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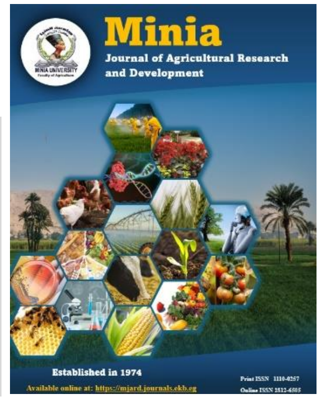


# Genetic Diversity and Essential Oil Composition of some Mentha species

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## ABSTRACT

This study investigated the physiological, biochemical, and genetic characteristics of various mint species, focusing on chlorophyll content, essential oil composition, and genetic relationships. Assessment of total chlorophyll content using SPAD revealed significant variability among species as *Mentha suaveolens* exhibiting the highest levels (36.6) however *M. piperita* had the lowest value (28.0). Essential oil analysis by GC-MS highlighted the presence of monoterpenes and sesquiterpenes in low concentrations across the species. Notably, *M. piperita* uniquely contained germacrene D, while *M. suaveolens* showed the highest amounts of β-caryophyllene. Dominant oxygenated monoterpenes were identified, with *M. spicata* rich in carvone (70.979%) and *M. longifolia* in menthol (21.851%). Genetic analysis using the ITS region yielded unique bands for all species, revealing varying degrees of genetic similarity with *M. spicata* and *M. longifolia* demonstrating the closest genetic relationship. Overall, the findings indicated a complex interplay between SAPD, essential oil composition diversity, and genetic relationships in *Mentha* species.

**Keywords:** *Mentha*, Essential oils, Chlorophyll and ITS primer

## INTRODUCTION

The genus Mentha, part of the Lamiaceae family, is a fast growing perennial plant that generally thrives in a wide range of agroclimatic conditions (Brickell and Zuk, 1997). The economic significance of *Mentha* is well established, as its essential oils, along with dried and fresh herb materials, are extensively used in cosmetics, beverages, confectionery, baking, pharmaceuticals, and pesticides. Numerous *Mentha* species are cultivated globally and are recognized as official drugs in various pharmacopoeias (Shaikh *et al.*, 2014). The morphological classification of *Mentha* genus poses challenges due to the occasional appointment of its members in other closely related genera like *Micromeria*, *Pulegium*, *Audibertia*, *Menthella*, *Thymus*, *Satureja*, *and Preslia*. However, *M. longifolia* L. (horse mint), which could be locally known as "Saudi mint," holds the distinction of being the most widely distributed *Mentha* one, thriving in Mediterranean regions,

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Europe, Australia, and North Africa. Moreover, previous phylogenetic analysis suggests that *M. longifolia* could serve as an ancestor to *M. spicata* and potentially contributes to the latter's organelle genome source (Bunsawat *et al.,* 2004). The origin of *M. spicata* L. (spearmint) traces back to Europe and the Mediterranean region, evolving into a globally cultivated species (Rita and Animesh, 2011). Based on morphological, cytological, and biochemical studies, some scientists propose that *M. spicata* arose from chromosome doubling in hybrids between the closely related species, *M. longifolia* and *M. suaveolens* (Aishima, 1936). However, other critical reviews challenge that concept (Harley and Brighton, 1977).

Leaf photosynthesis is considered a key component of canopy photosynthesis, which accounts for major variation in biomass and yield production (Peng, 2000). However, Hubbart *et al.* (2007) thought that it is necessary to recognize genetic factors that control leaf photosynthesis and to compare yield potential between related genotypes. Several putative QTLs have been detected for SPAD value or chlorophyll content (Yue *et al.*, 2006 and Kanbe *et al.*, 2008).

Ozguven *et al.* (2002) studied the variations in growth parameters and essential oil composition after a year of adaptation to a new geographical location of 10 different *Mentha* genotypes and ecotypes. Achieved results showed wide variations on herb biomass and essential oil (EOs) contents among these genotypes. The EOs of *M. spicata* ranged from 0.9 to 2.3%, exhibiting diverse chemotypes (Orav *et al.*, 2013 and Snoussi *et al.,* 2015). Principal constituents of *M. longifolia* EOs, including menthone, menthol, pulegone, menthofuran, and various terpenes, exhibit chemogeographical variations across different countries. Sharopov (2012) identified 25 chemical compounds, representing 98.59% of the total EOs components in *M. longifolia*, with trans and cis-dihydrocarvone, cis-piperitone epoxide, limonene, and 1, 8-cineole among the key compounds.

Assessing genetic diversity at the DNA level in *Mentha* species becomes imperative for developing molecular markers supporting genetic improvement programs. Studies utilizing RAPD (Khanuja *et al.*, 2000), AFLP fingerprinting (Gobert *et al.*, 2002), and ISSR (Smolik *et al.,* 2007 and Rabia *et al.*, 2015) might contribute to understanding genetic diversity within *Mentha* genus. Various DNA regions, originating from the nuclear genome (ITS) and the plastid one, such as matK, rbcL, rpoB, rpoC1, trnH-psbA, and ycf1, have been proposed as barcodes for diverse plant groups. Among these, ITS (particularly ITS2) stands out as one of the most frequently employed DNA fragments in plant molecular systematics at both the generic and species levels. The internal transcribed spacer (ITS) regions emerge as crucial components in molecular systematics within plant genera, owing to their general conservation within a species. The flanking highly conserved ribosomal genes provide an ideal foundation for universal primers in targeting and amplifying the ITS regions through PCR. Subsequently, the sequenced, analyzed, and compared ITS regions contribute to the production of evolutionary trees (Wang *et al.*, 2009).

## MATERIALS AND METHODS

### 1. The plant material

Four *Mentha* species (*M. longifolia*, *M. suaveolens, M. piperita, and M. spicata*) that shown in Fig. (1) Were adapted to the open field condition at Minia University, Nursery of Ornamental plants, (28.1244N and 30.3753E) and used for the present study.

**Fig. (1): Photos of the four studied *Menth* spp. from left to right; *M. piperita, M. longifolia, M. suaveolens,* and *M. spicata*.**

**2. Chlorophyll content**

Just before harvesting the herb, chlorophyll content was assessed using SPAD-502 portable Minolta chlorophyll meter (Spectrum Technologies, Inc., Plainfield, IL, U.S.), which has a 0.71 $cm^2$ measurement area and assesses chlorophyll based on absorbance measurements at 660 and 940 nm (Richardson *et al.*, 2002). Thirty separate measurements were made using the fifth-fully developed leaves from the top of 30 plants in each treatment.

**3. Essential oils distillation and analysis**

The herbs of these species were cut and air dried, then EOs were extracted using a clevenger apparatus for 2 hours according to European Pharmacopoeia (2010). Then the obtained EOs were dried using anhydrous sodium sulfate. The recognition of the EOs were determined by GC–MS that was carried out with Ds-Chrom 6200 Gas Chromatograph system (Donam Instrument, Korea) as described by Anwar et al. (2018). The percentage of each compound relative to total content was assessed from the area of the peak corresponding to each compound. Identification of EO components was adept based on comparison of authentic standards and by comparison of their mass spectral fragmentation patterns.

## RESULTS AND DISCUSSION

**1. Chlorophyll content**

The chlorophyll content, estimated as SPAD, exhibited significant variability ($P \leq 0.05$) among the different investigated mint species. *M. suaveolens* had the highest significant value (36.6), while *M. piperita* displayed the lowest one (28.0) (Fig. 2). This finding aligns with Khaliq *et al.* (2014) confirmation of a significant difference in chlorophyll content among *O. basilicum* ecotypes. Szulc *et al.* (2021) results declared association between genetic similarities of four maize cultivars based on SPAD.

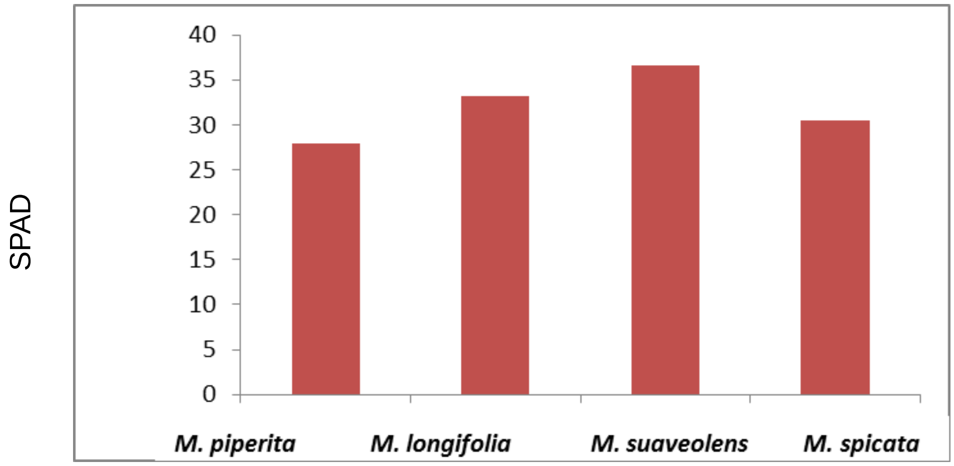


**Fig. (2): Total chlorophyll content of the four *Mentha* species**

## 2. Essential oils constitute

The chemical composition of the EOs identified by GC-MS are presented in Table (1). Generally, EOs of the investigated genotypes showed low concentrations (0.915 to 3.472%) of the monoterpenes α-pinene, β- pinene as well as sabinene. Data showed that spearmint did not contain β-pinene moreover, peppermint had not sabinene. Nevertheless, each genotype had only one sesquiterpene as a unique compound, for example, peppermint had Germacrene-D (6.040%) which was not detected in the other genotypes. However, *M. suaveolens*had the highest content of b-caryophyellene (3.356%) followed by spearmint (1.873%) and Saudi mint (0.878%).

Spearmint EO was rich in oxygenated monoterpenes carvone (70.979%) and limonene (10%) however, Saudi mint had (68.305%) and (13.505%) of these compounds, respectively. On the other had, the other two species did not contain carvone. Interstingly only speramint had 1,8 -Cineole (3.894%) similarly, peppermint was the only genotype had menthofuran (7.187%) and piperitone (3.351%) whereas cis-sabinol was found only in saudi mint at a concentration of (4.034%). So that these three compounds could be considered as a classifingy marker for these two species.

Regarding the oxygenated compounds, GC-MS showed that the most unbandent compounds in Saudi mint EOs were menthol, pulegone, and linalool (21.851, 12.407, and 11.459% respectively). Whereas peapermint EOs contaied pulgone, menthone, and menthol at 24.015, 19.753, and 16.819% as abundent compounds. These three compounds are meissing in the other two species, which means they are identical to previous species.

Usually, mint genotypes have either oxygenated monoterpene, e.g. carvone and its related compounds, or sesquterpene ones such as piperitenone, piperitone, pulegone, menthone but not both. Hefendhel and Murray (1976) suggested that oxygenetaed monoterpenes biosynthesis is under the control of a dominant gene C; however, the recessive one (cc) produces oxygenated sesquterpenes. Murray and Lincoln (1970) found that the dominant gene (I) led to the accumulation of linalool and/or linalyl acetate, but the recessive one (i) prevented the biosynthesis of the cyclic ketones compounds. Similary, Aziz and Craker (2010) concluded that menthol (34.29 %), isomenthyl acetate (30.47 %), and menthone (15.61 %) were the major components of the Egyptian pippermint EOs. Carvone and dihydrocarvone and their related compounds have been found as main components of spermint EOs, which had 2n = 48 or the triploid form 2n = 36 (Lawrence, 1978) which is probably a hybrid between *M. longifolia* (2n = 24) and *M. spicata* (2n = 48). Chauhan *et al.* (2010) and Hussain *et al.* (2010) found that carvone alone or carvone and limonene were the main constituents in a number of spearmint EOs. In addition, piperitone oxide and limonene (Koliopoulos *et al.*, 2010) were established as the main constituents.

**Table (1): EOs chemical composition of the four studied mint species**

| ***Compound percentage*** | ***M. piperita*** | ***M. longifolia*** | ***M. suaveolens*** | ***M. spicata*** |
|---|---|---|---|---|
| ***Monoterpene*** | | | | |
| α- pinene | 0.915 | 1. 335 | 1.140 | 2.358 |
| β- pinene | 2.738 | 2.355 | 1.327 | -- |
| sabinene | -- | 3.472 | 1.377 | 2.150 |
| ***Sesquiterpene*** | | | | |
| Germacrene-D | 6.040 | -- | -- | -- |
| b- caryophyllene, | -- | 0.878 | 3.356 | 1.873 |
| ***Oxygenated*** | | | | |
| 1,8- Cineole | -- | -- | -- | 3.894 |
| limonene | 5.109 | 6.129 | 13.505 | 10.00 |
| linalool | 4. 738 | 11.459 | -- | -- |
| menthone | 19.753 | 31.36 | -- | -- |
| cis- sabinol | -- | 4.034 | -- | -- |
| menthofuran, | 7.187 | -- | -- | -- |
| menthol | 16.819 | 21.851 | -- | -- |
| iso-menthol | 6.432 | 0.377 | -- | -- |
| pulegone | 24.015 | 12.407 | -- | -- |
| α- terpnol | -- | -- | 1.979 | 3.407 |
| piperitone | 3.351 | -- | -- | -- |
| carvone | -- | -- | 68.305 | 70.979 |
| **Total compounds (%)** | 92.359 | 94.322 | 90.989 | 94.661 |

## 3. Molecular analysis

### 3. 1. PCR Amplification of ITS Region

Tao *et al.* (2016) reported that the ribosomal internal transcribed spacer (ITS) of nuclear ribosomal DNA is a frequently used DNA marker and has been recommended as a primary plant DNA barcode in plant phylogenetics. Accordingly, the conserved sequence of the ITS region 1 of rDNA was amplified from the four mint species using two specific ITS primers (ITS-p5 and ITS-u2). As anticipated, all examined species produced a single, unique, and monomorphic band of approximately 800 bp (Fig. 3).

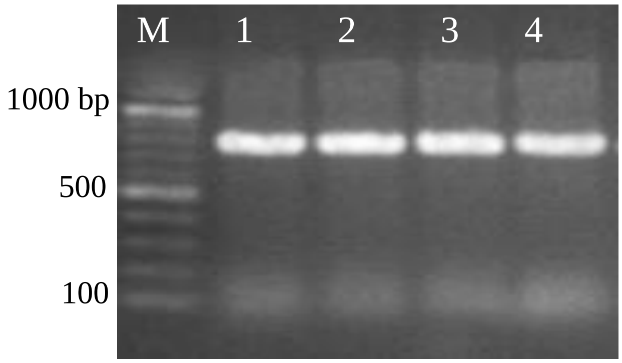


**Fig. (3): Electrophoretic pattern of PCR amplification product using ITS primers. M: ladder, 1- 4: *M. piperita, M. suaveolens, M. spicata* and *M. longifolia***

### 3. 2. DNA Sequencing

The amplified DNA fragments of the four species were sequenced using ITS-p5 and ITS- u4 primers. The sequence identity matrix of the four mint species (Table 2) shows varying degrees of similarity. *M. piperita* has sequence identities of 0.351 with *M. suaveolens*, 0.314 with *M. spicata* and 0.278 with *M. longifolia*. As shown in Table (2) *M. suaveolens* exhibits sequence identities of 0.369 with *M. spicata* and 0.358 with *M. longifolia*. *M. spicata* shows the highest sequence identity of 0.611 with *M. longifolia*. These results indicate that *M. spicata* and *M. longifolia* share the most similarity, while each of *M. piperita* and *M. longifolia* exhibits the least similarity among the species compared (Fig. 4). The results of the study show a clear relationship between the physiological, biochemical, and genetic characteristics of the Mentha species. The significant variability in chlorophyll content among the species, as revealed by SPAD analysis, may be linked to their adaptation to different environmental conditions, which is also reflected in their distinct biochemical properties. For instance, while *M. suaveolens* had the highest chlorophyll content, *M. piperita*, with the lowest chlorophyll content, was found to be rich in sesquiterpene hydrocarbons and uniquely contained germacrene D. This chemical diversity among species is further emphasized by the classification of species based on oxygenated monoterpene composition, where *M. longifolia* and *M. piperita* were high in menthone, menthol, and pulegone, while *M. suaveolens* and *M. spicata* were rich in carvone. Despite these biochemical differences, the genetic analysis showed that certain species, such as *M. spicata* and *M. longifolia*, are closely related, while others, like *M. piperita* and *M. longifolia*, exhibit more genetic divergence, suggesting that the observed chemical diversity is not necessarily tied to their genetic similarity.

The genetic analysis using the ITS region further clarified relationships among the species. The sequence identity matrix showed varying degrees of genetic similarity with *M. spicata* and *M. longifolia* sharing the highest similarity (0.611), potentially indicating close evolutionary ties. This aligns with previous phylogenetic findings by Gobert *et al*. (2006), which grouped *M. spicata* and *M. longifolia* within the same clade, though prior analyses were complicated by molecular noise and overlapping clades. The present study's clearer amplification results, producing a distinct monomorphic band, reduce ambiguity in interpreting these relationships. In contrast, *M. piperita* and *M. longifolia* displayed the least genetic similarity (0.278), despite their biochemical similarity, particularly in EO components such as menthol and menthone, suggesting that chemical profiles in *Mentha* do not always correlate with genetic proximity. This observation reinforces the hypothesis of convergent evolution, wherein certain adaptive biochemical traits may evolve independently across genetically distinct lineages. Overall, the study emphasizes the complexity of *Mentha* diversity, demonstrating that while genetic similarity provides clues to evolutionary lineage, it does not fully account for the biochemical traits that characterize each species. The findings highlight the unique value of combining genetic markers like ITS with biochemical analyses such as GC-MS to achieve a comprehensive understanding of diversity in *Mentha*.

**Table (2): Sequence identity matrix for the four *Mentha* species**

| Sequence Identity Matrix | *M. piperita* | *M. suaveolens* | *M. spicata* | *M. longifolia* |
|---|---|---|---|---|
| *M. piperita* | - | | | |
| *M. suaveolens* | 0.351 | - | | |
| *M. spicata* | 0.314 | 0.369 | - | |
| *M. longifolia* | 0.278 | 0.358 | 0.611 | - |

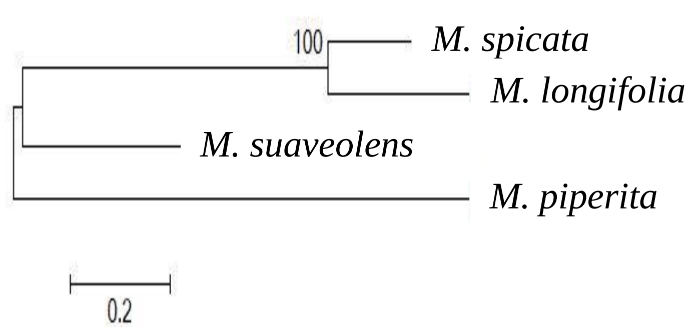


**Fig. (4): Dendrogram of the genetic distance among the four Mentha species**

## CONCLUSION

The study shows that *Mentha* species exhibit considerable diversity in chlorophyll content, EOs composition, and genetic identity. The clear differences in chlorophyll content reflect varying physiological responses, while the distinct chemical profiles of EOs highlight the potential for species-specific applications. The genetic analysis provides further insight into the evolutionary relationships among the species, suggesting that *M. spicata* and *M. longifolia* are more closely related than other species in the study. These findings contribute valuable information to the understanding of the diversity within the *Mentha* genus and its potential uses in various industries, such as pharmaceuticals and aromatherapy.

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**الملخص العربى**

# التنوع الوراثي وتركيب الزيوت العطرية لبعض أنواع النعناع

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ركزت هذه الدراسة على دراسة الخصائص الفسيولوجية والكيميائية الحيوية والجينية لاربعة أنواع مختلفة من النعناع، حيث تم تقييم محتوى الكلوروفيل، وتكوين الزيوت الأساسية، والعلاقات الجينية. أظهرت تقييمات إجمالي محتوى الكلوروفيل باستخدام جهاز SPAD تفاوتًا كبيرًا بين الأنواع؛ حيث أظهر *Mentha suaveolens* أعلى المستويات (٣٦.٦)، في حين سجل *M. piperita* أدنى قيمة (٢٨.٠). وكشف تحليل الزيوت الأساسية باستخدام جهاز GC-MS عن وجود الـــ monoterpenes والـــ sesquiterpenes بتركيزات منخفضة في الأنواع المدروسة. وتميز *M. piperita* بوجود مركب germacrene D بشكل فريد، بينما أظهر *M. suaveolens* أعلى التركيزات من مركب β-caryophyllene . وتم تحديد الـــ Dominant oxygenated monoterpenes، حيث احتوى *M. spicata* على نسبة عالية من الـــ carvone(٧٠.٩٧٩%)، واحتوى *M. longifolia* على نسبة عالية من الـــ menthol (٢١.٨٥١%). وكشفت التحليلات الجينية باستخدام منطقة ITS عن وجود حزمة فريدة بنفس الحجم لكل الأنواع ، إلا أن تحليل التتابع النيوكلوتيدي أظهر تبايناً في درجات التشابه الجيني بين الأنواع المدروسة، حيث كانت العلاقة الجينية الأقرب بين *M. spicata* و *M. longifolia*. بشكل عام، أشارت النتائج إلى تداخل معقد بين محتوى الكلوروفيل، وتنوع مكونات الزيوت الأساسية، والعلاقات الجينية في أنواع الـــ *Mentha*.