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Molecular fingerprinting of some Mentha species by sequencing and RFLP analysis of the 5S-rRNA non-transcribed spacer region

A. Capuzzo[a] & M. E. Maffei[a*]

Abstract

The genus *Mentha* is of particular economic importance. The development of new methods for the characterisation of *Mentha* species is crucial for their unequivocal identification. Amplification of the non-transcribed spacer (NTS) of the 5S-rRNA gene was used to characterise some *Mentha* species, which revealed a high-specific variability. Cloning and sequencing of all amplified NTS fragments enabled the discrimination among almost all species. *In silico* and experimental analyses identified specific restriction sites on the amplified 5S-NTS regions, facilitating the rapid and unambiguous discrimination of all the different species by polymerase chain reaction–restriction fragment length polymorphism. A direct comparison between essential oil composition and DNA fingerprinting confirmed a relationship between chemical and molecular data.

Keywords:

- *Mentha* species,
- molecular discrimination,
- essential oils,
- PCR–RFLP,
- 5S-rRNA NTS

Introduction

The genus *Mentha* L. (Lamiaceae) has been valued since ancient times because of its aromatic and therapeutic properties. *Mentha* is used for its antioxidant, tonic, digestive, antiseptic and refreshing properties (Dorman et al. 2003; Hayes et al. 2006; McKay & Blumberg 2006). *Mentha* is defined as a taxonomically complex genus (Harley & Brighton 1977; Tucker et al. 1980; Tucker & Naczi 2007); 18 species are reputed part of the genus and 11 natural hybrids are recognised as originating from the breeding of five Eurasian and African sexual species included in the section *Mentha* (i.e. *M. aquatica*, *M. arvensis*, *M. longifolia*, *M. spicata* and *M. suaveolens*) (Tucker & Naczi 2007). Many attempts have been made to clarify the relationships between species and hybrids and to introduce a unique classification of the genus. Over the past years, cytological and morphological data/features/traits/characteristics as well as essential oil composition analyses have been used for classification purposes. However, although cytological and morphological traits may be insufficient for a taxonomical discrimination, the chemical composition of an essential oil can vary depending on endogenous (e.g. anatomical, physiological and biochemical characteristics of the plant) and exogenous (e.g. climatic and environmental conditions) factors, which lead to the differentiation of ecotypes and/or chemotypes in plants of the same species (Barra 2009). In this context, as environmental conditions vary, a genotype may express different chemical phenotypes, whereas different genotypes, subjected to the same environmental pressures, may develop similar phenotypes. This is due to the fact that secondary metabolites are adaptive traits of an organism that

can be selectively expressed by environmental conditions (Wink 2003). To solve this problem, new approaches have been proposed to analyse the genus *Mentha*, using biomolecular data as support for taxonomical identification. Works based on chloroplast and nuclear DNA sequences (Gobert et al. 2002; Bunsawat et al. 2004) have been successfully used to confirm assumptions previously made by the use of conventional morphological and chemical analyses.
Recently, the phylogenetic relationships of many higher plant species have been elucidated and examined by sequence analysis of the 5S-rRNA gene spacer region (Gnavi et al. 2010a). The 5S-rRNA gene sequence is a key component of the ribosome complex in cells and cellular organelles except for the mitochondria of some species (Brown & Carlson 1997). In higher eukaryotes, the 5S-rRNA sequences are transcribed from hundreds to thousands of genes, which are separated by the coding sequences of 18S-rRNA and 26S-rRNA, and are organised in tandem repeats. The entire cluster sequence has portions coding the 5S-rRNA and spacer sequences called non-transcribed spacers (NTS), which can be distributed in one or more parts of the genome (Park et al. 2000). The high conservation level of the coding sequences is related to the precise and fundamental function of the 5S-rRNA-like components of the large subunit of the ribosomes of all eukaryotes. Some regions are more conserved than others, and this is explained by the mechanisms of transcriptional regulation (Negi et al. 2002).
The high degree of conservation of coding sequences and the high variability of the spacer regions allow the analysis of the organisation, evolution and multigene variability of many plant species (Scoles et al. 1988; Cox et al. 1992). Based on these considerations, NTS regions have been used to assess the inter- and intraspecific variabilities of many plant species, map 5S-rDNA arrays; assess the evolution of the genome and to perform phylogenetic reconstructions (Udovicic et al. 1995; Baker et al. 2000; Gnavi et al. 2010a). Furthermore, the use of characteristic restriction profiles [also called restriction fragment length polymorphism (RFLP)], coupled with capillary gel electrophoresis, allowed a further molecular discrimination of species, subspecies, hybrids and chemotypes (Bertea et al. 2005; Bertea et al. 2006; Rubiolo et al. 2009; Gnavi et al. 2010a, 2010b).
A biomolecular approach using sequencing and RFLP of the 5S-rRNA NTS was used in this study to unequivocally characterise and discriminate some of the most taxonomically and economically important species of the genus *Mentha*. The study describes the development of new, reliable, rapid, highly sensitive and easily applicable protocols based on molecular biological methods for the unequivocal determination of *Mentha* species.

Materials and methods

Plant materials

Mentha species (*M. arvensis* L. PI557584, *M. cervina* L. PI557634, *M. gattefossei* Maire PI557639, *M. pulegium* L. PI557771, *M. requienii* Benth PI557781, *M. spicata* L. PI557810, *M. suaveolens* Ehrh. PI557894) were kindly provided by the USDA Arctic and Subartic Plant Gene Bank of Palmer (Fairbanks, AK, USA) as rhizomes. Stolons of *M. longifola* and *M. aquatica* were collected from populations growing wild in the Piedmont region, Italy, and a voucher specimen has been deposited at the Hortus Botanicus Taurinensis Herbarium. Plants originated from rhizomes were grown in plastic pots with sterilised peat and vermiculite (v/v 4:1) at 23°C and 60% humidity using daylight fluorescent tubes at 270 $\mu E\ m^{-2}\ s^{-1}$ with a photoperiod of 16 h.

Genomic DNA extraction

Leaf samples of different plants originating from different rhizomes were pooled, frozen in liquid nitrogen and ground to a fine powder with a Tissue Lyser (Qiagen, Hilden, Germany). Genomic DNA was extracted from the ground powder using the Nucleospin Plant II Kit (Macherey Nagel,

Düren, Germany) following the manufacturer's instruction. The quantity and quality of the DNA were assessed by spectrophotometric analyses using the Nanodrop ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA).

PCR amplification, subcloning and sequencing

Approximately 20 ng of genomic DNA isolated from powdered leaf material of each sample was used as a template for polymerase chain reaction (PCR) amplification with forward primer 5S-P1 (5′-GTGCTTGGGCGAGAGTAGTA-3′) and reverse primer 5S-P2 (5′-TTAGTGCTGGTATGATCGCA-3′) flanking the NTS of the 5S-rRNA gene (Sugimoto et al. 1999; Gnavi et al. 2010a). The amplification was carried out in a 50-µl reaction mixture containing 5 µl of 10 × PCR buffer (Fermentas, Glen Burnie, MA, USA), 0.2 mM deoxynucleoside triphosphates (dNTPs), 20 pmol of forward and reverse primers and 0.5 U of Taq DNA polymerase (Fermentas). PCRs were carried out in a Whatman Biometra T-Gradient Thermalcycler (Whatman Biometra, Goettingen, Germany). Cycling conditions consisted of an initial 4 min at 94°C, followed by 30 s of denaturing at 94°C, 45 s of annealing at 54°C and 45 s of elongation at 72°C, repeated for 30 cycles and with 5 min of final extension at 72°C. One microliter of the amplification reaction mixture was analysed by capillary gel electrophoresis using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and the DNA 1000 LabChip Kit (Agilent Technologies) following manufacturer's instructions. The DNA 1000 LabChip Kit provides sizing and quantification of dsDNA fragments ranging from 25 to 1000 bp. PCR products were also analysed by a 2% agarose gel electrophoresis and visualised by ethidium bromide staining under UV. From this gel, bands ranging from 168 to 483 bp were purified using the Nucleospin Extract II Kit (Macherey Nagel) and then subcloned into pGEM-T Easy vector (Promega BioSciences, San Luis Obispo, CA, USA). The ligated products were transformed into *Escherichia coli* Subcloning DH5α Efficiency Competent Cells (Invitrogen, Paisley, UK). Colonies containing DNA inserts of the correct size were picked and grown overnight in 5 ml of Luria-Bertani liquid medium. The mini-preparation of plasmid DNAs was carried out using QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA, USA) following manufacturer's instructions. Plasmid DNAs were used as a template for sequencing at least twice both the strands of DNA. The sequences were detected by an ABI 377 automated sequencer according to the manufacturer's protocol (Applied Biosystems, Foster City, CA, USA).

Cluster analyses

Sequences were aligned with ClustalX2 software (Larkin et al. 2007) using default parameters to check the integrity of each sample sequence. Multiple sequences (one per sample) were then aligned by modifying the Gap Opening and Gap Extension Cost values to 15 and 1, respectively. From this last alignment, Phylogeny Tree was constructed using ClustalX2 Software. Neighbor Joining statistical method was selected and relationships were tested with 1000 Bootstrap replicates, considering gaps in the Pairwise Deletion option. *Salvia divinorum* 5S-NTS sequence (Bertea et al. 2006) (GenBank accession number DQ230979) was used as outgroup.
Essential oil cluster analysis was carried out on reference data from essential oil composition of the *Mentha* species under study. Data were analysed using Systat 10 using hierarchical clustering classification with Euclidean distance and Ward linkage.

PCR–RFLP

Purified PCR products of the 5S-rRNA gene spacer region were first digested in separate reactions with 10 U of *Bgl*I, *Msc*I, *Nde*I and *Xho*I (NEB, New England Biolabs, Ipswich, MA, USA) at 37°C for 1 h (3 h for *Nde*I), then inactivated by thermal treatment at 65°C for 15 min (except for *Taq*I,

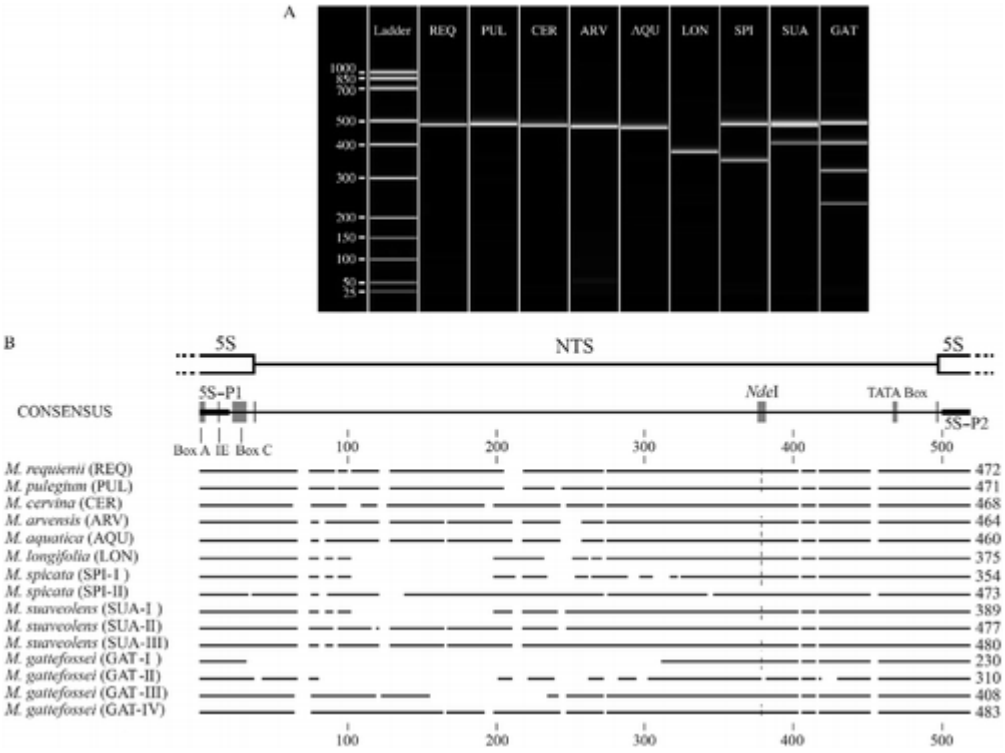
incubated at 65°C for 1 h, with the addition of 20 mM EDTA for inactivation). One microlitre of digestion reaction mixture was fractionated by Capillary Gel Electrophoresis (CGE) using the Agilent 2100 Bioanalyzer (Agilent Technologies) and DNA 1000 LabChip Kit (Agilent Technologies) following the manufacturer's instructions. Reproducibility was assessed by repeating digestions with different enzyme concentrations and timings, whereas for each set of runs, a different calibration curve was assessed by the DNA 1000 LabChip.

Results

DNA amplification, sequence and cluster analysis reveal differences in the 5S-rRNA NTS region of Mentha species

Two primers flanking the 5S-rRNA spacer region were used in the PCR analysis of genomic DNA isolated from different samples of *Mentha* species. The amplified fragments were in the range of approximately 160–490 bp (Figure 1A). Sequence analyses (NCBI GenBank accession numbers JF775651–JF775665) were aligned using ClustalX2 software (see Table S1). In general, the consensus sequence showed highly conserved regions in the first and last 60 bp along with a conserved region from 260 to 450 bp. Each fragment shown in Figure 1A was sequenced and named progressively (I, II, etc.) based on fragment length, as described in Figure 1B. A single DNA fragment, ranging from 450 to 490 bp, was present in five of nine species, whereas *M. longifolia* (Chambers & Hummer 1994) showed a single band of about 375 bp (Figure 1B). *M. spicata* (SPI) showed two fragments (354 and 473 bp), *M. suaveolens* (SUA) showed three fragments (389, 477 and 480 bp), whereas *M. gattefossei* (GAT) showed four fragments (230, 310, 408 and 483 bp) (Figure 1B).

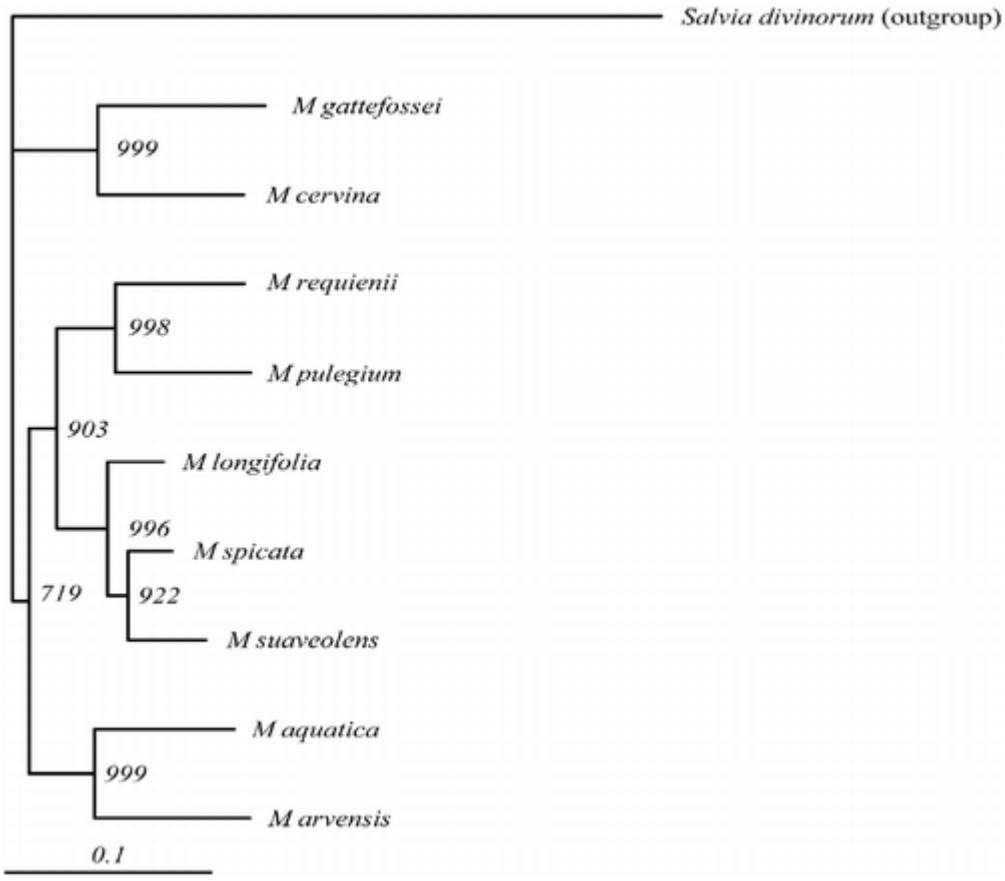
Figure 1 (A) Capillary gel electrophoresis of PCR products generated by primers flanking the spacer region of the 5S-rRNA gene using DNAs from different *Mentha* accessions. Single fragments were produced by 12 of 18 species and hybrids. *M. spicata* (SPI), *M. suaveolens* (SUA) and *M. gattefossei* (GAT) produced multiple fragments. (B) Sequence alignment diagram of 5S-rRNA spacer region fragments separated by CGE. Gaps (blank spaces) are introduced for the best alignment. In case of multiple fragments, sequences are named by bp size. Box A, IE, Box C and the TATA Box are indicated. The vertical dotted lines indicate the site of *Nde*I digestion.



The nucleotide sequence analysis revealed a 5S intragenic promoter sequence (from 1 to 32 bp of the consensus sequence, including the forward primer) composed of a partial sequence of Box A (1–4 bp), intermediate element (IE, 13–14 bp), Box C (23–32 bp, conserved in the majority of the sequences) and a TATA box inside the medium-conserved region (467–470 bp), approximately 30 bp upstream of the transcription start (represented by the trinucleotide GGG), which was highly conserved in the majority of sequences, preceded by a 20-bp GC-rich region and followed by a 10-bp AT-rich sequence (Figure 1B and Table S1).

Cluster analysis (neighbour-joining distances) showed a cluster linking *M. gattefossei* with *M. cervina* (Figure 2). Another cluster linked *M. arvensis* and *M. aquatica*. A third cluster was made of two subclusters: the first connected *M. requienii* and *M. pulegium*, whereas the second subcluster linked *M.spicata*, *M. suaveolens* and *M. longifolia* (Figure 2).

Figure 2 Cluster analysis was carried out on sequence data using ClustalX2 Software. The tree was rooted using *Salvia divinorun* as an outgroup. The analysis clearly separates *M. cervina* and *M. gattefossei* which have been classified in the section *Eriodontes* from the other *Mentha* species. Bootstrap values are indicated on the nodes.



RFLP analysis unequivocally discriminates Mentha species

To better discriminate species showing close bp fragment lengths, RFLP analysis was carried out. The *Taq*I restriction enzyme was able to discriminate most of the species under study (Figure 3), with the exception of *M. arvensis* (ARV) and *M. requenii* (REQ), which did not have sufficiently discriminating differences in fragment length. To better discriminate these two species, a specific restriction enzyme *Bgl*I was used. *Bgl*I enzyme digestion of the *M. requenii* 472-bp fragment (shown in Figure 1 and Table S1) produced two distinct fragments (266 and 206 bp) and the restriction enzyme was ineffective on *M. arvensis* (Figure 4).

Figure 3 Capillary gel electrophoresis of restriction fragments produced by the action of *Taq*I endonuclease on amplified PCR products. A complete discrimination was obtained for most of the species, with the sole exception of *M. requienii* (REQ) and *M. arvensis* (ARV), showing similar patterns. For abbreviations, see Figure 1(B).

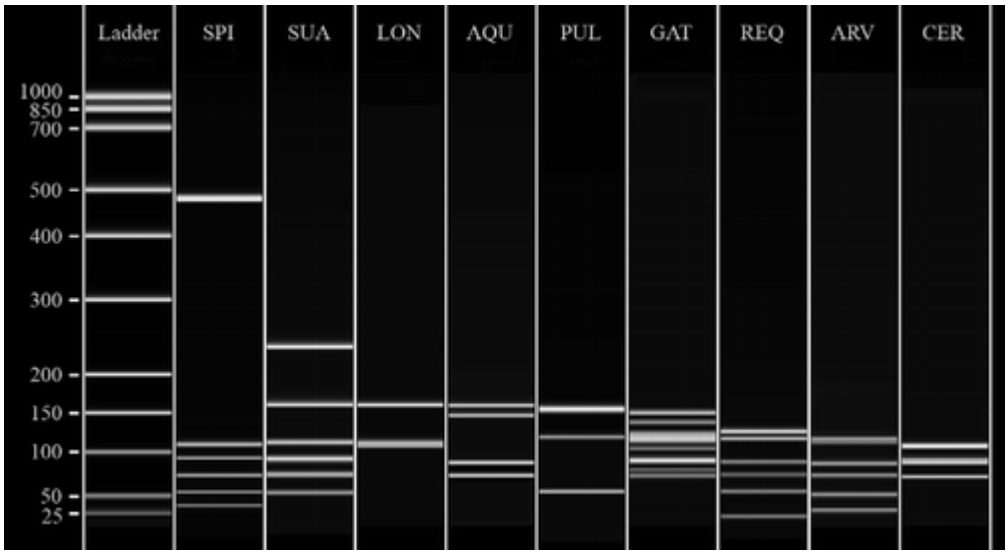
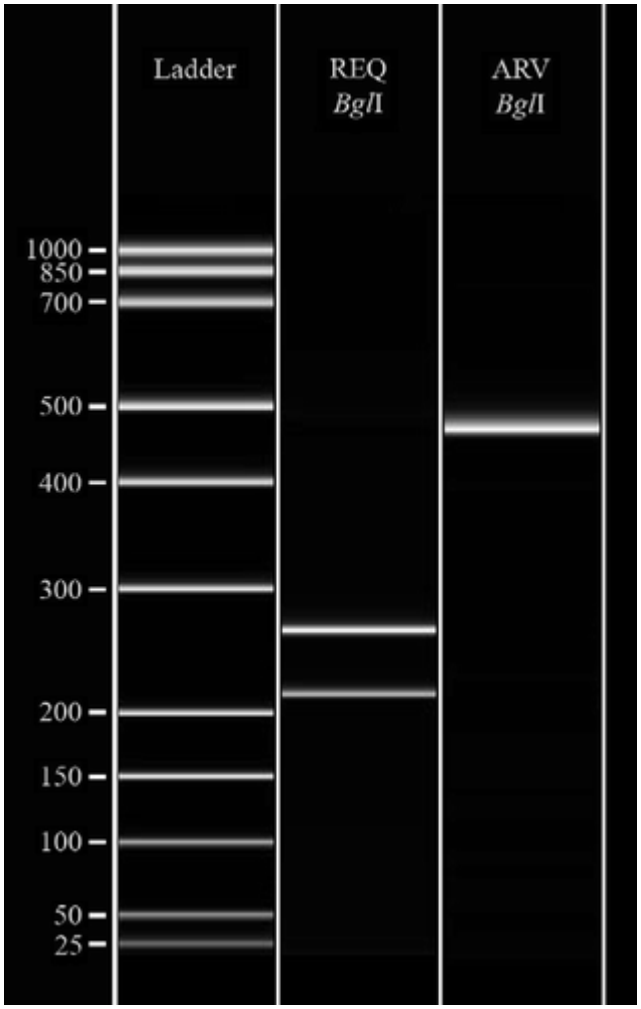
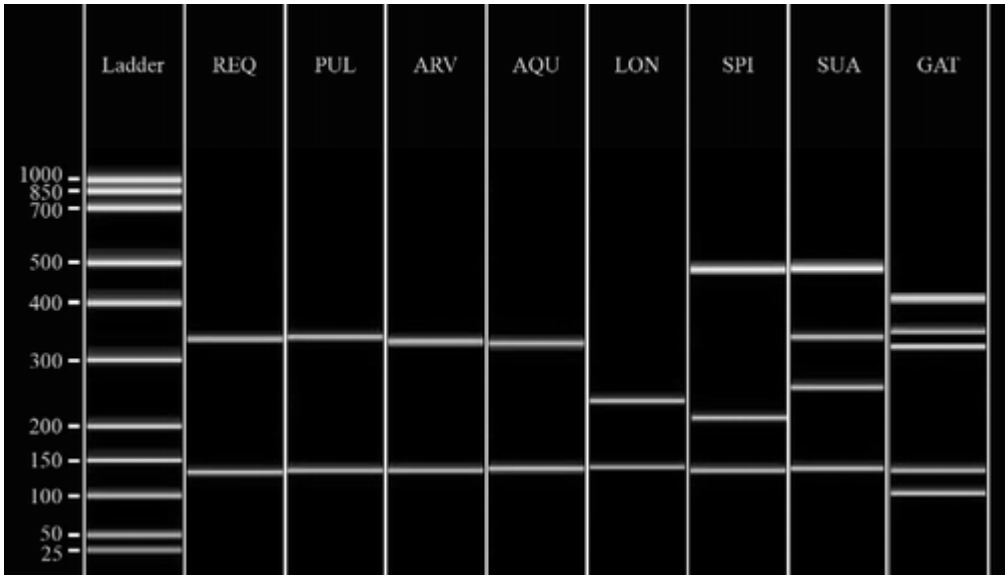


Figure 4 Capillary gel electrophoresis of the restriction fragments produced by the action of *Bgl*I endonucleases on *M. requieni* (REQ) and *M. arvensis* (ARV). *BglI* enzyme digestion of *M. requenii* produced two distinct fragments (266 and 206 bp), but the enzyme was ineffective on *M. arvensis*.



In an attempt to identify a molecular marker able to discriminate the genus *Mentha*, RFLP analysis was carried out using *Nde*I. Based on sequence analysis, the enzyme was found to cut between base 375 and 382 of the consensus sequence (Figure 1B and Table S1). Although this enzyme was unable to perform a cut on *M. cervina* (CER) sequence, in all other samples, *Nde*I generated a common fragment between 127 and 137 bp, in addition to other fragments (Figure 5).

Figure 5 Capillary gel electrophoresis of *Nde*I enzymatic digestion of the PCR products shows the generation of a common fragment between 127 and 137 bp, in addition to other fragments. *Nde*I enzymatic digestion was ineffective on *M. cervina* that is not included in the CGE (see discussion in the text). For abbreviations, see Figure 1B.



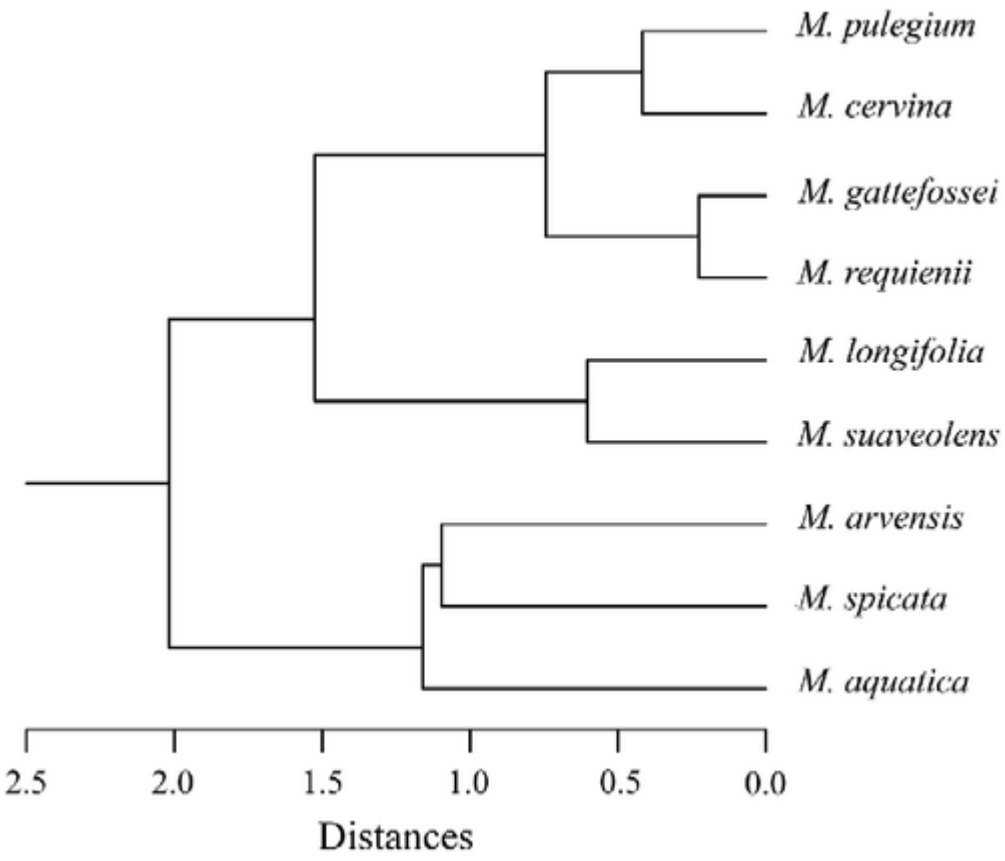
To validate the feasibility of this procedure, an *in silico* comparison was made with 5S-rRNA NTS sequences of other higher plants using the NCBI GenBank database [including species related to the genus *Mentha* such as *Salvia divinorum*, *S. officinalis*, *Perilla frutescens* var. *frutescens* and *P. frutescens* var. *purpurascens* (accessions DQ230979, DQ230980, EF673041 and EF673039, respectively) and unrelated species such as *Artemisia absinthium*, *Acorus calamus*, *Arabidopsis thaliana* and *Oryza sativa* (accessions EU816952, AY812747, NC003076 Cr.5 11184853–11185320, D26370, respectively)]. The *Nde*I restriction site was not found to produce the same specific fragment as in the *Mentha* accessions (data not shown).

Essential oil composition shows clustering patterns similar to DNA fingerprint

To assess whether molecular fingerprinting patterns obtained with the above analyses showed possible correlation with the essential oil composition of the *Mentha* species under study, a cluster analysis was done on reference data-sets. The *Mentha* species under study are characterised by the presence of various monoterpenes, including carvone (typical of *M. spicata*), piperitenone oxide (typical of *M. suaveolens* and some chemotypes of *M. longifolia*), pulegone (typical of *M. requienii*, *M. pulegium*, *M. gattefossei* and *M. cervina*), menthofuran (typical of *M. aquatica*) and 3-octanone (typical of *M. arvensis*) (Lawrence 2007a, 2007b). The cluster analysis was carried out only on the major essential oil components of the above species which revealed a close statistical linkage between species producing pulegone (*M. requienii*, *M. pulegium*, *M. gattefossei* and *M. cervina*) (Figure 6). Another cluster was made of two subclusters: one gathering *M. longifolia* and *M. suaveolens* (for their content in piperitenone oxide) and the other linking the remaining species (see

Table S2 for essential oil data and references). Several attempts to include also minor essential oil components in the cluster analysis did not provide a significant difference with respect to using only major components (data not shown).

Figure 6 Cluster analysis of the main essential oil components taken from reference data. A cluster gathers all species producing pulegone (*M. requienii, M. cervina, M. gattefossei* and *M. pulegium*). All other species are linked in the second cluster composed of a first subcluster linking *M. longifolia* and *M. suaveolens* (for their content of piperitenone oxide) and a second one linking all other species. Essential oil components and reference data are listed in Table S2.



Discussion

Previous studies on the sequences of plastid and nuclear DNA of the genus *Mentha* (Gobert et al. 2002; Bunsawat et al. 2004; Gobert et al. 2006; Saric-Kundalic et al. 2009) revealed important aspects of the evolutionary origin of this group of plants. In this study, sequencing of the 5S-RNA NTS region along with RFLP analysis allowed the complete molecular discrimination of some *Mentha* species.

Alignment analyses revealed significant differences between *Mentha* sequences, which were characterised by a high number of insertion/deletion events. In the case of *Mentha*, the high variability between different species indicates that the genus is still very vital, where vitality reflects the ability of species to produce interspecific hybrids. *M. aquatica*, *M. arvensis*, *M. longifolia*, *M. spicata* and *M. suaveolens* are the sexual species of the genus, characterised by a high interbreeding ability that generates much of the diversity observed (Harley & Brighton 1977; Gobert et al. 2006; Tucker & Naczi 2007).

Cluster analysis further confirms previous taxonomical classifications. *M. reqienii* and *M. pulegium* sequences are clustered in the same group. These two species belong to the same intrageneric division (section *Pulegium*), whereas *M. cervina* and *M. gattefossei* have been classified in the section *Eriodontes* (Tucker & Naczi 2007).

The analysis of nucleotide sequences was in agreement with previous observations in other plant species (Bhatia et al. 1993; Cloix et al. 2002; Negi et al. 2002), and showed that the 5S intragenic promoter sequence is composed of the partial sequence of Box A, IE and Box C. Another regulatory sequence observed was the TATA box, preceded by a GC-rich region and followed by an AT-rich sequence. These may contribute to facilitate the opening of the double strand of DNA in the transcription direction and to keep it closed in the opposite direction.

The use of restriction enzymes after amplification of the variable region of interest (NTS) has proved to be a fast, sensitive and reliable method for the distinction of different *Mentha* species. Although the restriction enzyme *Taq*I was not able to generate different restriction profiles (e.g. ARV, REQ), the use of *Bgl*I allowed to obtain an effective complete characterisation. In addition, the restriction reaction operated by *Nde*I allowed the identification of a potential genus-specific marker consisting of the 127–137 bp fragment. Regarding *M. cervina*, it was not possible to obtain the same digestion profile as in other samples. The latter fact points to an interesting and puzzling taxonomical problem. *M. cervina*, because of its morphological diversity with respect to other mints, has been classified in a separate section of the genus (*Eriodontes*) together with *M. gattefossei* (Tucker & Naczi 2007). The clustering of these two species further confirms their taxonomical diversity with respect to other mints.

A remarkable agreement was found between essential oil chemical data and molecular fingerprinting, with particular reference for the pulegone-producing species. Furthermore, *M. arvensis* and *M. aquatica* were clustered together both on chemical and molecular analyses, whereas *M. spicata* and *M. suaveolens* were found to belong to the same subcluster in both molecular and chemical clustering. The positive correlation between molecular and chemical data confirms previous reports obtained on different species of the same family and to different families (Bertea et al. 2005, 2006; Rubiolo et al. 2009; Gnavi et al. 2010a, 2010b).

In conclusion, these results clearly support the view that *Mentha* species show a remarkable variability, at both the genomic and gene product (secondary metabolites) levels. This study, by showing genomic difference in the 5S-rRNA spacer regions and consistent chemical variation in the terpenoid profile, enabled the unequivocal biomolecular fingerprinting discrimination of some *Mentha* species. Combined “omics” approaches are becoming a useful tool not only for basic science but also for industrial plant characterisation. Owing to the commercial relevance of several *Mentha* species, the identification of *Taq*I and *Nde*I sites can be used for rapid and precise species identification, complementing the essential oil chemical analysis.

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