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Molecular fingerprinting of peppermint (Mentha piperita) and some Mentha hybrids by sequencing and RFLP analysis of the 5S rRNA Non-Transcribed Spacer (NTS) region

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Abstract

Hybridization of species belonging to the genus *Mentha* is quite common. However, the indicators of hybridity are many and make *Mentha* hybrids' identification difficult. By using the same molecular strategy that allowed us to unequivocally identify some *Mentha* species, we amplified the Not-Transcribed-Spacer (NTS) of the 5S-rRNA gene to characterize the industrial crop peppermint, *M.* × *piperita* and some important *Mentha* interspecific hybrids: *M.* × *dalmatica*, *M.* × *dumetorum*, *M.* × *rotundifolia*, *M.* × *maximilianea*, *M.* × *smithiana*, *M.* × *verticillata*, *M.* × *villosa*. DNA amplification, sequence and cluster analysis revealed differences in the 5S-rRNA NTS region of *Mentha* hybrids. Peppermint and all other hybrids were unequivocally discriminated by RFLP analysis by using *Taq*I restriction enzyme, while a further discrimination between *M.* × *dumetorum* and *M.* × *verticillata* was obtained by *Xho*I restriction enzyme. Essential oil composition showed clustering patterns similar to DNA fingerprint, with a clear discrimination between plants producing menthofuran (e.g. *M. aquatica* and its related hybrids, including peppermint) and those containing piperitenone oxide (*M. longifolia* and its related hybrids).

Keywords

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

Introduction

The genus *Mentha* is defined as a taxonomically complex genus (Harley & Brighton 1977; Tucker et al. 1980); 18 species are reputed part of the genus and 11 natural hybrids are regarded as originating from the breeding of five Eurasian and African sexual species included in the section *Mentha* (Tucker & Naczi 2007). Hybridization of *Mentha* species is very common, but identification of these hybrids may be difficult. As pointed out by Tucker and Naczi (2007), several taxonomists have treated sterility as a criterion for hybrid origin, but several hybrids exhibit varying degrees of fertility.

Because the clarification of the relationships between species and hybrids is hard to achieve, over the past years, many attempts have been made for classification purposes (Barra 2009). New approaches have been proposed to analyse the genus *Mentha*, using biomolecular data as support for taxonomical identification (Shasany et al. 2005; Chen et al. 2012; Schanzer et al. 2012). Recently, amplification of the Not-Transcribed-Spacer (NTS) of the 5S-rRNA gene has been successfully used to characterize some *Mentha* species, revealing high specific variability. Cloning and sequencing of all amplified NTS fragments enabled the discrimination among almost all

Mentha species studied. *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 *Mentha* species by PCR–RFLP. Moreover, a direct comparison between essential oil composition and DNA fingerprinting confirmed a relationship between chemical and molecular data (Capuzzo & Maffei 2014). Furthermore, works based on chloroplast and nuclear DNA sequences (Gobert et al. 2002; Bunsawat et al. 2004; Sandionigi et al. 2012) have been successfully used to confirm assumptions previously made by the use of conventional morphological and chemical analyses.

Taxa of the genus *Mentha* have evolved in nature through natural hybridization and selection. Because most hybrids are unable to produce viable seeds, their propagation is through vegetative reproduction by using rooted stolons. While this procedure represents a disadvantage for propagation of plants as compared to seeds, it grants a higher uniformity of essential oils production with plants deriving from clonal propagation, as in the case of peppermint, a natural hybrid between *M. aquatica* and *M. spicata* (Lawrence 2007b). Because selection of actual hybrids through morphological traits and essential oil analyses is affected by phenotypic plasticity and environmental factors (Maffei 1988b, 1990), a biomolecular approach using sequencing and RFLP of the 5S-rRNA NTS was used in the present study to unequivocally characterize and discriminate peppermint (*M.* × *piperita*) and some of the most taxonomically and economically important hybrids of the genus *Mentha*. This study describes the development of reliable, rapid, highly sensitive and easily applicable protocols based on molecular biological methods for the unequivocal determination of *Mentha* hybrids.

Materials and methods

Plant materials

Mentha hybrids [*M.* × *dalmatica Tausch* PI557915 (*M. longifolia* × *M. arvensis*); *M.* × *dumetorum Schult*. PI557916 (*M. longifolia* × *M. aquatica*); *M.* × *rotundifolia* (L.) *Huds* PI637840 (*M. longifolia* × *M. suaveolens*); *M.* × *maximilianea F. W. Schult*. PI557936 (*M. aquatica* × *M. suaveolens*); *M.* × *piperita* L. PI557971 (*M. aquatica* × *M. spicata*); *M.* × *smithiana R. A. Graham* PI558001 (*M. aquatica* × *M. arvensis* × *M. spicata*); *M.* × *verticillata* L. PI558004 (*M. aquatica* × *M. arvensis*) and *M.* × *villosa Huds*. PI558008 (*M. spicata* × *M. suaveolens*)] were kindly provided by the USDA Arctic and Subartic Plant Gene Bank of Palmer (Fairbanks, AK, USA) as rhizomes. Data on hybrids progenitors are reported according to Tucker and Naczi (2007). Plants were grown in plastic pots with sterilized peat and vermiculite (4:1, v/v) at 23°C and 60% humidity using daylight fluorescent tubes at 270 $\mu E\ m^{-2}\ s^{-1}$ with a photoperiod of 16 h. In order to compare hybrids with their parental plants, data on *M. aquatica* and *M. longifolia* species were obtained from previous data (Capuzzo & Maffei 2014).

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 by 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 by 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 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; Capuzzo & Maffei 2014). The amplification was carried out in a 50-µl reaction mixture containing 5 µl of 10 × PCR reaction buffer (Fermentas, Glen Burnie, MA, USA), 0.2 mM dNTPs, 20 pmol of forward and reverse primers and 0.5 U of Taq DNA polymerase (Fermentas). PCR reactions 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 microlitre of the amplification reaction 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 visualized by ethidium bromide staining under UV. From this gel, bands ranging from 150 to 500 bp were purified by 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 performed by using QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA, USA), following manufacturer's instructions. Plasmid DNAs were employed as a template for sequencing at least twice both the strands of DNA. 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 performed on reference data from essential oil composition of the *Mentha* hybrids under study. Data were analysed by means of Systat 10 using hierarchical clustering classification with Euclidean distance and single linkage.

PCR–RFLP

Purified PCR products of the 5S-rRNA gene spacer region were first digested in separated reactions with 10 U of *Bgl*I, *Msc*I, *Nde*I, *Xho*I (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 reactions was fractionated by capillary gel electrophoresis (CGE) using the Agilent 2100 Bioanalyzer (Agilent Technologies) and DNA 1000 LabChip Kit (Agilent Technologies) following manufacturer's instructions. Reproducibility was assessed by repeating digestions with different

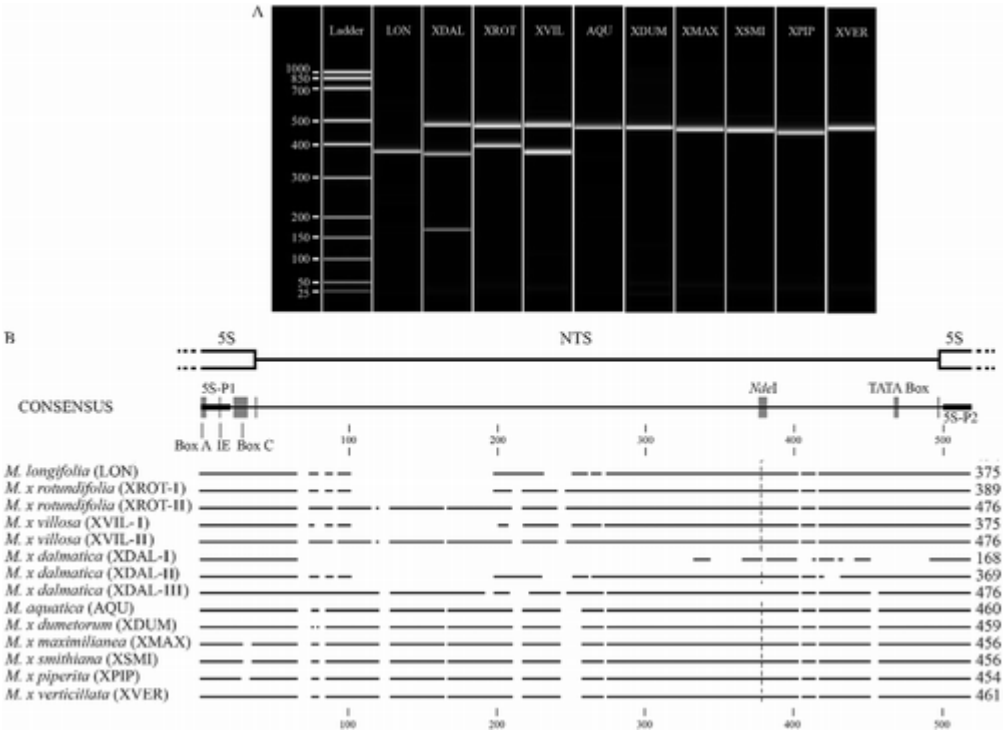
enzyme concentrations and timings, whereas for each set of runs a different calibration curve was assessed by the DNA 1000 LabChip.

Results

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

Two primers flanking the 5S-rRNA spacer region were used in the PCR analysis of genomic DNA isolated from different samples of *Mentha* hybrids. The amplified fragments were in the range of approximately 150–500 bp (Figure 1(A)). Sequence analyses (NCBI GenBank accession numbers from JF775666 to JF775678) were aligned using ClustalX2 software (see Supplementary 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 1(A), was sequenced and named progressively (I, II, etc.) based on fragment length, as described in Figure 1(B). A single DNA fragment, ranging from 450 to 490 bp, was present in all hybrids (Figure 1(B)). *M.* × *rotundifolia* and *M.* × *villosa* showed two fragments (389, 476 bp and 375, 476 bp, respectively), whereas *M.* × *dalmatica* showed three fragments (168, 369, 476 bp) (Figure 1(B)). Figure 1 also shows molecular data of the parental plants *M. longifolia* (showing a single band of about 375 bp) and *M. aquatica* (showing a single band of 460 bp) as reported by Capuzzo and Maffei (2014).

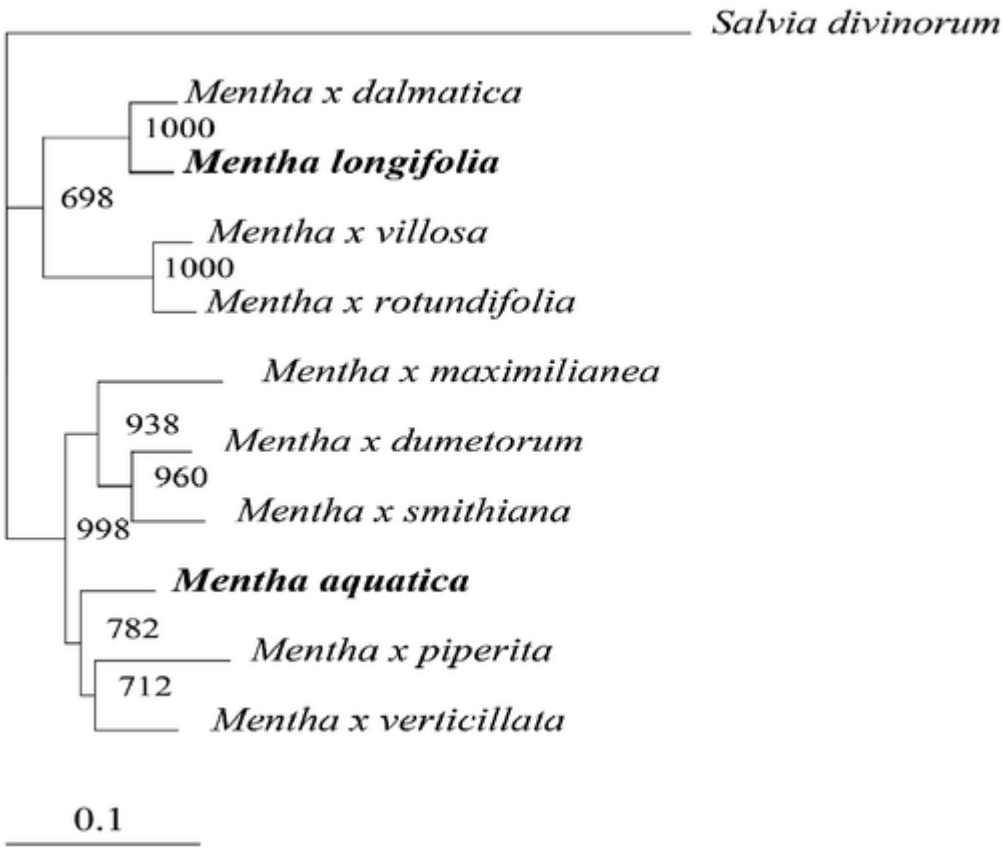
Figure 1 (A) Capillary gel electrophoresis of PCR products generated by primers flanking the spacer region of the 5S-rRNA gene using DNAs from different *Mentha* hybrid accessions. Single fragments were produced by 7 out of 10 species and hybrids. *M.* × *dalmatica* (XDAL), *M.* × *rotundifolia* (XROT) and *M.* × *villosa* (XVIL) 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.



As is typical of the genus *Mentha* (Capuzzo & Maffei 2014), the nucleotide sequence analysis revealed a 5 S 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 1(B) and Supplementary Table S1).

Cluster analysis (Neighbor-Joining distances) showed a cluster linking the parental plant *M. longifolia* with all its related hybrids (*M.* × *dalmatica*, *M.* × *villosa* and *M.* × *rotundifolia*), whereas a second cluster linked *M. aquatica* and the remaining related hybrids (Figure 2). In the latter cluster, a sub-cluster showed a close statistical linkage between *M.* × *dumetorum* and *M. smithiana* (Figure 2).

Figure 2 Cluster analysis performed on sequence data using ClustalX2 Software. The tree was rooted by using *Salvia divinorun* as an outgroup. The analysis clearly separates *M. longifolia* and *M. aquatica* and their related hybrids. *M. aquatica* and *M. longifolia* data are from Capuzzo and Maffei (2014). Bootstrap values are indicated on the nodes.



In addition, cluster analysis gathering all parental species (Capuzzo & Maffei 2014) and the hybrids sequences under study confirmed previous observations (Tucker & Naczi 2007) (see Supplementary Figure S1).

RFLP analysis unequivocally discriminates *Mentha* hybrids

To better discriminate hybrids showing close bp fragment lengths, RFLP analysis was performed. The *Taq*I restriction enzyme was able to discriminate most of the hybrids under study (Figure 3), with the exception of *M.* × *dumetorum* (XDUM) and *M.* × *verticillata*, which did not have sufficiently discriminating differences in fragment length. To better discriminate these two hybrids, a specific restriction enzyme *Xho*I was used. *Xho*I restriction enzyme digestion of the XDUM 459-bp fragment (shown in Figure 1) produced two fragments (307 and 152 bp) and the enzyme was ineffective on *M.* × *verticillata* (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 hybrids, with the sole exception of *M.* × *dumetorum* (XDUM) and *M.* × *verticillata* (XVER), showing similar patterns. For abbreviations, see Figure 1(B).

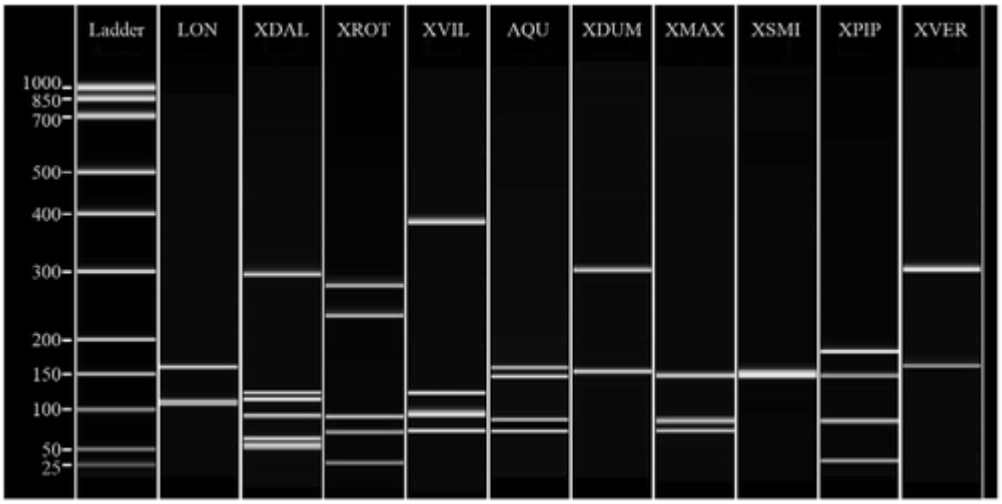
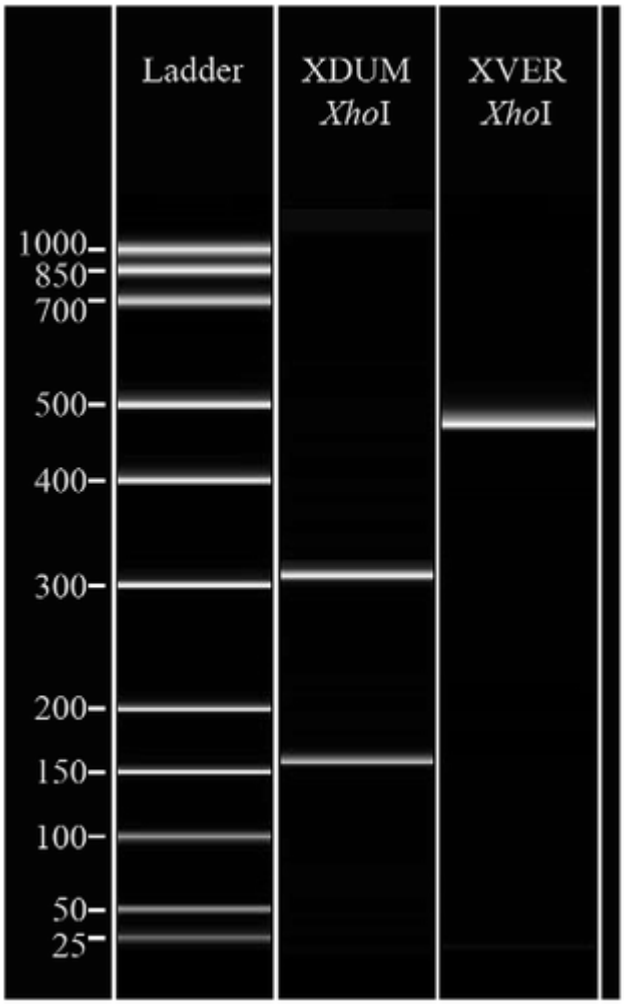


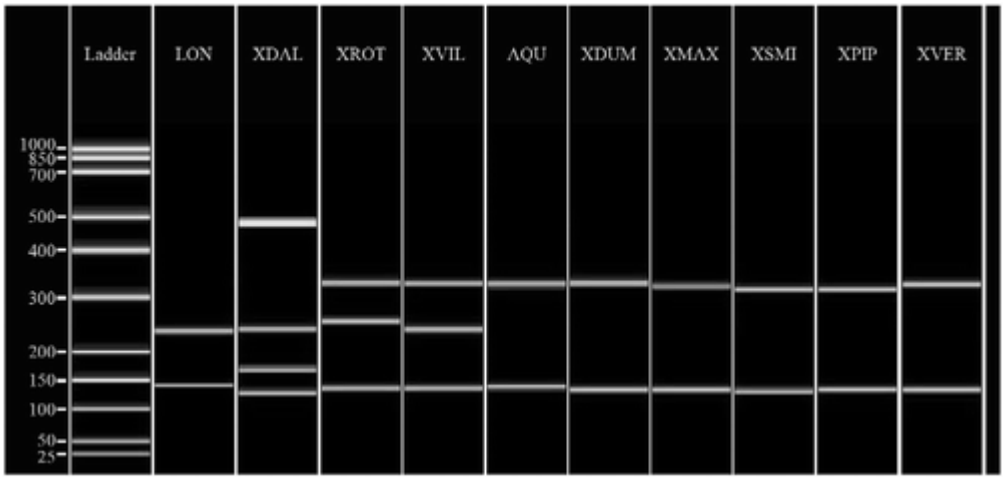
Figure 4 Capillary gel electrophoresis of the restriction fragments produced by the action of *Xho*I endonucleases on *M.* × *dumetorum* (XDUM) and *M.* × *verticillata* (XVER). *Xho*I enzyme digestion of *M.* × *dumetorum* produced two distinct fragments, but the enzyme was ineffective on *M.* × *verticillata*.



RFLP analysis using *Nde*I has proven to be an efficient molecular strategy able to discriminate the genus *Mentha* (Capuzzo & Maffei 2014). Based on sequence analysis on *Mentha* hybrids, the enzyme was found to cut between base 375 and 382 of the consensus sequence (Figure 1(B) and

Supplementary Table S1) and to generate in all hybrids a common fragment between 127 and 137 bp, in addition to other fragments (Figure 5).

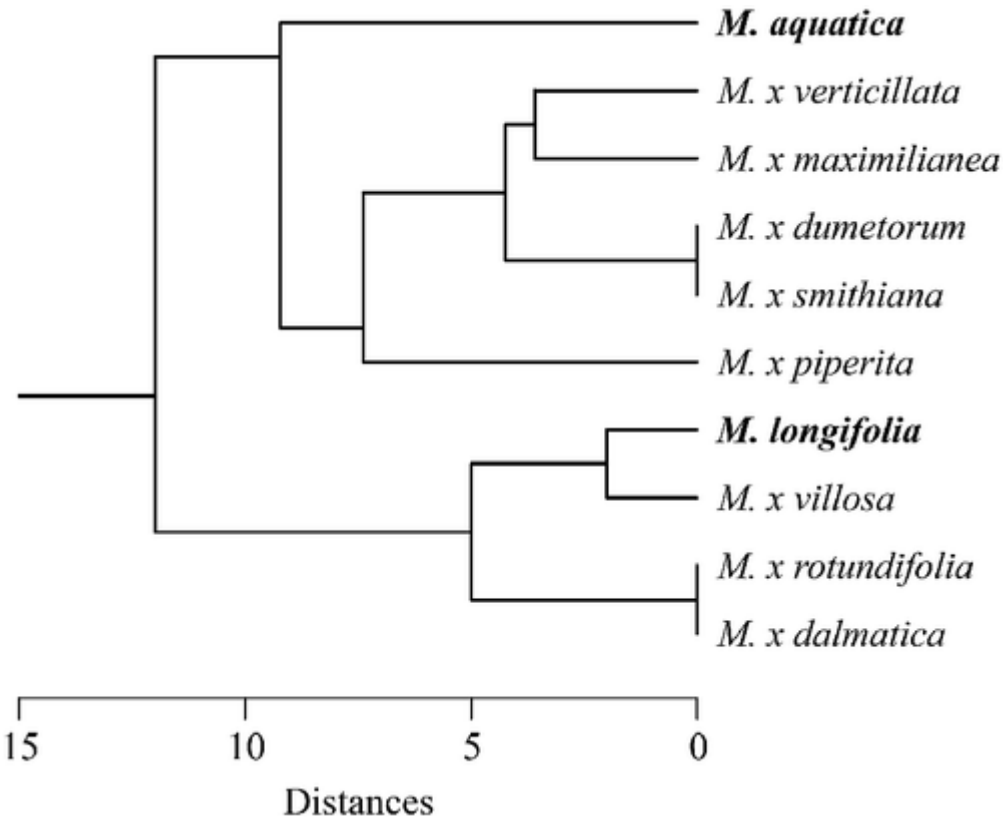
Figure 5 Capillary gel electrophoresis of *Nde*I enzymatic digestion of the PCR products shows the generation of a common fragment between 127 and 137 bp, in addition to other fragments. For abbreviations, see Figure 1(B).



Essential oil composition shows clustering patterns similar to DNA fingerprint

DNA fingerprinting patterns obtained with the above strategy have been shown to correlate with the essential oil composition of several *Mentha* species (Capuzzo & Maffei 2014). Therefore, we collected reference data on essential oil composition of the *Mentha* hybrids under study and calculated a cluster analysis by considering the main compounds. The *Mentha* hybrids under study are characterised by the presence of several monoterpenes. Menthofuran is a typical oxygenated monoterpene of the parent plant *M. aquatica*. This compound is also present in several hybrids deriving from crosses of *M. aquatica* with other sexual *Mentha* species, including *M.* × *dumetorum*, *M.* × *maximilianea*, *M.* × *piperita* and *M.* × *verticillata* (Lawrence 2007a, 2007b). On the other hand, the parental plant *M. longifolia* is characterised by the presence of the monoterpene oxygenated compounds menthone, pulegone, piperitenone and piperitenone oxide (Maffei 1988a). *M.* × *rotundifolia*, *M.* × *dalmatica* and *M.* × *villosa*, which all share *M. longifolia* as a parent plant, all show the presence of typical *M. longifolia* oxygenated monoterpenes (See Supplementary Table S2). A cluster analysis performed only on the major essential oil components of the above hybrids showed the presence of two main clusters: the first, comprising *M. aquatica*, is linked to a subcluster that links all other *M. aquatica* related hybrids; the second consists of two subclusters gathering *M. longifolia* and its related hybrids. A close statistical linkage is evident in the first cluster between *M.* × *dumetorum* and *M.* × *smithiana* (Figure 6). Several attempts to include also minor essential oil components in the cluster analysis did not provide significant differences as observed using only the major components (data not shown).

Figure 6 Cluster analysis of the main essential oil components taken from reference data. A cluster gathers all hybrids producing menthofuran along with its parental species *M. aquatica*. All other hybrids are linked in the second cluster including *M. longifolia* for their content of piperitenone oxide. Essential oil components and reference data are listed in Supplementary Table S2.



Discussion

In a previous study, sequencing of the 5S-RNA NTS region along with RFLP analysis allowed the complete molecular discrimination of some *Mentha* species (Capuzzo & Maffei 2014). This study confirmed the efficacy of the same molecular approach for the discrimination of some *Mentha* hybrids.

By comparing the results of cluster analysis with the chromosome numbers of the hybrids under study (Murray et al. 1971; Harley & Brighton 1977; Chambers & Hummer 1994), we were able to draw some conclusions. In hybrids related to *M. aquatica*, the close relationship with this parent correlates with the high genomic content of *M. aquatica* species ($2n = 96$ octaploid content) with respect to other *Mentha* species ($\leq 2n = 72$ hexaploid content) (Harley & Brighton 1977). One possible explanation may be the occurrence of introgression of the *M. aquatica* locus instead of others. This was also suggested for *M.* × *piperita* by Gobert et al. (2002). Interestingly, the opposite case could be observed in the closest relationship between *M.* × *dalmatica* (*Mentha arvensis* × *Mentha longifolia*) sequence and *M. longifolia*, which has a lower genomic content ($2n = 24$) with respect to the other parental *M. arvensis* species ($2n = 72$) (Harley & Brighton 1977; Capuzzo & Maffei 2014); this may represent the contribution of the *M. longifolia* genome, despite its small size and the evidence of how these mechanisms occur randomly. In addition, the presence of *M.* × *villosa* (*M. spicata* × *M. suaveolens*) sequences in *M. longifolia* group is interesting. These data support the assumption that the contribution of the parental genomes has been maintained by the hybrid generating two putative loci. Similar assumptions have been documented by Negi et al. (2002) about the origin of this genomic region in *Populus deltoides* and by Shibata and Hizume (2002) in *Allium cepa* and *A. schoenoprasum*. Moreover, the close relationships between the 5S NTS spacers, characterized by a high variability even among different varieties, supports the assumptions made about the origin of the different hybrids of this genus (Murray et al. 1971; Harley & Brighton 1977; Chambers & Hummer 1994; Bunsawat et al. 2004).

As found for *Mentha* species (Capuzzo & Maffei 2014), 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 5 S intragenic promoter sequence is composed of the partial sequence of Box A, IE and Box C. Even for *Mentha* hybrids, the TATA box, preceded by a GC-rich region and followed by an AT-rich sequence, might 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 presence of multiple amplicons in some of the taxa, as observed in other genera containing polyploids like the *Fragaria* genus (Liu & Davis 2011), suggests multiple copies for 5S-rDNA. This is probably generated during polyploidization in *Mentha*; however, further studies like *in situ* hybridization are necessary to elucidate their chromosomal localization.
The use of *Taq*I 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* hybrids. However, this enzyme was unable to discriminate between *M.* × *dumetorum* and *M.* × *verticillata*. The use of *Xho*I enabled an effective complete characterisation between the two hybrids.
As found for many other plants producing essential oils (Gnavi et al. 2010a, 2010b), a remarkable agreement was found between essential oil chemical data and molecular fingerprinting. This agreement was particularly interesting for peppermint, *M.* × *piperita*, the most important industrial crop of the genus. As peppermint oil adulteration is relatively common, the use of component ratios and pattern recognition techniques can detect and quantify adulteration (Lawrence 2007c).
However, despite the fact that peppermint seldom produces viable seeds, peppermint seeds are often present on the market. The molecular analysis of these seeds (i.e. much before the plant can produce essential oils) often demonstrates that they do not belong to peppermint (Maffei, in preparation). Therefore, our results can contribute to early detection by molecular identification of this important industrial crop.
The agreement between molecular and essential oil chemical data confirms previous reports obtained on different species of the same species, family and to different families (Bertea et al. 2005, 2006; Rubiolo et al. 2009; Gnavi et al. 2010a, 2010b; Capuzzo & Maffei 2014).
In conclusion, by showing genomic difference in the 5S-rRNA spacer regions and consistent chemical variation in the terpenoid profile, we provided evidence for the unequivocal biomolecular fingerprinting discrimination of some *Mentha* hybrids, including the industrial plant peppermint. Owing to the commercial relevance of this hybrid, the identification of *Taq*I sites can be used for rapid and precise hybrid identification, complementing the essential oil chemical analysis.

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