Morphological characterization, essential oil composition and DNA genotyping of *Ocimum basilicum* L. cultivars

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Abstract

The *Ocimum* genus includes more than 150 species. However, some attributions are difficult, due to the interference of man with selection, cultivation and hybridisation within the genus and to large morphological variation among the different species. A system of standardized descriptors, based on volatile oils, has been proposed, but its use is limited by the fact that several environmental factors may influence the plant chemical composition. In this paper, we experiment the usefulness of molecular markers of DNA polymorphism, based on AFLP analysis, to unravel disputed attributions. We conclude that the combined analysis of morphological traits, volatile oil composition and molecular markers represents the optimal approach to verify taxonomy and to correlate it with agronomic traits.

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Keywords: AFLP; Basil; DNA fingerprinting; Essential oil; *Ocimum basilicum* L.

1. Introduction

The *Ocimum* genus (Lamiaceae) comprises annual and perennial herbs and shrubs native to the tropical and subtropical regions of Asia, Africa and Central South America [1,2]. Although traditionally used as medicinal herb in the treatment of headaches, coughs, diarrhoea, worms and kidney malfunctions [3], basil has a long history as culinary herb, thanks to its foliage which adds a distinctive flavor to many foods. It is also considered to be a source of aroma compounds and essential oils containing biologically active constituents that possess insect repellent, nematocidal and antibacterial activity [4–6]. Basil essential oil has been extensively used in the flavouring of confectionery and baked goods, condiments, sausages and meat, salad dressing, non alcoholic beverages, ice creams; it has also found wide application in perfumery, as well as in dental and oral products [7].

The study of taxonomy of the genus is difficult, due to the interference of man with selection, cultivation and hybridisation. Pushpangadan and Bradu [8] recognize more than 150 species in the genus. However, most of their taxa are essentially based on leaf morphology and colour, which frequently depend on environmental conditions. For instance, leaves of the best known species, *Ocimum basilicum*, and of its close relatives varies from small and liniform to large and round and colours vary from yellow-green to grey-green, to red or to almost black. More recently, Paton et al. [9] proposed that only 65 species of *Ocimum* should be retained, and that other attributions should be considered synonyms or false attributions.

The still existing uncertainty in the classification within the genus depends on the fact that species identification relied on morphological characters whose expression is known to be affected by developmental and environmental
factors. To assist in classification a system of standardized descriptors based on volatile oil has proposed by Lawrence [5] and Grayer et al. [10] that classified the different basil chemotypes on the basis of the prevalent aromatic compound or the components major than 20%, respectively.

Although essential oils in different basil cultivars are variable, prevalent components are monoterpenes and phenylpropanoids [11,12]. Many Ocimum species contain primarily monoterpenoid derivatives such as limonene, camphor, 1,8-cineole, linalool and geraniol [13,14]. Others, including O. basilicum, contain primarily phenol derivatives, such as eugenol, methyl-2-thenyl-2-enyl, charicol, estragole, methyl-cinnamate, often combined with various amounts of linalool [15,16].

The chemotype classification based on just one major volatile oils is problematic because frequently one plant contains two or more compounds in nearly equal amounts. It is more convenient to consider the overall oil profile of major constituents, by identifying them above a fixed threshold level (e.g. 20% of total essential oil content).

In the last decade, new valuable tools, based on DNA analysis, have been made available for taxonomic studies [17–20]. The use of PCR-based tools allows detection of DNA polymorphism at random or specific loci in the genome. Their use has been instrumental in solving controversial taxon attributions by comparing genotypes independently from phenotypes. By identifying polymorphic sequences in the genomic DNA, these tools allow phylogenetic [21] and taxonomic [22] studies, as well as cultivar and clone identification [23–26].

In this work, we verified the capacity of the AFLP approach to analyse genetic distances among O. basilicum varieties and to verify the occurrence of any correlation among genetic distance, essential oil profile and morphological description.

<table>
<thead>
<tr>
<th>Code</th>
<th>Accession (commercial name)</th>
<th>Company</th>
<th>Leaf size</th>
<th>Leaf shape</th>
<th>Leaf margin</th>
<th>Leaf colour</th>
<th>Flower colour</th>
<th>Plant height (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Genovese gigante di Alborese</td>
<td>Galassi*</td>
<td>Medium</td>
<td>Ovate</td>
<td>Serrate</td>
<td>Pale green</td>
<td>White</td>
<td>40–45</td>
</tr>
<tr>
<td>B</td>
<td>Basilico a foglia fine</td>
<td>Galassi*</td>
<td>Small</td>
<td>Lanceolate</td>
<td>Entire</td>
<td>Green</td>
<td>White</td>
<td>20–25</td>
</tr>
<tr>
<td>C</td>
<td>Basilico a foglia gigante</td>
<td>SAIS2</td>
<td>Medium</td>
<td>Ovate</td>
<td>Serrate</td>
<td>Pale green</td>
<td>White</td>
<td>40–45</td>
</tr>
<tr>
<td>D</td>
<td>Basilico a foglia lattuga</td>
<td>Dotto3</td>
<td>Large</td>
<td>Ovate</td>
<td>Serrate</td>
<td>Pale green</td>
<td>White</td>
<td>30–40</td>
</tr>
<tr>
<td>E</td>
<td>Basilico gigante</td>
<td>SAIS2</td>
<td>Medium</td>
<td>Ovate</td>
<td>Serrate</td>
<td>Pale green</td>
<td>White</td>
<td>30–35</td>
</tr>
<tr>
<td>F</td>
<td>Basilico Genovese foglia gigante</td>
<td>SAIS2</td>
<td>Medium</td>
<td>Ovate</td>
<td>Serrate</td>
<td>Pale green</td>
<td>White</td>
<td>40–45</td>
</tr>
<tr>
<td>G</td>
<td>Basilico Genovese foglia piccola</td>
<td>SAIS2</td>
<td>Medium</td>
<td>Ovate</td>
<td>Serrate</td>
<td>Pale green</td>
<td>White</td>
<td>30–35</td>
</tr>
<tr>
<td>H</td>
<td>Basilico Genovese fiore violato</td>
<td>Galassi*</td>
<td>Medium</td>
<td>Ovate</td>
<td>Serrate</td>
<td>Pale green</td>
<td>Violet</td>
<td>30–35</td>
</tr>
<tr>
<td>I</td>
<td>Basilico fine verde</td>
<td>Hortus4</td>
<td>Small</td>
<td>Lanceolate</td>
<td>Entire</td>
<td>Green</td>
<td>White</td>
<td>25–30</td>
</tr>
</tbody>
</table>

All are commercial accessions cultivated in the Liguria region (Italy).

* Galassi s.n.c., via 1 Maggio, 3, P.O. Box 55, I-47035 Gambettola, FC, Italy.
* SAIS spa: Società Agricola Italiana Sementi, via Ravenate 214, Cesena, Italy.
* Dotto, via Lavatano 15, I-33050 Montegiliano, UD, Italy.
4 Hortus srl, via Emilia 1820, I-47020 Longiano, PR, Italy.

2. Material and methods

2.1. Plant material

Seeds of nine variety of O. basilicum L. obtained from local markets were sown and grown in the greenhouse for 3 months at 25–28°C. Cultivars used for this study represent most of the commercially basil plants used for culinary purposes in Italy. Names and morphological characteristics of each cultivar are listed in Table 1. Plants at flowering stage were collected and aromatic compounds from fresh material were extracted by distillation and analysed by GC–MS.

2.2. Essential oil extraction

Three grams of fresh leaves were added to 150 ml of water in a 500 ml flask. The mixture was hydro-distilled until 50 ml were recovered. The distillate was extracted three times with freshly distilled ethyl ether. The solvent was removed at room temperature and the essential oil diluted with ethyl acetate to 5 mg/ml. An aliquot was injected into the Capillary GC–MS chromatograph. For each cultivar three different extractions were performed.

2.3. GC–MS analysis

Capillary GC–MS measurements were carried out on a HP-5MS (0.25 mm x 30 m) column coupled directly to a quadruple MS. Carrier gas: He; flow rate: 1 ml/min; split 1: 49; injection point: 250°C; oven: initial temperature 60°C for 4 min; ramp: 5°C min⁻¹; final temperature 210°C; electron energy: 70 eV. Quantitative data were obtained from normalized area values. The identification of compounds was based on comparison of their relative retention times with those of standards, by coelution and MS analysis.
Table 2

Primer used for AFLP analysis

<table>
<thead>
<tr>
<th>Name</th>
<th>DNA sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>E + 01</td>
<td>5′-GACTGCTGTACCAATTC-3′</td>
</tr>
<tr>
<td>E - 01</td>
<td>5′-GATGAACCTGCTAGTAAA-3′</td>
</tr>
<tr>
<td>E32</td>
<td>5′-GACTGCTGTACCAATTCG-3′</td>
</tr>
<tr>
<td>E33</td>
<td>5′-GACTGCTGTACCAAATTCG-3′</td>
</tr>
<tr>
<td>E34</td>
<td>5′-GACTGCTGTACCAAATTC-3′</td>
</tr>
<tr>
<td>M34</td>
<td>5′-GACTGCTGTACCAAATTC-3′</td>
</tr>
<tr>
<td>M35</td>
<td>5′-GATGAACCTGCTAGTAAA-3′</td>
</tr>
<tr>
<td>M36</td>
<td>5′-GATGAACCTGCTAGTAAA-3′</td>
</tr>
<tr>
<td>M37</td>
<td>5′-GATGAACCTGCTAGTAAA-3′</td>
</tr>
<tr>
<td>M38</td>
<td>5′-GATGAACCTGCTAGTAAA-3′</td>
</tr>
<tr>
<td>M40</td>
<td>5′-GATGAACCTGCTAGTAAA-3′</td>
</tr>
</tbody>
</table>

2.4. AFLP analysis

Young leaves (1–2 cm long) were harvested from rooted cuttings, frozen in liquid nitrogen and ground into a fine powder. Genomic DNA was extracted from about 150 mg of fresh leaves using the GenElute™ Plant Genomic DNA Kit (Sigma, Saint Louis, USA).

AFLP was performed as described in Vos et al. [26], except that genomic DNA (200 ng) was digested (3 h) with EcoRI (0.5 U) and MseI (0.5 U). DNA fragment was ligated (with T4 DNA ligase) to EcoRI (5 pmol) and MseI (50 pmol) adapters in a final volume of 50 μL. Ligation was performed at 37 °C for 3 h. The resulting mixture was used as template in a pre-amplification reaction that contained DNA primers (E + 01 and M + 01 of Table 2) complementary to the core of the EcoRI and MseI adapter, respectively. The 50 μL pre-amplification mixture contained 20 μL of “digested/ligated DNA”, 50 ng of the selected primers, 30 ng of MseI adapter, respective.

The nine O. basilicum accessions listed in Table 1 were used for chemical and genetic analysis. These cultivars are the most commonly utilized in the Mediterranean area for culinary and ornamental use. In particular, cultivars A, C, E, G, F are largely used in Italy for fresh consumption and for the production of “pesto”, a typical Italian sauce known for its unmistakable taste and aroma. Plants had been grown in equal condition and harvested leaves used for chemical and molecular analysis were of the same age and at a comparable developmental stage.

Eleven different components were identified by the GC/MS analysis in the nine O. basilicum accessions. Considerable variation in the chemical composition was recorded in the analysed varieties as showed in Table 3. The dominant constituent in all samples was linalool, ranging between 19 and 38% of total oils. This agrees with data produced by other authors [12]. Eugenol was also prevalent in almost all analysed cultivars, except in the case of the D accession, where its percentage was considerably reduced. D is the only cultivar containing methylchavicol (8.50%). Cineole, terpineol and farnesene were found in all the cultivars. Cineole can be considered the third main component (except in the case of accession D, where it was the second main component). Terpineol...
Table 3

<table>
<thead>
<tr>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cineole</td>
<td>10.89 ± 2.70</td>
<td>13.07 ± 3.19</td>
<td>7.09 ± 2.96</td>
<td>11.57 ± 3.19</td>
<td>1.11 ± 0.78</td>
<td>9.65 ± 2.96</td>
<td>1.11 ± 0.78</td>
<td>11.19 ± 2.96</td>
<td>0.78 ± 1.24</td>
</tr>
<tr>
<td>Linalool</td>
<td>11.96 ± 0.34</td>
<td>29.58 ± 4.20</td>
<td>18.96 ± 1.24</td>
<td>26.36 ± 1.24</td>
<td>4.20 ± 0.37</td>
<td>26.40 ± 1.24</td>
<td>4.20 ± 0.37</td>
<td>23.98 ± 1.24</td>
<td>4.65 ± 0.37</td>
</tr>
<tr>
<td>Camphora</td>
<td>13.32 ± 0.77</td>
<td>0.70 ± 0.57</td>
<td>0.62 ± 0.48</td>
<td>0.78 ± 0.57</td>
<td>0.33 ± 0.24</td>
<td>1.14 ± 0.48</td>
<td>0.33 ± 0.24</td>
<td>0.17 ± 0.48</td>
<td>0.11 ± 0.24</td>
</tr>
<tr>
<td>Limonene</td>
<td>13.50 ± 0.06</td>
<td>0.03 ± 0.09</td>
<td>2.79 ± 0.09</td>
<td>4.48 ± 0.10</td>
<td>0.09 ± 0.04</td>
<td>2.76 ± 0.09</td>
<td>0.09 ± 0.04</td>
<td>0.14 ± 0.09</td>
<td>0.10 ± 0.04</td>
</tr>
<tr>
<td>Terpineol</td>
<td>13.60 ± 0.34</td>
<td>1.91 ± 0.74</td>
<td>1.46 ± 0.37</td>
<td>1.98 ± 0.37</td>
<td>0.34 ± 0.19</td>
<td>1.08 ± 0.37</td>
<td>0.34 ± 0.19</td>
<td>2.25 ± 0.37</td>
<td>0.31 ± 0.19</td>
</tr>
<tr>
<td>Methylchavicol</td>
<td>13.70 ± 0.59</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Chavicol</td>
<td>14.40 ± 0.26</td>
<td>–</td>
<td>6.27 ± 0.11</td>
<td>–</td>
<td>–</td>
<td>1.34 ± 0.04</td>
<td>–</td>
<td>1.34 ± 0.04</td>
<td>–</td>
</tr>
<tr>
<td>Eugenol</td>
<td>16.16 ± 3.04</td>
<td>23.10 ± 3.69</td>
<td>21.64 ± 3.69</td>
<td>3.34 ± 0.19</td>
<td>8.00 ± 0.11</td>
<td>24.43 ± 3.69</td>
<td>8.00 ± 0.11</td>
<td>24.04 ± 3.69</td>
<td>4.69 ± 0.11</td>
</tr>
<tr>
<td>Methyleugenol</td>
<td>16.67 ± 0.05</td>
<td>0.10 ± 0.04</td>
<td>0.32 ± 0.04</td>
<td>0.33 ± 0.04</td>
<td>0.04 ± 0.02</td>
<td>0.33 ± 0.04</td>
<td>0.04 ± 0.02</td>
<td>0.33 ± 0.04</td>
<td>0.09 ± 0.02</td>
</tr>
<tr>
<td>Farnesene</td>
<td>17.33 ± 0.72</td>
<td>9.75 ± 0.72</td>
<td>10.10 ± 0.34</td>
<td>12.60 ± 0.34</td>
<td>0.75 ± 0.10</td>
<td>10.30 ± 0.34</td>
<td>0.75 ± 0.10</td>
<td>13.29 ± 0.34</td>
<td>0.11 ± 0.03</td>
</tr>
</tbody>
</table>

* Other compounds: ocimene, germacrene, naphtalene.

Three independent individuals of each accession were used for essential oils analysis.

Minor components absent in some of the cultivars.

In order to define relationships among the accessions based on essential oil content, the data of Table 3 were used to construct a dendrogram showing Euclidian distance (Fig. 1). This evidenced large variability within the nine analysed accessions. The closest chemical profiles were those of accessions A, C, G, and F. Table 1 shows that these varieties are characterized by medium large and ovate leaves.

The E accession, which can be found close to the A–C–G–F cluster of Fig. 1, showed comparable morphological characteristics.

Accessions H and I, which are generally used as ornamental plants and not for food, cluster in the same branch of the dendrogram of Fig. 1, but show different morphological traits. Accessions B and D, which are set apart from all other accessions, are characterized by peculiar letuce-like leaves, and by slim leaves, respectively. In conclusion, the results show that all analysed accessions can be recognized with the combined use of essential oil profile and morphological traits, but morphological and chemical traits are both influenced by environmental factors. A previous study on some of these cultivars showed large variability in aromatic compound composition depending on growth stage [16,30,31]. Factors which may potentially affect essential oil percentage are conditions under which plants have been grown, drying, extraction procedure and age [15,32]. For instance, essential oil compositions may differ, in the same genotype, depending on the light regime [33] or on whether plants have been grown in the greenhouse or in open field [34]. It is worth noting that plants at the growth stage (10–12 cm in height) at which they are used for “pesto” contain methyleugenol at high concentration. This finding is important and should be investigated further, in view of possible risks for human health, as it is has been shown to be carcinogenic [35].

Eugenol and methyleugenol content is strictly related with plant height: methyleugenol is predominant in plants up to 10 cm height, while eugenol is prevalent in taller plants [16].

To verify if the limitations evidenced in the use of phenotypic and chemical data for taxonomic purposes, can be overcome by molecular analysis, DNA extracted form the nine O. basilicum accessions was analysed by AFLP. Polyacrilamide gel electrophoresis of the amplification products, revealed 163 bands, 83 of which were polymorphic. Results, summarized in the dendrogram of Fig. 2 define genetic relationships among the analysed accessions and also among the five analysed individuals within each accession. The similarity index among accessions varies from 0 (full genomic similarity) to 0.18. The dendrogram separated all analysed cultivar, with the exception of C and G, which are thus considered genetically closely related. Accessions B and D show high genetic similarity with C and G. Accessions A, H, I, E, and F are clearly separated from each other.
and from the other accessions. In the case of accession B, C, D, and G no genetic variability was detected among individuals within the cultivar, suggesting that these can be considered as clones. The low genetic variability observed in these cultivars may be explained with the consideration that reproduction and propagation of *O. basilicum* is preferentially autogamous and that this leads to drastic genetic variability reduction upon auto-pollinations. The reduction of genetic variability was high in the accessions used in agriculture possibly because breeders use to select few genotype for seed production. Only individuals of the A, E, H, and I cultivars showed consistent genetic variability. We underline that the H and I accession are not used in food production while A and E cultivars are used in cultivation, but probably their seeds were derived from a mixture of parental plants. Based on our results we suggest that the availability of molecular tools to characterize and classified different cultivars showed consistent genetic variability. DNA genotyping offers the unique capacity to classify accessions regardless of environmental condition and plant growth stage. Morphological characters, which are the easiest to determine, may only provide a primary classification. In essential oil containing plants, chemical composition is sometimes important, especially for food production [10]; however, since environmental (e.g. growth stage) and genetic (e.g. mutations) factors may affect essential oil composition [13], oil content cannot provide hints for varietal classification.

This relevant aspect of this study is that it shows that genomic similarity does not necessarily reflect similarity or difference in output traits, such as oil composition, or agronomic traits. For example, cultivars B and D are quite different in their oil composition but genetically very similar. Thus, AFLP dendrograms may be used for universal taxonomic studies, while dendrograms based on end-use related traits, such as oil composition, may be of practical interest, but do not necessarily correlate with taxonomy.

**Fig. 1.** Dendrogram, produced with the UPGMA method, showing Euclidian distance among the nine accessions of *Ocimum basilicum* L. The dendrogram was based on the essential oil data matrix of Table 3. The accessions were those listed with alphabetic letters in Table 1.
Fig. 2. The UPGMA dendrogram computed using [28] genetic distance matrix based on AFLP data. Five samples for each O. basilicum cultivar were analysed. The values on the branches were the result of 2000 bootstrap replications. Only bootstrap values >50% are indicated.

References


