8	*O. basilicum*	Thai basil 'Queenette'	46.16	7.25	5.50	18.75	0.00	0.00	0.00	0.00	0.17	0.63	0.00	1.95	0.00	0.00	0.00	0.00	0.00
9	*O. basilicum*	Dark opal	58.79	6.32	1.92	0.93	0.00	0.00	0.00	0.00	0.00	1.17	0.00	7.71	0.97	0.00	0.00	0.39	0.00
10	*O. africanum*		26.42	0.25	66.54	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.31	0.00	0.62	0.00	0.37	0.00
11	*O. africanum*		7.20	0.83	1.12	0.00	31.21	21.80	0.36	0.00	0.00	0.00	0.00	0.88	0.00	1.71	14.54	3.69	0.00
12	*O. americanum*		7.70	0.00	0.42	0.00	23.19	17.25	2.26	0.00	0.00	0.00	0.00	0.00	0.00	0.00	8.31	6.62	0.00
13	*O. americanum*		12.15	0.57	0.65	0.00	28.58	20.16	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.52	7.15	7.12	0.00
14	*O. americanum*		0.00	0.55	0.79	0.00	34.87	25.10	1.25	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	6.35	0.00
15	*O. gratissimum*		12.09	39.74	0.97	0.42	0.00	0.00	12.17	0.00	0.00	0.00	0.00	1.08	0.00	0.00	0.00	10.10	0.00
16	*O. gratissimum*		0.75	0.58	0.31	0.71	0.00	0.00	0.00	39.73	14.94	0.00	0.00	0.00	0.00	0.00	0.00	1.52	0.00
17	*O. kilimandscharicum*		0.35	0.00	0.30	0.00	0.00	0.00	0.00	0.00	0.43	56.97	0.00	14.63	0.00	0.00	0.00	0.00	0.00
18	*O. tenuiflorum*		2.78	2.90	3.35	0.00	0.00	0.00	6.58	0.00	0.00	0.00	51.98	5.14	0.00	0.87	0.00	4.15	0.00
19	*O. tenuiflorum*		3.71	5.12	21.62	0.00	0.00	0.00	3.78	0.00	0.00	0.00	25.89	21.77	0.00	0.00	0.00	2.77	0.00
20	*O. tenuiflorum*		0.41	4.83	14.69	0.13	0.00	0.48	4.37	0.00	0.00	0.00	26.68	24.74	0.00	0.00	0.00	3.57	0.00
21	*O. tenuiflorum*		0.00	7.56	17.18	0.00	0.00	0.00	2.92	0.00	0.00	0.00	24.60	24.90	0.00	0.00	0.00	2.97	0.00
22	*O. campechianum*		5.13	5.53	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	20.31	14.00	0.00	0.00	8.25	11.10

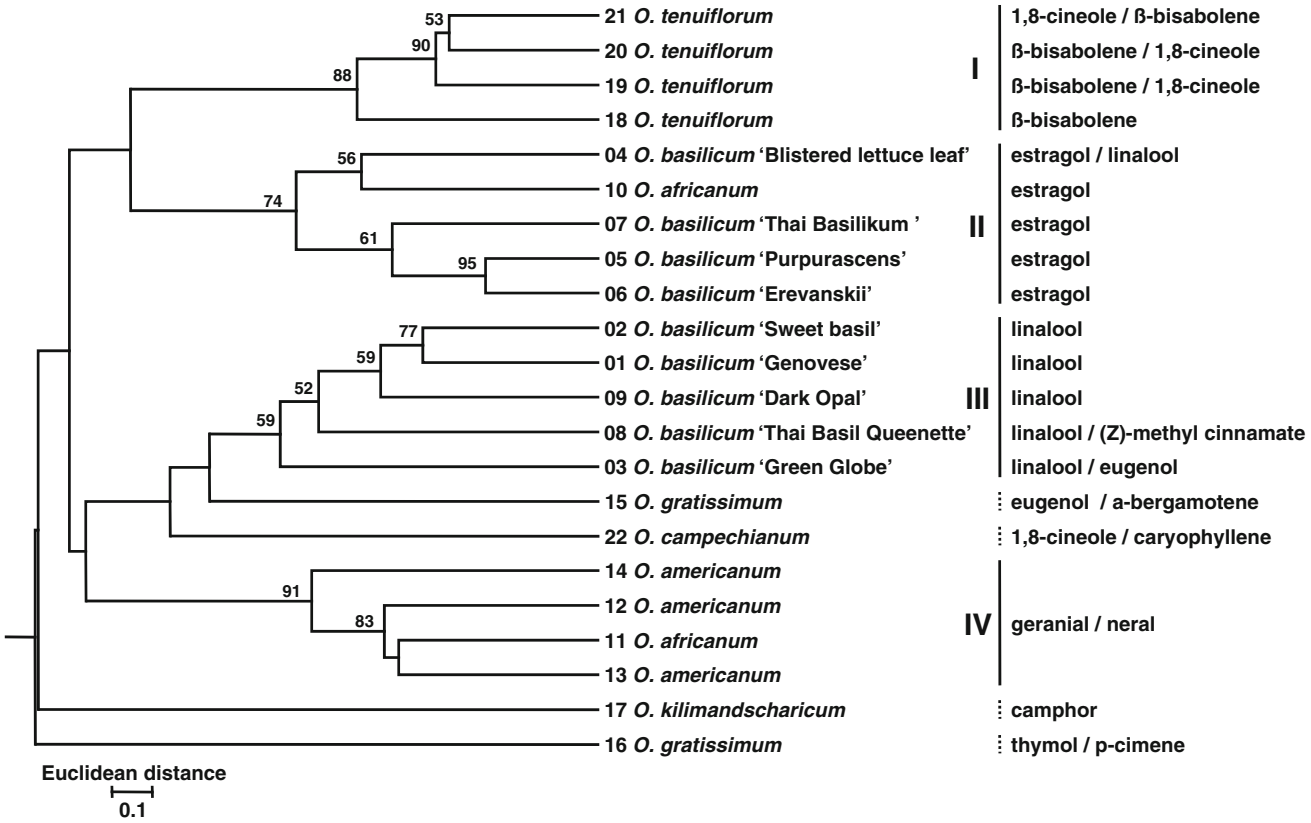

Fig. 1 Dendrogram obtained by cluster analysis of the composition of essential oils of 22 *Ocimum* accessions. Boostrap support values higher than 50% of 1,000 replicates are given above the branches. Chemotypes are indicated on the *right*. The clusters are designate by *Roman numerals*

On the basis of all compounds presenting oil, *O. campechianum* (22) and *O. gratissimum* (15) accessions were clustered together, but with a low BS, with *O. basilicum* accessions rich in linalool. The main compounds of *O. campechianum* (22) essential oil were 1,8-cineole (20.31%) and caryophyllene (14%). *O. gratissimum* had two chemotypes: (1) eugenol/α-bergamotene (accession 15 with 39.74% eugenol and 12.17%/α-bergamotene) and (2) thymol/p-cymene chemotype (accession 16 with 39.73% thymol and 14.94% p-cymene). However, this *O. gratissimum* accession (16) did not cluster with any other accessions based on the chemical composition. The fourth cluster was composed of *O. americanum* accessions (12, 13, 14), including one *O. africanum* accession (11) rich in geranial (23.19–34.87%) and neral (17.25–25.10%). Camphor (56.97%) was the main compound of *O. kilimandscharicum* (17) essential oil.

Genetic relationships

Four AFLP primer combinations generated 1,734 polymorphic DNA fragments. Using AFLP data, all the accessions were successfully separated. The pairwise distances based on those data ranged from 0.193 between two accessions belonging to *O. americanum* (12 and 13) to 0.875 between *O. basilicum* 'Sweet basil' (02) and *O. campechianum* (22); the average accession distance was 0.635. Dice's distance between pairs of accessions belonging to the same species was high for accessions belonging to *O. gratissimum* (0.744), but was low for accessions belonging to *O. tenuiflorum* (0.233). Matrix correlation based on Dice's distances and biochemical dissimilarity was relatively low ($r = 0.404$), but highly significant ($P < 0.001$ after 1,000 permutations). Genetic relationships were determined by neighbor-joining cluster analysis based on Dice's distance matrix between accessions. By AFLP data four clusters were resolved with bootstrap support values of 100%, while the *O. campechianum* accession was separated out of all groupings (Fig. 2).

The parsimony analysis of the AFLP data, from 22 accessions, contained 1,734 polymorphic fragments; 1,158 were parsimony-informative. The g1 statistic for this data set was −0.63, indicating significant phylogenetic signal. An unweighted parsimony analysis yielded a single most parsimonious tree. The tree had a length of 3,790 steps, consistency index (CI) of 0.458, retention index (RI) of 0.571, and rescaled consistency index (RC) of 0.261. The

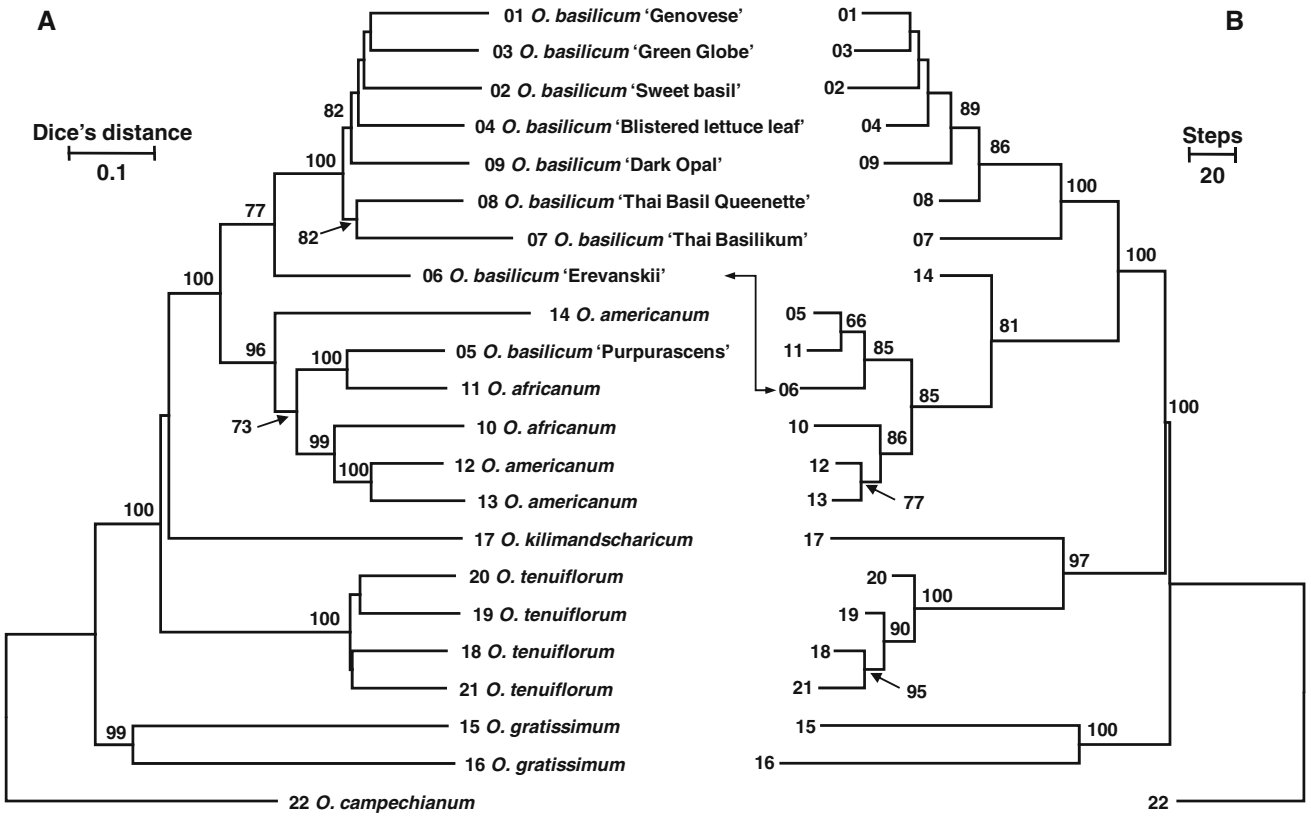

Fig. 2 **a** Phylogram of the neighbor-joining tree based on Dice's distances and **b** phylogram of the most parsimonious tree (1,123.42 steps) recovered from the AFLP analysis of 22 *Ocimum* accessions after one round of character reweighting. Trees were rooted using the *O. campechianum* accession. Boostrap support values higher than 50% of 1,000 replicates are given above the branches

successive weighting approach produced a topologically identical tree. Tree length was reduced to 1,123.42 steps with CI = 0.803, RI = 0.837, and RC = 0.674. Subsequent rounds of reweighting have yielded the same tree. Four major clusters, separating *O. basilicum*, *O. americanum/O. africanum*, *O. tenuiflorum*, and *O. gratissimum*, with very high bootstrap support values appeared in both NJ and MP trees, while the *O. campechianum* accession was separated out of all groupings (Fig. 2). In the NJ tree and MP tree, two incongruences, concerning the *O. basilicum* 'Erevanskii' (06) accession and *O. kilimandscharicum* (17) accession, could be noted.

To depict the reticulate relationships among taxa, a neighbor-net diagram derived from AFLP was produced as presented in Fig. 3. Splits with a weight <0.01 (34 out of 76) were omitted to aid legibility. The fit value was 98.599, indicating that the phylogenetic signal in the data is represented almost perfectly well by the split graph.

The neighbor-net diagram indicated a possible solution for the two above-mentioned incongruences, while based on the same dissimilarity matrix, it allowed the visualization of apparently conflicting data, such as caused by interspecies hybridization. According to NJ analysis the *O. basilicum* 'Erevanskii' (06) accession belonged to the *O. basilicum* cluster, while according to the parsimony analysis, it belonged to the *O. americanum/O. africanum* cluster. Nevertheless, the results represented on the neighbor-net diagram indicated a hybrid origin of the *O. basilicum* 'Erevanskii' (06) accession involving *O. basilicum* and *O. africanum* as putative parental species. Concerning the *O. kilimandscharicum* (17) accession, it was clustered together with *O. basilicum*, *O. africanum*, and *O. americanum* accessions as a sister taxon according to the NJ tree, but with low BS. On the other hand, on the MP tree it was clustered together with *O. tenuiflorum* accessions also as a sister taxon. In the neighbor-net diagram *O. kilimandscharicum* accession was clearly separated from both *O. basilicum* and *O. americanum/O. africanum* as well as the *O. tenuiflorum* cluster, but it is closer to the *O. tenuiflorum* cluster.

Discussion

For each *Ocimum* sp. accession included in this study, chemotypes were compared to their respective genotypes. Investigations on genetic diversity, taxonomic and phylogenetic relationships, as well as essential oil composition

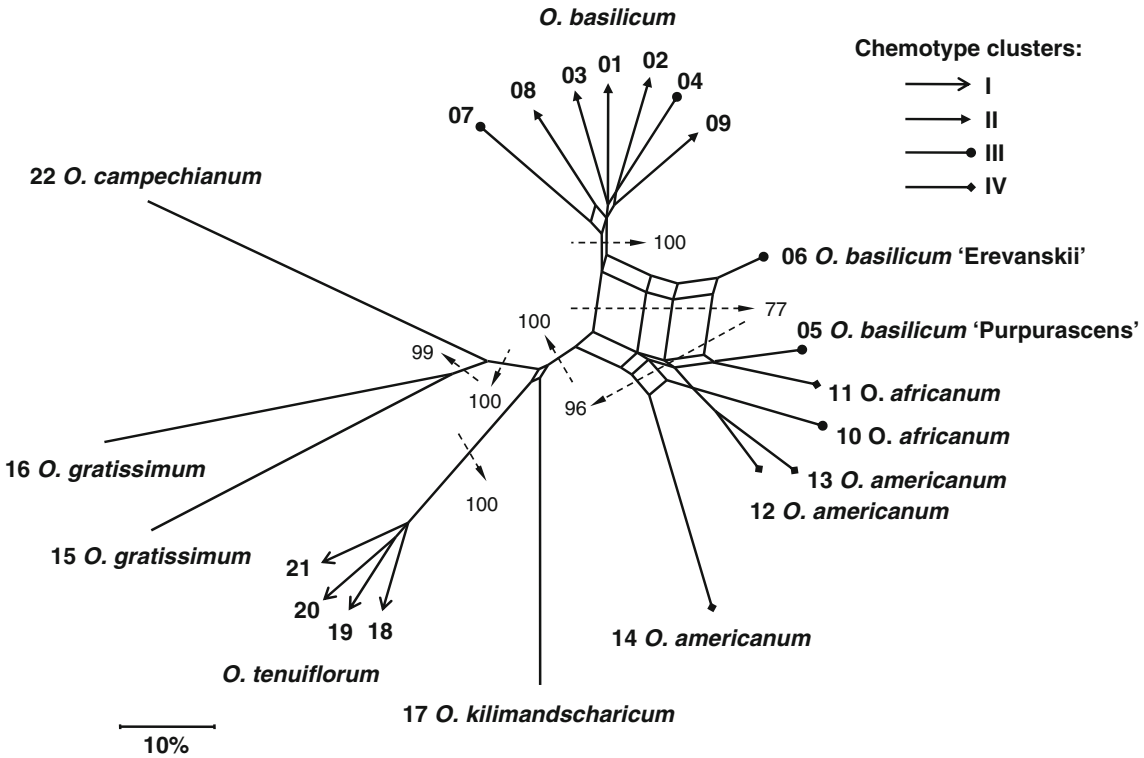

Fig. 3 Neighbor-net diagram based on AFLP data of 22 *Ocimum* accessions. Boostrap support values (higher than 50% of 1,000 replicates) were derived from neighbor-joining analysis. Chemotype clusters are indicated at the branch tips and designated by cluster numbers given in Fig. 1

and morphological variability of some *Ocimum* species have been reported earlier (Carović-Stanko et al. 2011; Carović-Stanko et al. 2010a, b; De Masi et al. 2006; Singh et al. 2004; Satovic et al. 2002; Vieira et al. 2003). However, the present study combines these different approaches and also analyzes relationships among the most outspread *Ocimum* species as well as among the most widespread *O. basilicum* cultivars.

According to the AFLP data analysis, the *O. campechianum* accession was separated out of all groupings and also had a different essential oil composition with 1,8-cineole and β-caryophyllene as the main compounds. Moreover, according to the results of Vieira and Simon (2000), *O. campechianum* accessions were very rich in 1,8-cineole, while one accession exhibited a distinct chemical profile, with β-caryophyllene as the major constituent and with a lower relative percentage of 1,8-cineole. All of our results showed high distinction of *O. campechianum* from other investigated species.

According to phenotypical traits revealed in this study, *O. tenuiflorum* was clearly distinguished from the other investigated species. It has three chemotypes: β-bisabolene chemotype, β-bisabolene/1,8-cineole chemotype, and 1,8-cineole/β-bisabolene chemotype. Zheljazkov et al. (2008a) reported that the major oil constituents of *O. tenuiflorum* were eugenol and methyl chavicol (estragole), while Kothari et al. (2005) reported that methyl eugenol is the major oil constituent. Such discrepancies in major oil constituents published so far for *O. tenuiflorum* can be explained by the different genotypes included in the research by the different authors and/or cultivated under different conditions. Concerning the AFLP analyses, the examined *O. tenuiflorum* accessions had separate positions from the rest of the species on phenetic trees as well as the *O. gratissimum* accessions. The separate position of *O. gratissimum* and *O. tenuiflorum* could be explained by their belonging to the *Sanctum* group, while *O. basilicum*, *O. americanum*, and *O. africanum* belong to the *Basilicum* group (Khosla 1995). Moreover, *O. gratissimum* and *O. tenuiflorum* have a smaller number of chromosomes ($2n = 40$ and $2n = 36$, respectively) compared to *O. basilicum* and *O. americanum* ($2n = 48$ and $2n = 72$, respectively) (Carović-Stanko et al. 2010a). Additionally, according to our results, *O. gratissimum* had two chemotypes: eugenol/α-bergamotene and thymol/p-cymene, which were also reported by Vieira and Simon (2000). According to Hiltunen and Holm (1999), Hegnauer (1966) divided *O. gratissimum* oils into three chemotypes: thymol, citral, and eugenol types, while according to Pino et al. (1996), *O. gratissimum* oil is rich in thymol, p-cimene, and β-caryophyllene. Such differences could also be explained by the different genotypes included in the research by

different authors and/or cultivated under different conditions.

O. kilimandscharicum was the only species included in this research that is rich in camphor, which is in agreement with the results of Hegnauer (1966) (according to Hiltunen and Holm 1999). Furthermore, according to genetic characterization, it was clustered together with *O. basilicum*, *O. africanum*, and *O. americanum* accessions as a sister taxon according to the NJ tree with low BS. On the other hand, on the MP tree it was clustered together with *O. tenuiflorum* accessions with high BS (97%). According to results presented on the neighbor-net diagram, *O. kilimandscharicum* was closer to *O. tenuiflorum*, although, Paton (1992) grouped it together with *O. basilicum* and *O. americanum* in one sub-section (*Ocimum* subsect. *Ocimum*). Nevertheless, on the basis of the presented results we cannot precisely define its taxonomic position.

O. basilicum accessions as well as *O. americanum* and *O. africanum* accessions were separated from all other species included in this study with 100% bootstrap support. Various studies have pointed out that *O. basilicum* occurs in many subspecies, botanical varieties, and forms, which differ from each other both in the morphology and chemical composition of the essential oil, showing widespread intraspecies variability. Essential oils in different *O. basilicum* cultivars are variable; however they contain primarily phenylpropenes, such as eugenol, methyleugenol, chavicol, estragole, and methyl-cinnamate, often combined with various amounts of linalool (Grayer et al. 1996; Zheljazkov et al. 2008b). According to our analyses *O. basilicum* has five chemotypes: linalool, linalool/eugenol, linalool/(Z)-methyl cinnamate, estragol/linalool, and estragol. As early as 1930 Guillaumin (according to Hiltunen and Holm 1999) classified *O. basilicum* oil into four categories: (1) linalool-methyl chavicol (estragole) type, (2) camphor type, (3) methyl cinnamate type, and (4) eugenol type. Sobti and Pushpangadan (1982) reported the existence of five different chemotypes, including a new eugenol chemotype. According to the results of Vieira and Simon (2000), *O. basilicum* is rich in linalool, methyl chavicol, and methyl (E)-cinnamate. Accessions reported in their paper were found to be linalool/methyl cinnamate rich and linalool/methyl chavicol rich. On the basis of more than 200 analyses of oils extracted from *O. basilicum*, Lawrence (1993) delineated four essential oil chemotypes (methyl chavicol, linalool, methyl eugenol, and methyl cinnamate) and also numerous subtypes. Zheljazkov et al. (2008b), on the basis of the oil content of 38 *O. basilicum* accessions, have reported seven chemotypes: linalool, linalool-eugenol, methyl chavicol, methyl chavicol-linalool, methyl eugenol-linalool, methyl cinnamate-linalool, and bergamotene. Such differences in chemotypes could be explained by different cultivars and varieties included in the research by different authors, while *O. basilicum* is rich in cultivars and cultivated all over the world. It has to be pointed out that the volatile oil constituents exhibited an excellent tool for discrimination among the investigated accessions, but the use of secondary metabolites as an intraspecific marker needs to consider other factors, such as environmental conditions and plant development stage (Vieira et al. 2001). On the other hand, our work was conducted under the same conditions on the same field where all the accessions were grown at the same time, and leaves and flowers were collected and dried at the same growth stage. In this manner, the variables due to pedoclimatic and growth stage factors were controlled. Then again, the revealed chemotypes in *O. basilicum* are not always in agreement with the determined genotypes, meaning that chemotypes classified on the basis of essential oil composition are not sufficient for the taxonomy studies.

O. africanum and *O. americanum* accessions together with the *O. basilicum* 'Purpurascens' (05) accession according to NJ analyses have formed a separate cluster. According to MP analyses the *O. basilicum* 'Erevanskii' (06) accession was also a member of the *O. americanum*/*O. africanum* cluster. Such clustering could be explained by their very close phylogenetic relationships or by the hybrid origin of *O. africanum* (10), *O. basilicum* 'Purpurascens' (05), and *O. basilicum* 'Erevanskii' (06) accessions according to the chromosome number and genome size, as reported previously by Carović-Stanko et al. (2010a). Additional evidence for a hybrid origin of *O. africanum*, *O. basilicum* 'Purpurascens,' and *O. basilicum* 'Erevanskii' accessions could be their essential oil composition. A connection between chemotypes and genotypes is presented in the neighbor-net diagram (Fig. 3). *O. africanum* has two chemotypes, geranial/neral (accession 11) and estragol (accession 10), whereas all studied *O. americanum* accessions belong to the geranial/neral chemotype. This could be additional evidence that *O. americanum* is one of the parents of *O. africanum*. Furthermore, the estragol chemotype found in *O. africanum* and *O. basilicum* 'Purpurascens' and *O. basilicum* 'Erevanskii' accessions supported the theory (Carović-Stanko et al. 2010a) where *O. africanum* is one of the parents of these two *O. basilicum* accessions.

The results revealed a certain degree of correspondence between phenotypic and molecular data among the investigated *Ocimum* accessions, indicating that phenotypic trait analysis can be a sound basis for further differentiation by molecular markers. *O. africanum*, *O. americanum*, and *O. basilicum* phylogenetically are more closely related to each other than the other species included in research, *O. gratissimum*, *O. tenuiflorum*, *O. campechianum*, and *O. kilimandscharicum*. In order to elucidate the genetic

basis for the chemical profiles observed within each species, different approaches, such as association mapping or the development of the genetic linkage map and subsequent quantitative trait loci (QTL) analysis, could be pursued to determine the genetic basis of the variation in the chemical composition observed within each species.

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