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# Genome size and ISSR markers for *Mentha* L. (Lamiaceae) genetic diversity assessment and species identification

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ABSTRACT

The systematics of the *Mentha* genus is very complicated and still unclear. This is mainly due to the existence of variation in basic chromosome number, frequent interspecific hybridization, cytomixis, polymorphism in morphology and essential oil composition under different environmental conditions, colonial mutant propagation, as well as the occurrence of polyploidy, aneuploidy and nothomorphs. Morphological traits, chemical composition, and even caryological analysis failed in discrimination of *Mentha* species. Therefore, the aim of this study was to combine genome size estimation with ISSR markers for quick and precise species identification, determination the inter- and intraspecific variation within this genus, as well as description of relationships between genotypes. Genome size estimation revealed, that investigated *Mentha* species possessed very small and small genomes. Additionally, for 13 *Mentha* species, this is the first report on genome size. Within one species different values of genome size occurred, which indicated polyploidy and aneuploidy. The smallest genome (0.63 pg/2C) was detected in *M. gattefossei*, while the largest in *M.* × *verticillata* (4.15 pg/2C). Flow cytometric measurements of genome size allowed to distinguish some species and cytotypes, and thus it can be used as a first step in species identification. More precise and detailed characterisation were provided by ISSR markers. All the primers used in ISSR-PCR, revealed to be useful in species identification, while primers $(GACA)_4$, $(GTG)_6T$, $(CTC)_4RC$, $(GA)_6CC$ enabled to distinguish *M. aquatica*, *M. canadensis*, *M. longifolia*, *M. pulegium* and *M. spicata* cytotypes, and primers $(GACA)_4$, $(GA)_6CC$ and $(GA)_8YC$ to identify *M.* × *gracilis* and *M.* × *piperita* cultivars. The phylogenetic analysis revealed the existence of seven main clusters and two species (*M. requienii* and *M.* × *smithiana*) being not clustered to any of the created groups within the *Mentha* genus. This analysis mostly confirms the latest taxonomical classification of the *Mentha* genus, however some exceptions are discussed. The identification system of combined genome size estimation, and ISSR markers proved to be useful for species in *Mentha* genus.

## 1. Introduction

The genus *Mentha* L. (mint) is one of the most important taxa of the Lamiaceae family, and it comprises from 18 to 30 species, among which about 11–13 are hybrids. Species in the genus are almost exclusively perennial, herbaceous plants, which are distributed all over the five continents, mainly in temperate and sub-temperate regions of the world. Many *Mentha* species are widely cultivated for their pleasant aroma and flavor, since their essential oils are used in pharmaceutical, cosmetics, and perfumery applications, as well as for culinary purposes (Gobert et al., 2002; Kumar et al., 2015). This make many *Mentha* species very valuable for industry, as mint oil is a supplement for wide spectrum of products. In medicine and pharmacy mint essential oils are usually used for antipruritic, astringent, antiseptic, and antimicrobial purposes, and for treating neuralgia, myalgia, headaches, and migraines (Hendriks, 1998; Cowan, 1999; Iscan et al., 2002). The major components of mint oil are menthol, menthofuran, carvone, linalool, and linalyl acetate (Bakkali et al., 2008; Kumar et al., 2015). The most significant species for the industry are: *Mentha canadensis* (Japanese mint), *M. cardiaca* (spearmint), *M. spicata* (native spearmint), *M. pulegium* (corn mint), *M.* × *gracilis* (Scotch spearmint) and *M.* × *piperita* (peppermint; Smolik et al., 2007; Hua et al., 2011; Rodrigues et al., 2013).

The systematics of the *Mentha* genus is very complicated and still unclear, mainly due to the variation in basic chromosome number, frequent interspecific hybridization, cytomixis, polymorphism in morphology and essential oil composition under different environmental conditions, colonial mutant propagation, as well as the occurrence of polyploidy, aneuploidy and nothomorphs (Gobert et al., 2002; Tucker and Chambers, 2002; Tyagi, 2003; Denslow and Poindexter, 2009; Tucker, 2012; Jabeen et al., 2012). This is the cause of the existence of

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numerous synonymous names (over 3000 published names in the genus), and still uncertain taxonomy of the genus. To solve this problem, cytological and molecular methods such as: chromosome counting, AFLP, RFLP, RAPD, CAPS, ISSR, SSR markers and polymorphism of limonene synthase gene have been applied to support the taxonomical affiliation, and to establish phylogenetic relationships between species/accessions (Khanuja et al., 2000; Fenwick and Ward, 2001; Gobert et al., 2002; Smolik et al., 2007; Jabeen et al., 2012; Wang et al., 2013; Kumar et al., 2015). Despite numerous research conducted on mint species, most of them have concentrated on one, or at least several species/accessions, and there is a lack of comprehensive studies covering wide range of species and accessions. So far, hybrid origin of many *Mentha* species has been confirmed, together with the occurrence of intra- and interspecific variation in *Mentha* genus (Khanuja et al., 2000; Tucker and Chambers, 2002; Smolik et al., 2007; Rahimmalek, 2011; Kumar et al., 2015). Regarding taxonomy, various *Mentha* species classifications have been proposed (Briquet, 1897; Harley and Brighton, 1977; Tucker and Naczi, 2007).

A lot of efforts have been made in mint breeding, where both, traditional and biotechnological methods were used towards improvement of the yield and quality of essential oils, incorporation of disease and pest tolerance, together with the increasing of propagule productivity (Bhat et al., 2002). Therefore better understanding of the relationships between existing genotypes of *Mentha* and their correct identification, is crucial for precise description of germplasm, selection of appropriate material for breeding, along with conservation purposes and taxonomy (Khanuja et al., 2000; Kazemi and Hajizadeh, 2012). Correct species/cultivars identification can be used by breeders and propagators to ensure, that propagated materials are true-to-type (Fenwick and Ward, 2001).

The aim of the study was to establish genome size (2C DNA content), and ISSR molecular markers for 34 *Mentha* accessions, to determine the inter-and intraspecific variation within this genus, relationships between genotypes and to define markers, which help in the species identification. Additionally, for 13 *Mentha* species this is the first report on genome size.

## 2. Materials and methods

### 2.1. Plant material

Thirty four *Mentha* accessions, representing 18 species were used in the study. Rhizomes and seeds were obtained from GRIN-ARS-USDA gene bank, or commercially purchased (Table 1). Plant material were grown in 1:2 sand and commercial humus mixture in 12 cm pots, in a growth chamber with 16/8 h (day/night) photoperiod at 26/18° C.

### 2.2. Flow cytometry

Young leaves of all mint accessions were collected and prepared for the flow cytometric analysis, according to the procedure described by Rewers and Jedrzejczyk (2016). Plant material was chopped with the presence of 1 ml of Galbraith's buffer (45 mM $MgCl_2$, 30 mM sodium citrate, 20 mM 3-(N-morpholino)propanesulfonic acid, 0.1% (v/v) Triton X-100, pH 7.0; Galbraith et al., 1983), with the addition of propidium iodide (PI, 50 µg/ml) and ribonuclease A (RNase A, 50 µg/ml), as well as 1.0% (w/v) polyvinylpyrrolidone (PVP-10). The nuclei suspension was analysed using a CyFlow SL Green (Partec GmbH, Münster, Germany) flow cytometer, equipped with a high-grade solid state laser, with green light emission at 532 nm, as well as with side (SSC) and forward (FSC) scatters. For each sample, the DNA content of 5000–7000 nuclei was measured, using linear amplification. The obtained histograms (CV = 4.04-6.10%; Table 2) were evaluated using FloMax program (Partec GmbH, Münster, Germany). Leaves of *Zea mays* CE-777 (5.43 pg/2C; Lysak and Doležel, 1998) and *Petunia hybrida* P × Pc6 (2.85 pg/2C; Marie and Brown, 1993) were used as internal standards (Table 2). Analyses were performed on six individuals per genotype. Nuclear DNA content was calculated, using the linear relationship between the ratio of the 2C peak positions of *Mentha* genotypes, and the internal standard on the histogram of fluorescence intensities. The 2C DNA contents (pg) were transformed to megabase pairs of nucleotides, using the following conversion: 1 pg = 978 Mbp (Doležel and Bartoš, 2005). The results were estimated using a one-way analysis of variance, and a Duncan's test ($P < 0.05$; Statistica v. 10, StatSoft, Poland).

**Table 1**
The origin of *Mentha* accessions used in the study.

| Species | Taxon | Material | Accession no. or purchase company | Origin |
|---|---|---|---|---|
| *M. aquatica* | cv. Eau de Cologne | rhizomes | PI 557993 | USA |
| *M. aquatica* | var. *citrata* | rhizomes | PI 557989 | USA |
| *M. aquatica* | | rhizomes | PI 557572 | Germany |
| *M. australis* | | rhizomes | PI 617476 | Australia |
| *M. canadensis* | | seeds | PI 557606 | Japan |
| *M. canadensis* | | rhizomes | PI 617495 | China |
| *M. canadensis* | | rhizomes | PI 277803 | Brazil |
| *M. gattefossei* | | rhizomes | PI 557639 | Morocco |
| *M. japonica* | | rhizomes | PI 617475 | Japan |
| *M. longifolia* | | seeds | PI 177465 | Turkey |
| *M. longifolia* | ssp. *capensis* | rhizomes | PI 557767 | South Africa |
| *M. longifolia* | | rhizomes | PI 557758 | India |
| *M. longifolia* | ssp. *hymalaiensis* | rhizomes | PI 557768 | Nepal |
| *M. longifolia* | ssp. *typhoides* | rhizomes | PI 557770 | Syria |
| *M. pulegium* | | seeds | PI 557774 | Sweden |
| *M. pulegium* | | seeds | PI 203305 | Chile |
| *M. requienii* | | rhizomes | PI 557781 | France |
| *M. rotundifolia* | | seeds | Rocalba | Spain |
| *M. spicata* | | rhizomes | PI 294099 | Guatemala |
| *M. spicata* | ssp. *condensata* | rhizomes | PI 557888 | USA |
| *M. spicata* | | rhizomes | PI 557885 | USA |
| *M. suaveolens* | ssp. *insularis* | rhizomes | PI 557911 | France |
| *M. suaveolens* | ssp. *suaveolens* | rhizomes | PI 557891 | Unknown |
| *M.* × *dalmatica* | | rhizomes | PI 557913 | USA |
| *M.* × *gracilis* | cv. Scotch Spearmint | rhizomes | PI 557935 | USA |
| *M.* × *gracilis* | cv. Double Mint | rhizomes | PI 557922 | USA |
| *M.* × *piperita* | | rhizomes | PI 557916 | Netherlands |
| *M.* × *piperita* | f. *lavanduliodora* | rhizomes | PI 557952 | Brazil |
| *M.* × *piperita* | cv. White Peppermint | rhizomes | PI 557953 | Mexico |
| *M.* × *piperita* | cv. Variegated Peppermint | rhizomes | PI 557974 | USA |
| *M.* × *smithiana* | | rhizomes | PI 558001 | UK |
| *M.* × *suavis* | | rhizomes | PI 557936 | UK |
| *M.* × *verticillata* | | rhizomes | PI 558004 | Netherlands |
| *M.* × *villosa* | var. *alopecuroides* | rhizomes | PI 558009 | USA |

### 2.3. DNA extraction

Genomic DNA was extracted from 0.12 g of fresh leaf material, from five randomly selected individual plants per genotype, using a Plant DNA GPB Mini Kit (GenoPlast Biochemicals, Poland). Leaf material was prepared according to the protocol described by Rewers and Jedrzejczyk (2016). DNA quality and quantity were established by spectrophotometric measurements and agarose gel electrophoresis. Only samples of high quality were used for ISSR-PCR analysis.

### 2.4. ISSR-PCR

The ISSR analysis were performed in a mixture containing 30 ng of genomic DNA template, 5 µl of 2xPCR Master Mix Plus (0.1 U/µl Taq DNA polymerase, 4 mM $MgCl_2$, 0.5 mM of each dNTPs; A&A Biotechnology, Poland), 10 µM primer (Genomed, Poland) and sterile, deionized water to a final volume of 12.5 µl. The PCR reactions were