Synergistic Effects of Combining Morphological and Molecular Data in Resolving the Intraspecific Classification in *O. basilicum* L.

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Summary

High levels of both morphological and chemical variability exist within the *O. basilicum* L. species. Long-term traditional uses and wide distribution throughout the world, as well as traditional selection and breeding efforts, have contributed to variability within the species. Morphological traits according to UPOV descriptor list and AFLP markers were utilized to define the extent of existing variation in the species analyzing 24 accessions. Phenotypic dissimilarities between pairs of accessions were calculated and the UPGMA dendrogram was constructed. A number of clearly defined clusters have been detected, giving a good representation of traditional taxonomic relationships. Genetic relationships were determined by Neighbour-Joining cluster analysis based on Dice's distance matrix between accessions. Generally, morphologically similar accessions grouped together and a high congruence between trees was observed. Our analyses revealed a certain degree of correspondence between morphological and molecular data among *O. basilicum* L. accessions. Both AFLP markers and morphological descriptors can contribute in resolving existing problems concerning intraspecific classification in *O. basilicum*.

Key words

AFLP, basil, morphological descriptors, UPOV

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Introduction

The genus *Ocimum* L. comprises between 30 and 160 species (Paton et al. 1999). The most widely grown are *O. africanum* Lour (= *O.* x *citriodorum* Vis.) *O. americanum* L. (= *O. canum* Sims.), *O. basilicum* L., *O. gratissimum* L. and *O. tenuiflorum* L. (= *O. sanctum* L.).

Basil (*Ocimum basilicum* L.) has been acclaimed for its diversity as a source of essential oils, its flavour and delicacy as spice, and its beauty and fragrance as an ornamental (Simon et al., 1990). It is extensively used in pharmacy, perfume and food industries for its natural aroma and flavour (Darrah, 1980). Within *O. basilicum* there are five main botanical varieties (var. *basilicum* L., var. *difforme* Benth., var. *minimum* L., var. *purpurascens* Benth., and var. *thyrsiflorum* /L./ Benth.) with overlapping variations in leaf size and colour, flower colour, growth characteristics and aroma (Morales et al., 1993).

In order to describe newly bred cultivars a descriptor list based on morphological traits was developed by the International Union for the Protection of New Varieties of Plants (UPOV, 2003). The descriptor list based on highly heritable and easily scorable morphological traits has been used in characterization of accessions entering the Collection of Medicinal and Aromatic plants of the Department of Seed Science and Technology, Faculty of Agriculture, University of Zagreb, Croatia included in the Croatian Plant Genetic Resources Database (http://cpgrd.zsr.hr).

Amplified fragment length polymorphism markers (AFLPs; Vos et al., 1995) are highly efficient for quantitative assessment of genetic relationships and diversity by generating genetic data from a large number of loci without requiring prior knowledge of DNA sequence (Belaj et al., 2003). They have been used to characterize genetic diversity in a number of medicinal and aromatic plants including *Ocimum* (Labra et al., 2004; Carović-Stanko et al., 2010a).

The aim of this study was to (A) group basil accessions into morphotypes based on morphological descriptors, (B) determine the genetic relationships among accessions using AFLP markers, and (C) compare the patterns of variability obtained by both analyses.

Materials and methods

Plant material. A total of 24 *O. basilicum* accessions have been included in both morphological and molecular analyses. All investigated taxa are listed in Table 1. 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/).

Morphological analyses. Morphological research was carried out during the year 2006 in the field trial. Each accession was represented by 12 plants in order to assess a set of 27 morphological traits according to UPOV descriptor list (2003).

Molecular analyses. Total genomic DNAs were extracted from fresh leaves using DNeasy Plant Mini Kit (Qiagen®). The quality and quantity of the extracted DNAs were checked on 0.8% agarose gels with λ DNA as a standard (Boehringer Manheim). The DNA concentrations were measured using QubitTM fluorometer (Invitrogen®).

Table 1. Accessions of *Ocimum basilicum* used in research

No.	Accession*	Species	Variety	Cultivar
1	MAP00294	*O. basilicum*	var. *basilicum*	Genovese
2	MAP00232	*O. basilicum*	var. *basilicum*	Sweet basil
3	MAP01642	*O. basilicum*		Green Gate
4	MAP00576	*O. basilicum*		Grosses gruenes
5	MAP01619	*O. basilicum*		Oriental Basil
6	MAP01648	*O. basilicum*		Green Globe
7	MAP01655	*O. basilicum*		Pistou
8	MAP00560	*O. basilicum*		Fine verde
9	MAP01654	*O. basilicum*		Napoletano Basil
10	MAP00559	*O. basilicum*	var. *difforme*	Blistered lettuce-leaf basil
11	MAP01653	*O. basilicum*		Mammoth Basil
12	MAP01623	*O. basilicum*	var. *difforme*	Difforme
13	MAP00586	*O. basilicum*	var. *thyrsiflorum*	Thai-Basilikum
14	MAP00146	*O. basilicum*	var. *purpurascens*	No. 3193
15	MAP01629	*O. basilicum*	var. *purpurascens*	Mexican Basil
16	MAP01658	*O. basilicum*		Oriental Breeze
17	MAP01644	*O. basilicum*		Anise Basil
18	MAP01639	*O. basilicum*		Ararat
19	MAP01649	*O. basilicum*		Cinnamon Basil
20	MAP01657	*O. basilicum*		Thai Basil 'Queenette'
21	MAP00284	*O. basilicum*		Dark Opal
22	MAP01650	*O. basilicum*		Osmin
23	MAP01652	*O. basilicum*		Rubin Basil
24	MAP01640	*O. basilicum*	var. *minimum*	Bush Basil

*Accession number from The Collection of Medicinal and Aromatic Plants, Zagreb, Croatia available at: http://cpgrd.zsr.hr

The AFLP protocol followed Vos et al. (1995) with several modifications. Restriction digestion and adapter ligation were performed simultaneously on 200 ng of genomic DNA in total volume of 33 µl. 15 units of high concentration restriction enzyme EcoRI (Fermentas®) and three units of high concentration restriction enzyme Tru1I (= MseI) (Fermentas®) were used for DNA digestion. For ligation of 25 pmol EcoRI and 25 pmol Tru1I double-stranded nucleotide adapters, three units of high concentration T4 DNA ligase (Fermentas®) were applied. Digestion and ligation were performed for 2 h at 37°C and 14 h at 23 °C, respectively. Pre-amplification and amplification were performed in GeneAmp PCR System 9600. A four primer combinations were selected for amplification (FAM-EcoRI-ACT + Mse-CAG, NED-EcoRI-AGA + Mse-CAG, VIC-EcoRI-ACG + Mse-CGA, and PET-EcoRI-ACC + Mse-CGA). Samples were analysed using ABI3130 DNA sequencer (Applied Biosystems®). The presence or absence of fragments was scored on the chromatograms with the GeneMapper 4.0 Software (Applied Biosystems). All fragments between 50 and 500 bp were scored. The obtained peaks were automatically transposed into a binary matrix. Peaks which height exceeded absolute value 50, adjusted in the Peak Amplitude Threshold Settings of GeneMapper 4.0 Software, were scored as present (1), otherwise were scored as absent (0). An accession of *O. americanum* L. was included in molecular analyses to be used as an outgroup.

Data analyses. Phenotypic dissimilarities between pairs of accessions were calculated using the proportion-of-shared-alleles distance (Bowcock et al., 1994) as implemented in MICROSAT (Minch, 1997). In our case, as each accession can have only one state for a given trait, the results obtained by using the proportion-of-shared-alleles distance formula are identical to those

obtained by 1 - simple matching coefficient: $D_{SM} = 1 - S_{SM} = 1 - (m / n)$, where m is the number f morphological traits shared between a pair of accessions and n is the total number of traits. Cluster analysis based on dissimilarity matrix was performed using the unweighted pair-group method (UPGMA; Sneath and Sokal, 1973) as implemented in NEIGHBOR programme of the PHYLIP ver. 3.6b software package (Felsenstein, 2004). The reliability of the UPGMA topology was assessed via bootstrapping (Felsenstein, 1985) with over 10,000 replicates generated by MICROSAT and subsequently used in NEIGHBOR and CONSENSE programmes in PHYLIP.

AFLP amplified fragments were scored for the presence (1) or absence (0) of homologous bands to create binary matrices. Pairwise distances were calculated using Dice coefficient (Dice, 1945). Cluster analyses were conducted using Neighbour-joining algorithm (Saitou and Nei, 1987) as implemented in TREECON for Windows ver. 1.3 b (Van de Peer & De Wachter, 1994). Statistical support of the branches was tested with bootstrap analyses (Felsenstein, 1985) using 1,000 pseudoreplicats. Neighbour-joining tree was rooted using *O. americanum* accession (25).

Cophenetic correlation between matrices based on morphological and AFLP data was calculated and Mantel's test (Mantel, 1967) including 1,000 permutations was performed as implemented in NTSYS-pc ver. 2.1 (Rohlf, 2000).

The analysis of molecular variance (AMOVA; Excoffier et al., 1992) was used to partition the total AFLP diversity (1) between groups of morphotypes (Green vs. Purple), among morphotypes [True, Small-leaf, Lettuce-leaf, Purple (A), Purple (B), Purple (C)] and within morphotypes of *O. basilicum*, (2) among morphotypes within Green-basil group, and (3) among morphotypes within Purple-basil group. The analyses were performed on Dice's distance matrix among individuals treating an AFLP profile as a haplotype (Huff et al., 1993) in Arlequin ver. 2.000 (Schneider et al., 2000). The variance components were tested statistically by non-parametric randomisation tests using 10,000 permutations. Pairwise comparisons examined with AMOVA resulted in values of ϕ_{ST} that are equivalent to the proportion of the total variance that is partitioned between two morphotypes and could be interpreted as the distance average between types (Huff, 1997; Gustine & Huff, 1999).

Results and discussion

Twenty-three out of 27 morphological traits were polymorphic among the accessions. Uninformative, i.e. monomorphic traits were: number of flowering shoots, serration of leaf blade margin, hairiness of bracts and colour of style. Average phenotypic dissimilarity between all pairs of accessions, calculated using the proportion-of-shared-alleles distance, was 0.551, ranging from 0.000 to 0.957. Two accessions 'Genovese' (01) and 'Grosses Gruenes' (04) were indistinguishable. The UPGMA analysis revealed five clearly defined clusters representing basil morphotypes: True basils, Small-leaf basils, Lettuce-leaf basils, Purple basils A (var. *purpurascens* morphotype) and Purple basils B ('Dark Opal' morphotype), giving a good representation of traditional taxonomic relationships (Fig. 1). These results are mostly in agreement with Darrah's (1980) classification and

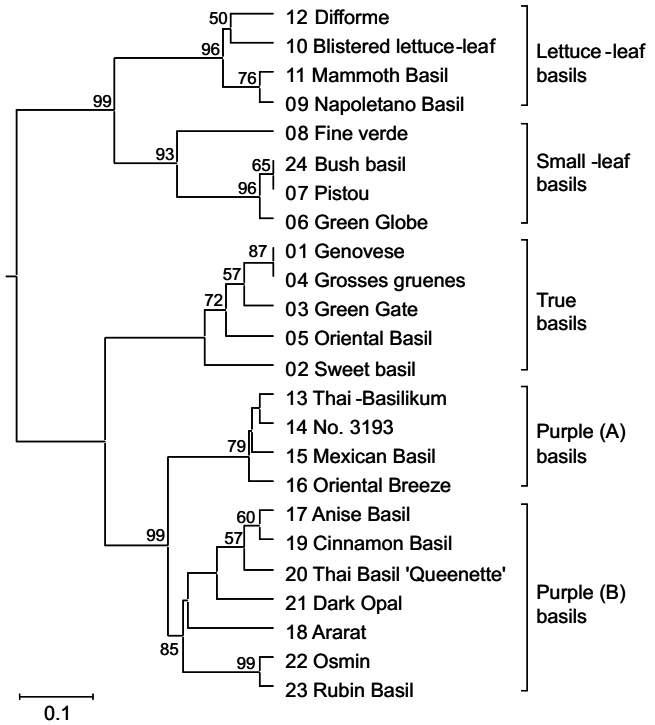

Figure 1. UPGMA dendrogram based on 23 morphological traits in 24 accessions of *Ocimum basilicum*. Numbers above branches indicate bootstrap support values over 50% in 1,000 pseudoreplicates.

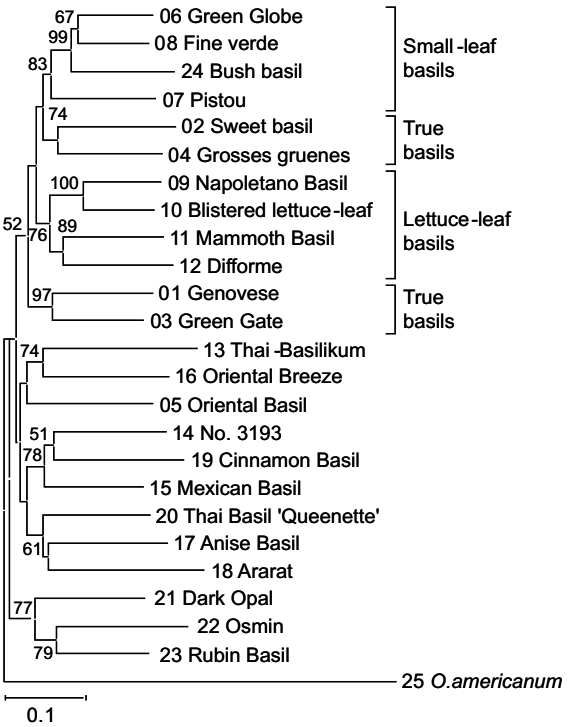

Figure 2. Neigbour-joining tree based on AFLP markers in 25 *Ocimum* accessions. Tree was rooted using *Ocimum americanum* accession. Numbers above branches indicate bootstrap support values over 50% in 1,000 pseudoreplicates.

Table 2. AMOVA analysis for the partitioning of AFLP diversity (1) between groups of morphotypes (Green vs. Purple), among morphotypes [True, Small-leaf, Lettuce-leaf, Purple (A), Purple (B)] and within morphotypes of *Ocimum basilicum* as well as (2) among morphotypes within Green-basil group, and (3) among morphotypes within Purple-basil group

Analysis	Source of variation	df	Variance components	Percentage of variation	ϕ-statistics	p(ϕ)
(1)	Between groups (Green vs. Purple)	1	0.012	6.21	0.062	~ 0.0000
	Among morphotypes within groups	3	0.013	7.16	0.076	~ 0.0000
	Within morphotypes	19	0.162	86.63	0.134	~ 0.0000
(2)	Among morphotypes within Green-basil group	2	0.024	14.42	0.144	~ 0.0000
	Within morphotypes	10	0.141	85.58		
(3)	Among morphotypes within Purple-basil group	1	–0.0003	–0.17	–0.002	0.479
	Within morphotypes	11	0.186	100.17		

p(ϕ) - ϕ-statistics probability level after 10,000 permutations

Table 3. Pairwise ϕ_{ST} between morphotypes of *Ocimum basilicum*

Morphotypes	(1)	(2)	(3)	(4)	(5)
(1) True basils		**	**	*	**
(2) Small-leaf basils	0.129		*	*	**
(3) Lettuce-leaf basils	0.126	0.189		*	**
(4) Purple basils (A)	0.087	0.145	0.131		ns
(5) Purple basils (B)	0.079	0.167	0.149	-0.002	

Lower diagonal: ϕ_{ST} values; Upper diagonal: p-values after 10,000 permutations (** - $p < 0.01$; * - $0.01 < p < 0.05$; ns - $p > 0.05$)

classification suggested by Carović-Stanko et al. (2010b), who classified the *O. basilicum* cultivars in six morphotypes: True, Small-leaf, Lettuce-leaf, Purple basil (A), Purple basil (B) and Purple basil (C) morphotype.

Four AFLP primer combinations generated a total of 993 polymorphic markers. With the AFLP analysis we managed to separate all accessions and the Dice distance ranged from 0.189 to 0.483. By Neighbour-Joining analysis, *O. basilicum* accessions were separated into three main clusters (Fig. 2). First cluster represented a group of Green morphotypes (True, Small-leaf, Lettuce-leaf), while the other two clusters comprised accessions belonging to Purple morphotypes, but not in accordance to morphological grouping into Purple A and Purple B morphotype. Within a cluster of Green morphotypes, both Small-leaf and Lettuce-leaf morphotypes were monophyletic while True basils morphotype was polyphyletic.

The cophenetic correlation between matrices based on morphological and AFLP data was relatively low (0.27), but significant ($p = 0.002$).

The analysis of molecular variance (AMOVA) (Tab. 2) revealed a significant level ($p < 0.0001$) of genetic variation both between groups of morphotypes (Green vs. Purple; 6.21%), and among morphotypes within groups (7.16%), while the most of the diversity was attributed to variation within morphotypes (86.63%). Within Green basil group, a percentage of total variation attributable to differences among morphotypes amounted to 14.42% ($p < 0.0001$). On the other hand, AMOVA showed no evidence of genetic differentiation between accessions belonging to morphotypes Purple A and Purple B ($p = 0.479$). In accordance with Neighbor-Joining tree no significant structure was found considering two purple morphotypes (A and B). To check the genetic distance between morphotypes Pairwise ϕ_{ST} was used. Not surprisingly, all the distances, except between Purple basil A and Purple basil B, were significant, confirming the existence of clear phenotypical dissimilarity (Tab. 3). Overall, our results confirm a general understanding of high morphological variation in basil (Simon et al., 1999; Labra et al., 2004).

Conclusions

Results revealed a certain degree of correspondence between morphological and molecular data among *O. basilicum* L. accessions. Hence, both morphological descriptors and AFLP markers can contribute in resolving existing problems concerning intraspecific classification in *O. basilicum*. Morphological trait analysis can provide an inexpensive and reliable classification of accessions and it seems to be a sound basis for further differentiation by molecular markers. Genetic analysis, by AFLP makers, mostly was in accordance with morphology.

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