Chromosome number and ploidy level of balm (*Melissa officinalis*)

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**Abstract**

**Background:** Lemon balm (*Melissa officinalis* L.) is of increasing importance resulting in rising growth area. Improved knowledge on the genome structure, number of chromosomes in connection with the taxonomical structure of balm is indispensable for improved new varieties.

**Results:** A collection of 40 balm accessions (*M. officinalis*) was characterized by flow cytometry and FISH (18/25S and 5S rDNA) to determine the chromosome number and ploidy level. Three different types were found: diploid genotypes with $2n = 2x = 32$ chromosomes; tetraploid $2n = 4x = 64$ chromosomes and triploid $2n = 3x = 48$ chromosomes. A haploid base number of $x = 16$ chromosomes is likely. First time described triploid accessions are sterile but cytologically and morphologically stable for many years. Triploids express better winter hardiness and regeneration after harvesting cuts as well as bigger leaves and internodes.

**Conclusions:** A basic chromosome number of $x = 16$ is reported for the first time for the species *M. officinalis*.

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Die wachsende Bedeutung von Zitronenmelisse (*Melissa officinalis* L.) führt zur Ausdehnung des hierfür erforderlichen Anbauumfanges. Ein verbesserter Kenntnisstand der Genomstruktur, der Chromosomenzahl und der hiermit in Zusammenhang stehenden taxonomischen Struktur der Melisse sind unerlässliche Voraussetzungen für verbesserte, neue Sorten.

Eine Kollektion von 40 Melisseherkünften (*M. officinalis*) wurde durchflusszytometrisch und durch FISH (18/25S and 5S rDNA) untersucht, um den Ploidiegrad und die Chromosomenzahl zu ermitteln. Drei unterschiedliche Typen wurden konnten bestimmt werden: diploide Genotypen mit $2n = 2x = 32$ Chromosomen; tetraploide mit $2n = 4x = 64$ Chromosomen und triploide mit $2n = 3x = 48$ Chromosomen. Die haploide Chromosomenzahl ist mit $x = 16$ anzunehmen. Die erstmalig beschriebenen triploiden Herkünfte sind steril aber zytologisch und morphologisch über viele Jahre stabil. Sie zeigen eine bessere Winterhärte und einen schnelleren Wiederaufwuchs nach Ernteschnitten, wie auch größere Blätter und Internodien.

Die Basischromosomenzahl von $x = 16$ wird erstmalig für die Art *M. officinalis* beschrieben.

**Background**

Lemon balm (*Melissa officinalis* L.) is an old crop and is used for phytopharmaceuticals, as an aromatic plant and in traditional folk medicine. A wide spectrum of secondary metabolites exists in lemon balm. For the medicinal use, the active ingredients essential oil with lemon fragrance and rosmarinic acid are necessary [6, 2, 3, 13].

*M. officinalis* belongs to the family Labiatae (syn. Lamiaceae). This crop plant is grown worldwide but its origin is not well-defined, however the Mediterranean Region or western Asia is considered as the area of origin [9]. The subspecies *M. officinalis* ssp. *officinalis* and ssp. *altissima* (Sibth. & Sm.) Arcangeli are distinguishable especially by the shape of calyx and density of different types of hairs [5, 20]. The middle tooth of the three upper lip teeth of fruiting calyx is broadly triangular for ssp. *officinalis* whereas it is inconspicuous, truncate or...
emarginated for ssp. *altissima*. Pignatti [17] classified ssp. *altissima* as species *M. romana*.

The chromosome base number in Labiatae ranges from \(x = 5\) to 11, but also \(x = 13, 15, 17\) and 19 occur [10]. For higher numbers, polyploidy is assumed followed by structural rearrangements. Chromosome numbers of 32 and 64 were reported for *M. officinalis* ssp. *officinalis* and ssp. *altissima*, respectively [20]. Ssp. *altissima* has been suggested as an ancestor of the cultivated diploid ssp. *officinalis* despite being tetraploid [9]. Davis [5] separated the ssp. *inodora* (Bornm.) Bornm. based on the long patent hairs on stems and fairly distinct, triangular middle tooth of upper calyx lip and mentioned intermediates between all three subspecies. Pignatti [17] described beside *M. officinalis* L. \((2n = 32)\) the species *M. romana* Miller as synonymous with ssp. *altissima* because of the absence of any offspring \((2n = 64)\). Darlington and Wylie [4] presented the haploid chromosome number for *M. officinalis* with \(x = 8\).

Lemon balm is of increasing importance, resulting in rising growth area and investigations for improved new varieties. Better winter hardiness, higher content of essential oil and higher yield of *M. folium* (lemon balm leaves) are of special interest in these programmes. Adaptation of technologies for acceleration of the breeding process using doubled haploid lines has been initiated. Therefore, the knowledge on the genome structure and number of chromosomes in connection with the taxonomical structure of *M. officinalis* is indispensable. In order to determine the ploidy level of \(40\) accessions and the chromosome number of the haploid *M. officinalis* genome, we employed flow cytometry and FISH using rDNA-specific probes. Beside di- and tetraploid accessions, first time triploid accessions \((2n = 3× = 48)\) have been identified for *M. officinalis*.

**Results and discussion**

Genome size determination by flow cytometry of *M. officinalis* revealed for \(23\) accessions a diploid, four accessions a triploid and \(13\) accessions a tetraploid ploidy level (Table 1).

To confirm the ploidy predictions, chromosome numbers were determined and multicolour FISH was performed with \(18/25S\)- and \(5S\) rDNA-specific probes (Table 2). In *M. officinalis* \(5S\) and \(18/25S\) rDNA were localized on different chromosomes. Unlike this result in some other genera e.g. *Helianthus*, *Brassica* and *Alstroemeria* [18, 19, 1] localization of \(5S\) and \(18/25S\) rDNA on the same chromosome was found. Analysis of six selected accessions revealed for the putative diploid genotypes a chromosome number of \(32\) and two chromosome pairs exhibiting either \(18/25S\) rDNA- or \(5S\) rDNA-specific signals (Fig. 1a, b). Putative triploid accessions contained \(48\) chromosomes and revealed six distinct hybridization signals: three \(18/25S\) rDNA and three \(5S\) rDNA (Fig. 1c, d). The last category exhibited \(64\) chromosomes and eight signals: four \(18/25S\) rDNA and four \(5S\) rDNA sites (Fig. 1e, f).

In accessions showing six or eight hybridization signals, the intensity of rDNA signals varied. Metaphases of accessions showing six signals displayed one strong and two weak \(18/25S\) rDNA signals (Fig. 1c). The same was true for \(5S\) rRNA sites. Metaphases of genotypes showing eight rDNA signals for each marker, one chromosome pair displayed a strong and one pair a weak hybridization signal (Fig. 1e). Hence accessions with \(32\) chromosomes and one pair of \(18/25S\) rDNA and \(5S\) rDNA signals are diploid \((2n = 2× = 32)\). Accessions with \(48\) chromosomes and three rDNA signals are triploid \((2n = 3× = 48)\), and accessions with \(64\) chromosomes and two pair of rDNA signals are tetraploid \((2n = 4× = 48)\). Therefore, a chromosome basic number of \(x = 16\) in the genus *Melissa* is likely. In contrast, Darlington and Wylie [4] postulated a basic chromosome number of \(x = 8\) and a somatic number of \(2n = 32\), without giving any information about investigated subspecies. Later on, Tutin et al. and Pignatti [20, 17] reported a chromosome number of \(2n = 32\) for *M. officinalis* ssp. *officinalis*, *M. officinalis* ssp. *altissima* and *M. romana* has \(2n = 64\) chromosomes [17, 20]. The reports of Heidari et al. [11] and Murin [15] of \(2n = 32\) chromosomes and Löve [14] of \(2n = 64\) chromosomes for *M. officinalis* provide no information about the analysed subspecies.

Two scenarios regarding the origin of triploid balm can be postulated: an unreduced gamete of a diploid plant formed triploid offspring after fertilization with a haploid gamete. Alternatively, a tetraploid parent hybridized with diploid parent and formed triploid offspring. The different signal intensity of either \(5S\) or \(18/25S\) rDNA sites in triploid balm could be explained by the different copy number of parental rDNA repeats.

**Phenotype characterization of triploid balm**

The plant phenotype of the four triploid accessions was characterized. Stems of the specimen BLBP 78, BLBP 88, BLBP 111 and BLBP 113 reached 120 to 140 cm (ssp. *officinalis* 50 to 80 cm, [17]) with tendency of laying down and entangling. The size of leaves \((7.6\) cm length, standard deviation \(s\) 0.59 and \(5.4\) cm width, \(s\) 0.56) was bigger than diploid ssp. *officinalis* type leaves \((6.9\) cm length, standard deviation \(s\) 0.51 and \(4.5\) cm width, \(s\) 0.42). The internodes are longer \((9.4\) cm, standard deviation \(s\) 0.99) in comparison with diploid ssp. *officinalis* \((6.5\) cm, \(s\) 0.78) accessions (Table 3). Triploid accessions had very good cold resistance and regenerated faster after winter and harvesting cuts (results not shown). The colour of leaves
Table 1 Determined ploidy level of balm accessions (*Melissa officinalis*) from Leibniz institute for plant genetics and crop plant research at Gatersleben, Germany (IPK) and bavarian state institute for agriculture at Freising, Germany (LfL) based on flow cytometry.

| Accession No. | COL | Taxonomical group of *Melissa officinalis* L<sup>1</sup> | Origin<sup>2</sup> | Ploidy level |
|---------------|-----|------------------------------------------------------|-----------------|-------------|
| BLBP 5        | LfL | *M. officinalis* L.                                   | southern France | diploid     |
| BLBP 8        | LfL | *M. officinalis* L.                                   | Spain           | diploid     |
| BLBP 19       | LfL | *M. officinalis* L.                                   | Germany         | diploid     |
| BLBP 27       | LfL | *M. officinalis* L.                                   | Germany         | diploid     |
| BLBP 33       | LfL | *M. officinalis* L.                                   | botanical garden Halle, Germany | diploid |
| BLBP 48       | LfL | *M. officinalis* L.                                   | Germany         | diploid     |
| BLBP 49       | LfL | *M. officinalis* L.                                   | Unknown         | diploid     |
| BLBP 50       | LfL | *M. officinalis* L.                                   | Unknown         | diploid     |
| BLBP 78       | LfL | *M. officinalis* L.                                   | France          | diploid     |
| BLBP 87       | LfL | *M. officinalis* L.                                   | Georgia, landrace | diploid |
| BLBP 88       | LfL | *M. officinalis* L.                                   | botanical garden Liege, France | triploid |
| BLBP 111      | LfL | *M. officinalis* L.                                   | Hungary         | triploid    |
| BLBP 113      | LfL | *M. officinalis* L.                                   | France          | triploid    |
| MELI 1        | IPK | *M. officinalis* L.                                   | Unknown         | diploid     |
| MELI 2        | IPK | *M. officinalis* L.                                   | Unknown         | diploid     |
| MELI 4        | IPK | *M. officinalis* L.                                   | East Germany    | diploid     |
| MELI 5        | IPK | *M. officinalis* L. ‘Erfurter Aufrechte’              | East Germany    | diploid     |
| MELI 6        | IPK | *M. officinalis* L.                                   | Germany         | diploid     |
| MELI 7        | IPK | *M. officinalis* L. ‘Citra’                           | Unknown         | tetraploid  |
| MELI 8        | IPK | *M. officinalis* L.                                   | Georgia         | diploid     |
| MELI 10       | IPK | *M. officinalis* L.                                   | France          | diploid     |
| MELI 11       | IPK | *M. officinalis* L. ‘ital. Melissa, Cedronella’       | Italy           | diploid     |
| MELI 12       | IPK | *M. officinalis* L.                                   | Italy           | tetraploid  |
| MELI 13       | IPK | *M. officinalis* L.                                   | Georgia         | diploid     |
| MELI 14       | IPK | *M. officinalis* L. subsp. altissima (Sibth. & Sm.) Arcang. | Italy          | tetraploid  |
| MELI 15       | IPK | *M. officinalis* L. subsp. altissima (Sibth. & Sm.) Arcang. | Italy          | tetraploid  |
| MELI 16       | IPK | *M. officinalis* L. subsp. officinalis                 | Unknown         | diploid     |
| MELI 17       | IPK | *M. officinalis* L.                                   | Greece          | tetraploid  |
| MELI 18       | IPK | *M. officinalis* L. subsp. altissima (Sibth. & Sm.) Arcang. | Unknown       | tetraploid  |
| MELI 19       | IPK | *M. officinalis* L.                                   | Italy           | tetraploid  |
| MELI 20       | IPK | *M. officinalis* L.                                   | Italy           | tetraploid  |
| MELI 21       | IPK | *M. officinalis* L. subsp. altissima (Sibth. & Sm.) Arcang. | Albania        | tetraploid  |
| MELI 22       | IPK | *M. officinalis* L.                                   | Turkey          | tetraploid  |
| MELI 23       | IPK | *M. officinalis* L. subsp. altissima (Sibth. & Sm.) Arcang. | Italy          | tetraploid  |
| MELI 24       | IPK | *M. officinalis* L. subsp. altissima (Sibth. & Sm.) Arcang. | Italy          | tetraploid  |
| MELI 25       | IPK | *M. officinalis* L. ‘Zitronenmelisse’                  | Unknown         | diploid     |
| MELI 26       | IPK | *M. officinalis* L.                                   | Armenia         | diploid     |
| MELI 27       | IPK | *M. sp.*                                              | Italy           | diploid     |
| MELI 28       | IPK | *M. officinalis* L.                                   | Italy           | tetraploid  |
| D9597         | IPK | *M. sp. ‘Zitronenmelisse’                             | Unknown         | diploid     |

<sup>1</sup>COL: collection

<sup>2</sup>taxonomical classification according to information of collection holders
was bluish to greyish green in comparison to green leaves of ssp. officinalis (Fig. 2). Young leaves of triploid accessions have indumenta similar to ssp. altissima (Fig. 2) whereas according to Tutin et al. [20], leaves of diploid ssp. officinalis are glabrescent or sparsely hairy above, glandular-puberulent and more or less sparsely hairy beneath. Adult leaves of triploid accessions are more similar to ssp. officinalis. Stems of triploid accessions are greyish- or whitish-tomentose beneath with similarity to ssp. altissima. The triploid accessions are not lemon-scented. They had a soap-like, nauseating scent. These plants had an inconspicuous formation of flowers but do not produce any seed, neither under conditions of isolation nor by open pollination, likely due to meiotic problems. These first time described triploid accessions were propagated by cuttings and are cytologically and morphologically stable for at least six years.

## Table 2 Level of ploidy, number of chromosomes and signals resulting from FISH with 18/25S rDNA and 5S rDNA probes in accessions of balm (Melissa officinalis)

| Accession | Number of chromosomes | Signals of 18/25S rDNA | Signals of 5S rDNA | Ploidy |
|-----------|-----------------------|------------------------|-------------------|--------|
| BLBP 48   | 32                    | 2                      | 2                 | diploid |
| MELI 1    | 32                    | 2                      | 2                 | diploid |
| BLBP 78   | 48                    | 3                      | 3                 | triploid |
| BLBP 113  | 48                    | 3                      | 3                 | triploid |
| MELI 14   | 64                    | 4                      | 4                 | tetraploid |
| MELI 22   | 64                    | 4                      | 4                 | tetraploid |

## Conclusions

The basic chromosome number of \( x = 16 \) is reported for the first time for the species *M. officinalis* and for family Labiatae.

This is the first characterization of triploids in *M. officinalis*. These triploid accessions are sterile but cytotically and morphologically stable. The length and width of the leaves and the length of internodes exceeded the comparable data for diploid accessions but are not significant for all characters.

For exact origin analysis of triploids as well as the characterization of allotetraploid or autotetraploid character of tetraploids analysis of meiotic chromosome pairing is necessary. Chromosome specific molecular markers would open the chance to ascertain the level of similarity of homoeologous groups of chromosomes. This is a prerequisite for better characterization of phylogenetic distance of *M. officinalis* ssp. altissima in comparison to ssp. officinalis.

## Material and methods

### Plant materials

A set of 40 accessions of *M. officinalis* have been characterized, of which 27 and 13 were provided from the Federal ex-situ Collection of Agricultural and Horticultural Plants of the Leibniz Institute for Plant Genetics and Crop Plant Research at Gatersleben, Germany (IPK) and the Bavarian State Institute for Agriculture at Freising, Germany (LfL) respectively. LfL collection contained old varieties and breeding material from middle and western Europe, the IPK collection includes mainly landraces and wild types from eastern and middle Europe (Table 1).

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**Fig. 1 a-f**: Mitotic metaphase chromosomes of balm (*Melissa officinalis*) after FISH with 18/25S rRNA and 5S rRNA-specific probes. Above (a, c, e): FISH, red 18/25S rDNA, green 5S rDNA; arrows mark weak signals, below (b, d, f): DAPI stained chromosomes; a: diploid MELI 1, \( 2n = 2x = 32 \); c: triploid BLBP 78, \( 2n = 3x = 48 \); e: tetraploid MELI 22, \( 2n = 4x = 64 \). The size bars equals 5 \( \mu \)m
All IPK accessions were grown from seeds whereas all LfL accessions were propagated by cuttings starting with a single plant. Radish (*Raphanus sativus* L.) was used as genome size marker in flow cytometry.

**Evaluation of ploidy level by flow cytometry**

Measuring of relative DNA amount of nuclei occurred by flow cytometry (Facs calibur, Becton Dickinson, BD) with a red fluorescence laser as basis for detection of ploidy level. For each probe, leaf material was chopped with razor blades in 500 μl nuclei extraction buffer (CyStain PI absolute P, Sysmex) and stained with the corresponding staining buffer, containing 5 % polyvinylpyrrolidone 25 (Serva) and 0.6 % propidium iodide (Serva). Immediately after staining, the nuclei suspension was filtered using a 5 ml polystyrene round-button tube with a cell-strainer cap (BD). For reference, radish was measured in separate sample after five samples of balm.

**Chromosome preparation**

*M. officinalis* seeds were germinated and the cell division synchronized with 1.25 mM hydroxyurea for 17 h. For vegetative multiplied accessions (LfL), root tips from potted plants were used. In contrast to Pan et al. [16], the recovery time after hydroxyurea treatment was 24 h at 6 °C. Root tips were fixed in ethanol-acetic acid (3:1, 24 h) and stored in 70 % ethanol at −20 °C. After washing with aqua dest. root tips were digested with an enzyme mixture (4 % cellulase, ‘Onozuka R-10’, Serva and 1 % pectlyase Y-23 (Seishin Pharmaceutical)) in 75 mM KCl and 7.5 mM Na-EDTA, (pH 4.0 for 36 min. at 37 °C, [12]). Softened root tips were squashed in 45 % acetic acid. After removal of the coverslip by freezing (−84 °C) the slides were air dried overnight at 24 °C and stored at −20 °C.

**Fluorescence in situ hybridization**

The 18S-5.8S-25S rDNA loci were detected with a 220 bp-long 25S repeat-specific probe labelled with biotin-16-dUTP (Boehringer Mannheim) during polymerase chain reaction (PCR) amplification of the genomic DNA of *Allium ampeloprasum* with primers designed according to the sequence published by Yokota et al. [21]. For the localization of 5S rRNA genes, a 117 bp fragment obtained after PCR amplification from the same genomic DNA with specific primers coding for this region [8] was used. The labelling of this amplified probe was performed with digoxigenin-11-dUTP (Boehringer Mannheim). The hybridization mixture contained 80 ng of each DNA probe (5S and 25S rDNA) and 10 μg of salmon-sperm DNA in 20 μl of hybridization buffer (50 % deioinized formamide, 10 % dextran sulphate, 2 x SSC) per slide [19].

The FISH procedure was performed according to Fuchs and Schubert [7] with the following modifications: prior to hybridization, slides were incubated in 50 ng/μl of DNase-free RNase in 2 x SSC for 1 h at 37 °C, washed three times in 2 x SSC for 5 min and treated with 0.5 ng/μl of proteinase K for 10 min at 37 °C, followed by three times washing in 2 x SSC for 15 min. The slides were then postfixed in 4 % paraformaldehyde for 10 min, washed three times in 2 x SSC for 15 min, dehydrated in a graded ethanol series (70, 80 and 96 %) at −20 °C, and air-dried. The hybridization mixture (probe) was denaturated (80 °C, 7 min), incubated on ice (about 5 min), dropped onto

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**Table 3** Length and width of second leaf and length of second internode from base of stem for balm (*Melissa officinalis*) of different ploidy level

| ploidy   | leaf length [cm] | leaf width [cm] | internodes [cm] | n^a |
|----------|------------------|-----------------|-----------------|-----|
|          | Min   | mean | max | s^a | Min   | mean | max | s^a | Min   | mean | max | s^a |
| diploid  | 6.2   | 6.9  | 7.5 | 0.51 | 4.0   | 4.5  | 5.1 | 0.42 | 5.8   | 6.5  | 8.3 | 0.78 |
| triploid | 6.9   | 7.6  | 8.1 | 0.59 | 4.7   | 5.4  | 6.0 | 0.56 | 8.4   | 9.4  | 10.5| 0.99 |

^a standard deviation

^ab number of accessions; For each accession at least ten plants were measured.

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**Fig. 2** leaf colour and density of pubescence in balm (*Melissa officinalis*); a: diploid, b: triploid and c: tetraploid. The size bars equals 1 cm.
slides, covered with coverslips, and sealed with rubber cement. Probes and chromosomes were denatured together on a heated desk (7 min, 80 °C). The slides were then incubated overnight at 37 °C in a humidity chamber. After hybridization and removing the coverslips, the slides were washed in 2 x SSC at 37 °C three times for 5 min each, followed by three 5 min stringent washes in 0.3 x SSC at 60 °C and then blocked for 30 min at 37 °C with a solution of 4 x SSC, 3 % BSA and 0.1 % Tween 20. The biotinylated probe was detected with 10 ng/μl of streptavidin-Cy3 (Dianova) and amplified with two steps of 10 ng/μl of biotinylated anti-streptavidin (Vector) and 10 ng/μl strepavidin-Cy3. Together with the first amplification step of the biotin labelled probe, the detection of the digoxigenin labelled probe with 9 ng/μl of anti-digoxigenin-fluorescein (Roche) was done and then amplified with 8 ng/μl anti-sheep-fluorescein (Dianova). Chromosomes were counterstained and embedded in 15 μl of DAPI-VECTASHEILD antifade solution (Vector Laboratories). Images were captured for each fluorescence dye separately with a cooled CCD camera system Axiocam (Zeiss) on a microscope Axioimager Z1 (Zeiss) with the following filter combinations: 02 (DAPI), 10 (FITC) and 20 (Cy3). Pseudocoloration and merging of images were done with software of the Isis program (Metasystems).

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
JK carried out the flow cytometry, measurement of leaves and internodes, chromosome preparation and performed the statistical analysis and drafted the manuscript. OS carried out fluorescence in situ hybridization. UK participated in the flow cytometry. FM conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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