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TAXONOMICAL STUDIES ON FOUR-*MENTHA* SPECIES GROWN IN EGYPT THROUGH MORPHO-ANATOMICAL CHARACTERS AND SCOT GENETIC MARKERS

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Abstract

Four species grown in Egypt and belong to genus *Mentha* (family Lamiaceae) were studied. These species represented by one wild specie with two subspecies; *M. longifolia* subsp. *typhoides* and subsp. *schimperi* and three cultivated ones; *M. spicata, M. sativa* and *M. peperita*. The aim of this study was to determine the taxonomic relationships among these species through leaf morphological characters. Stomata and trichome patterns were examined under Scanning Electron Microscope (SEM), in addition to the observation of the anatomical characteristics of leaf, petiole and stem by using the optical microscope. The analysis of volatile oil by GC-MS was carried out to identify the differences in the chemical constituent level among the studied species. At the molecular level, ten SCOT markers were utilized to examine the genetic diversity and phylogeny among the four *Mentha* species. 190 amplified fragments were scored with sizes ranging from 250 to 3000 bp. The similarity percentages ranged 25.7% to 45.5 % between the four species. These results revealed significant differences among the studied *Mentha* species. They were clustered into three groups based on the UPGMA analysis. These results prove the accuracy of SCOT markers in estimating the genetic diversity, identification and phylogeny of *Mentha* species.

Key words : Anatomy, Lamiaceae*, Mentha,* SCOT, SEM, volatile oil.

Introduction

Mints are perennial, rarely annual, aromatic plants which belong to more than 18 species and 11 hybrids in the genus *Mentha* that is distributed across Africa, Asia, and North America. The genus *Mentha* is a member of family Lamiaceae, order Lamiales (Tucker & Naczi, 2007). The Lamiaceae family has been divided into eight subfamilies (Harley *et al.,* 2004), and *Mentha* within the subfamily Nepetoidae and tribe Menthaea which includes important medicinal genera (Tucker & Naczi, 2007; Fialová *et al.,* 2015). Some *Mentha* species, such as *Mentha spicata* L. and *Mentha longifolia* L., can naturally cross pollinated producing different hybrids (Tucker & Naczi, 2007). Mints are mainly cultivated for essential oils which have a high capacity of antioxidant, anti-inflammatory and anticancer agents (Kanatt *et al.,* 2007; Juergens *et al.,* 2003). Many investigators reported that the main essential oil chemical constituents are carvone, pulegone, menthone, limonine and cis-piperitone oxide in *M. longifolia*, and *M. spicata* (Younis and Beshir 2004; Gitibarzin *et al.,* 2014; Bardaweel *et al.,* 2018) whereas *M. pipertita* is rich in menthol and menthofuran compared to other components (Rohloff 1999; Gavahian *et al.,* 2014). The taxonomy of the genus *Mentha* has been in constant change for more than 100 years (Tucker *et al.,* 2007). Although the genus *Mentha* is now redefined to include more than 30 species based on a molecular phylogenetic analyses and chromosome numbers as well as major components of essential oil (Bunsawat *et al.,* 2004; Tucker & Naczi, 2007), the taxonomy of this genus is still unclear.

Molecular phylogenetic studies were used to indicate the relative relationships among plant species (Sramko *et al.,* 2016), along with the traditional morphological methods. For the purpose of phylogenetic studies, molecular markers are important tools to provide more accurate assessments of genetic variation and genotype

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identification through DNA fingerprinting since they are independent of environmental conditions and the developmental stage of the organism (Khanuja *et al.,* 2000; Semagn *et al.,* 2006; Patwardhan *et al.*, 2014; Nidaf 2017). Many investigators used different popular DNA marker methods in genetic diversity analysis, most notably are random amplified polymorphic DNA (RAPD) (Williams *et al.,* 1990), amplified fragment length polymorphism (AFLP) (Vos *et al.*, 1995), and inter simple sequence repeat (ISSR) (Blair *et al.,* 1999). There are several other applications of molecular markers in plants such as analysis of genetic diversity within crop germplasm (Collard *et al.,* 2005), Quantitative Trait Loci (QTL) mapping for agronomical important traits (Gupta *et al.,* 1999; Gostimsky *et al.,* 2005), estimation of plant evolution (Ahuja *et al.,* 2010)**,** genome editing (Nadeem *et al.,* 2018), and examining biotic and abiotic stresses (Bhau *et al.,* 2016).

In *Mentha*, DNA marker methods have recently been used to answer the complex questions of the genetic and taxonomic relationships among genotypes (Ibrahim, 2017) as well as the natural hybridization (Dhawan *et al.*, 2018). Also, the genetic diversity in *Mentha cervina* was analyzed using ISSRs markers and proved to be useful in genetic diversity and phylogeny studies (Rodrigues *et al.,* 2013). Moreover, *M. longifolia* has been used as a model species for *Mentha* genetic research especially as its genome has been reported as beneficial resources for metabolic engineering and molecular breeding (Vining *et al.,* 2016). However, the molecular markers are not fully utilized to explore the genetic diversity and phylogeny in *Mentha*.

Recently, Start Codon Targeted (SCOT) markers system has become the best choice for molecular markers used in genetic diversity studies (Collard and Mackill, 2009). It was developed based on the short conserved sequence around the Translational Start Site (TSS) which is conserved in all plant genes (Bhattacharyya *et al.*, 2013). It is independent of environmental conditions and the developmental stage of the organism (Nidaf *et al.*, 2017). It is simple because its PCR products were resolved by performing agarose gel electrophoresis. Also, it is highly reproducible due to the use of longer primers which were designed following the short conserved region flanking the initial codon (ATG) compared to arbitrary markers like RAPD (Collard and Mackill, 2009).

Thus, in this study, taxonomic relationship among four species of *Mentha* grown in Egypt, namely; *M. longifolia* subsp. *typhoides* and subsp. *schimperi; M. spicata*; *M. sativa* and *M. piperita* were evaluated using the morphological leaf characteristics; shape, margin, apex and base. Furthermore, anatomy of lamina, leaf petiole and stem were carried out, along with scanning electron microscopy analyses to measure the morphological characters of upper and lower leaf surfaces. Also, SCOT markers were performed to assess DNA finger prints of the four studied species of *Mentha* grown in Egypt in order to validate the genetic diversity and evaluate the phylogeny among them. This could be useful in taxa identification and breeding programs. In addition, the obtained volatile oil was analyzed using GC-MS technique to identify the chemical components in our samples.

Materials and Methods

Plant material and leaf morphological studies

Mentha plants are represented in this study by one wild species with two subspecies; *M. longifolia* subsp. *typhoides* and subsp. *schimperi* and three cultivated species; *M. spicata, M. sativa* and *M. peperita*. The plants were grown at the Research Station, Faculty of Pharmacy, Cairo University, Giza, Egypt during two successive seasons, 2017 and 2018.

Morphological characteristic of leaf

Leaf samples were collected to measure the following morphological characters; shape, margin, apex, base, and color according to Tsukaya (2006).

Scanning electron microscopy analyses

For scanning the leaf surface patterns, ten mature fresh specimens were chosen for scanning electron microscopy analyses (SEM). The procedure was performed as described by Claugher in 1990. The chosen specimens were fixed in 5% glutaraldehyde in 0.1 M phosphate buffer (PB) pH 7 for 2 hours followed by washing in PB for 5 times, 10 minutes each. The samples were then dehydrated in different concentrations of ethanol (30, 50, and 70%) with suspending the solution for one hour each. Finally, the specimens were transferred to a critical point drier, then, they were coated with gold/ palladium (Au/Pd) using a JFC–1100E Ion sputter (JEOL) in a sputter coater instrument. Photographs of morphological structure of the upper and lower lamina surface were captured with different magnifications by means of Scanning Electron Microscope (SEM), model; a JEOL-JSM-T-100, Central Laboratory, National Information and Documentation Center (NIDOC), Dokki, Giza, Egypt**.** Terminology of leaf surface sculptures followed by Murley (1951) and Claugher (1990). Investigation and identification criteria of the studied taxa were based on the authentic flora and taxonomic references, among of them; Hedge (1992) and Harley *et al.,* (2004).

Anatomical study

Lamina, leaf petiole and stem specimens (8 weeks old) were taken from the 5th internodes. Specimens were killed and fixed for at least 48 hrs. in F.A.A. (10 ml formalin, 5 ml glacial acetic acid and 85 ml ethyl alcohol 70%). Dehydration the specimens was performed by soaking in a normal butyl alcohol series after washing with 50 % ethyl alcohol. After that, the specimens were embedded in paraffin wax at melting point (56°C). Paraffin wax sections were taken with a thickness of 20 µ. The sections were double stained with crystal violet-erythrosin, cleared in xylene and mounted in Canada balsam (Nassar and El-Sahhar, 1998) and examined under optical microscope.

Extraction of essential oils

Leaf samples of the four selected *Mentha* species were collected at flower stage to extract the essential oil. The samples were air-dried and then, subjected to hydro distillation using a Clevenger apparatus for 3hrs for the isolation of essential oils according to the protocol described by Baser (1999). The yield of the extracted essential oil was recorded, and two replicate extractions were performed. The extracted essential oils were stored in the freezer at -4°C until GC-MS analysis after dehydration in anhydrous sodium sulfate.

GC-MS analysis

The obtained essential oil samples were dried over anhydrous sodium sulfate and were subjected to GC-MS analysis a TRACE GC Ultra Gas Chromatographs (THERMO Scientific Corp., USA), coupled with a THERMO mass spectrometer detector (ISQ Single Quadrupole Mass Spectrometer) at the Laboratory of Medicinal and Aromatic plants, National Research Center as described by Adams 1995 to determine the main chemical constituents. The system was equipped with a TG-WAX MS column (30 m × 0.25 mm i.d., 0.25 μm film thickness). Helium was used as carrier gas at a flow rate of 1.0 mL/min. The temperature was 40°C for 1 min rising at 4°C/min to 150°C and then, holding for 6 min. After that, the temperature was raised at 4°C/min to 210°C and held for 1min. Diluted samples (1:10 hexane, v/v) of 0.2 µL of the mixtures were injected. Mass spectra were obtained by electron ionization (EI) at 70 eV, using a spectral range of m/z 40-450. The components of the oils were identified by comparison of KI, Kovats indices in reference to n-alkanes (C9-C22) and retention indicates with Wiley library.

Molecular analysis

Genomic DNA extraction

Total genomic DNA was extracted from young leaves of the different *Mentha* species according to the method described by Nidaf khan *et al.,* (2017) for *Mentha* species. High purity and quantity of DNA were provided by this method, as determined by gel electrophoresis and Gel Doc™ XR+ system with Image Lab™ software Bio-Rad®, before the DNA samples were subjected to PCR amplification using the SCOT primers.

Primer Design and SCOT-PCR amplification

SCOT primers were designed based on a conserved sequence surrounding the ATG initial codon derived from previous studies by (Joshi *et al.,* 1997; and Sawant *et al.,* 1999), Table 1. All primers were 18-mer and ranged in GC content between 50% and 72%. There were no degeneracies. A total of ten SCOT primers were developed by Biotic Serve Company. All the PCR reactions were carried out in a total volume of 50 µl containing 30 ng of template DNA, 0.5 U of MyTaq Red DNA polymerase, 1xTaq Red reaction buffer and 10 pmol of primer. The reaction program was set at 94°C for 4 min, followed by 43 cycles of 94°C for 1 min, 50-55°C for 1 min according to each primer and 72°C for 2 min with final extension at 72°C for 5 min. All PCR amplification DNA patterns were analyzed on 1.7% a garose gel in 1x TAE buffer stained with ethidium bromide and visualized under UV light and photographed using a Gel Doc™ XR+ system with Image Lab™ software Bio-Rad®.

Data analysis

PCR-amplified fragments obtained by SCOT primers were scored as absent (0) or present (1). Only clear bands were scored. Pair-wise similarity matrices were generated by Jaccard's coefficient of similarity (Rohlf, 1998). A dendrogram was constructed based on the UPGMA (Unweighted Pair Group Method of Arithmetic average) algorithm to illustrate the genetic relationships between accessions and cluster analysis according to (Sneath and Sokal 1973).

Results and Discussion

Leaf morphological characters

Data presented in Table 1 and Fig. 1 indicated that all studied species have simple and opposite leaves. *M. spicata*, *M. piperita* and *M. sativa* have petiole, while *M. Longfolia* subsp. *schimperi* and subsp. *typhoides* were sessile. Leaf shape was varied among the studied *Mentha* species which it was lanceolate in *M. spicata*, ovate to lanceolate in *M. sativa* and *M. longfolia* subsp. *typhoides*, narrowly lanceolate in *M. longfolia* subsp. *schimperi*, and ovate in *M. piperita* (Table 1 and Fig. 1). Also, leaf base was varied; truncate in *M. spicata*,

Table 1: Diagnostic characters of the studied *Mentha* species.

Characters	*M. longifolia* subsp. *typhoides*	*M. longifolia* subsp. *schimperi*	*M. sativa*	*M. spicata.*	*M. piperita.*
Leaf	Ovate -	Narrowly	Ovate -	Lanceolate	Ovate
Shape	lanceolate	Lanceolate	lanceolate	Serrate	Serrate
Margin	Serrate	Serrate to entire	Serrate		
Apex	Acute	Acute	Acute	Acute	Acute
Base	Clasping	Clasping	Not clasping	Truncate	Cordate
Color	Gray	Green	Green	Green	Green
Petiole	Sessile	Sessile	Petiolate	Petiolate	Petiolate
Upper epidermis:					
Stomatal type	Diacytic	Anomocytic	Anomocytic	Anomocytic	Diacytic
Stomatal level	Raised	Superficial	Superficial & Semi raised	Superficial	Superficial & Semi raised
Sculpture pattern	Reticulate	Rugose	Rugose &Reticulate	Pusticulate	Pusticulate
Trichomes type	Glandular and non-glandular	Glandular and non-glandular	Glandular	Glandular	Glandular
Lower epidermis:					
Stomatal type	Anomocytic	Anomocytic	Anomocytic	Diacytic & Anomocytic	Diacytic & Anomocytic
Stomatal level	Raised	Semi raised	Superficial	Semi raised	Superficial
Sculpture pattern	Reticulate	Rugose	Ruminate	Reticulate	Reticulate & Rugose
Trichomes type	Glandular and non-glandular	Glandular and non-glandular	Glandular	Glandular	Glandular

Fig.1: External morphology of *Mentha* species leaves. Key: 1- *M. longifolia* subsp. *typhoides* 2- *M. longifolia* subsp. *schimperi* 3- *M. sativa 4- M. spicata 5- M. piperita.*

cordate in *M. piperita*, not clasping in *M. sativa*, and hardly clasping in *M. longfolia* subsp. *schimperi* and subsp. *typhoides*. Meanwhile, all *Mentha* species have serrate leaf margin, and acute leaf apex. Leaf color is green in all *Mentha* species except in *M. longfolia* subsp. *typhoides* (Table 1 and Fig. 1). Quite similar results were recorded by Abd El Maksoud and Azer (2014) on *M. longifolia* subsp. *typhoides* and subsp. *schimperi*, and *M. sativa*. Moreover, Kieltyka-Dadasiewicz *et al.,* (2017) studied the same leaf morphological characters of *M. piperita* which was in agreement with the present-results.

Scanning electron microscopic investigations

Upper lamina surface

M. spicata and *M. longfolia* subsp. *schimperi* have anomocytic type of stoma with superficial level, while *M. sativa* has anomocytic type with superficial and semiraised levels. On the other hand, *M. piperita* has diacytic type with superficial and semiraised stoma levels, whereas *M. longfolia* subsp. *typhoides* exhibited diacytic ones with raised level (Table 1 and Fig.2). Pusticulate sculpture pattern was detected in both *M. spicata* and *M. piperita*, whereas *M. sativa* has rugose and reticulate patterns. *M. longfolia* subsp. *schimperi*

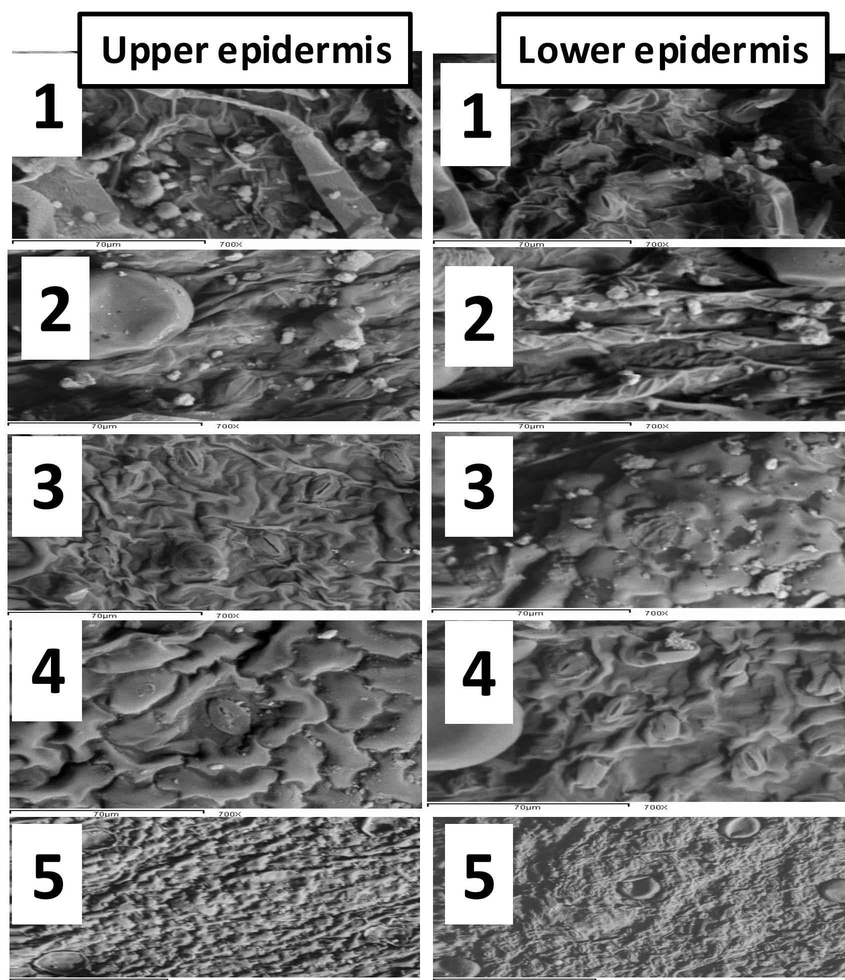

Fig. 2: Scanning electron micrographs of upper and lower epidermis in the leaf of *Mentha* species. Key: 1- *M. longifolia* subsp. *typhoides,* 2- *M. longifolia* subsp. *schimperi* 3- *M. sativa,* 4- *M. spicata* 5- *M. piperita.*

and subsp. *typhoides* have rugose and reticulate sculpture patterns, respectively. In addition, *M. longfolia* subsp. *schimperi* and subsp. *typhoides* exhibited glandular and non-glandular trichomes with globulate and smooth orientations, respectively, while *M. spicata*, *M. piperita* and *M. sativa* have only glandular trichomes with smooth orientation (Fig. 2).

Lower lamina surface

Mentha spicata has diacytic and anomocytic stoma types with semi raised level, whereas *M. piperita* exhibited diacytic and anomocytic ones with superficial stomata level. *M. sativa* has anomocytic type with superficial level (Table 1 and Fig. 2). Furthermore, the stomatal type in *M. longfolia* subsp. *schimperi* was anomocytic with semiraised level. Also, anomocytic type of stomata was recorded in *M. longfolia* subsp. *typhoides* but with raised level. Reticulate sculpture pattern was observed in *M. spicata* and *M. longfolia* subsp. *typhoides* while rugose sculpture pattern was recorded in *M. longfolia* subsp. *schimperi*. Both reticulate and rugose sculpture patterns were detected in *M. piperita*. In *M. sativa*, it was notably observed that sculpture pattern was distinct from the other species which exhibited ruminate sculpture pattern. Like in upper epidermis, trichomes were glandular with smooth orientation recorded in *M. spicata*, *M. piperita* and *M. sativa* and non-glandular lower epidermis globulate and smooth orientation exhibited in *M. longfolia* subsp. s*chimperi* and subsp. *typhoides,* respectively.

Diacytic and anomocytic stomata were reported by Abd El Maksoud and Azer (2014) in *M. longifolia* subsp. *typhoides*, *M. longifolia* subsp. *schimperi*, and *M. sativa* while Kieltyka- Dadasiewicz (2017) in *M. piperita* being in agreement with the presented findings. It has been indicated that stomata are present on both lamina surfaces in Lamiacea, except in *Clinopodium vulgare* and *Stachys schtschegleevii* (Metcalf and Chalk 1979; Zarinkamar 2007). Stomatal types are varied depending on the species in *Mentha* (Xie *et al.,* 2013). Although the morphological measurements like width and length of leaf have been indicated before (Kieltyka- Dadasiewicz 2017), the characterization of the stomata morphological features, like stomatal types, level, and patterns, are not present in many reports. Also, trichome morphology has been investigated in many Lamiacea species including the genus *Mentha* (Colson *et al.,* 1993; Werker *et al.,* 1993). Comparison among *Mentha* species have been studied by characterizing the glandular and non-glandular trichomes where in both lamina surfaces (Moon *et al.,* 2009) due to their importance in essential oil secretion (Sharma *et al.,* 2003; Rehman *et al.,* 2016). Moreover, it has been showed that the trichome morphology in *Mentha* species is similar to that found in various species belonging to Lamiacea family (Turner *et al.,* 2000). Previously, the trichome morphology has been studied to identify its correlation with the essential oil components in *M. piperita* (Maffei *et al.,* 1986). Later, the evolution of scanning electron microscopy analyses encouraged the scientists to investigate the structural features of glandular and non-glandular trichomes (Turner *et al.,* 2000; Choi and Kim 2013). There are three types of glandular trichomes which are capitate, piltate, and sub sessile glandular trichome.

Non-glandular trichomes have also three types; simple unicellular, uniseriate, and branched trichomes (Moon *et al.,* 2009). It has been shown that glandular trichomes, smooth ornamentation and non-glandular on upper and lower epidermis are detected in *M. longifolia* subsp. *typhoides*, *M. longifolia* subsp. *schimperi*, and *M. sativa* (Abd El Maksoud and Safwat A. Azer (2014). In *M. spicata*, capitate glandular trichomes were observed on a daxial surface whereas single non-glandular trichomes with peltate trichomes were detected in *M. piperita*.

Generally, although the epidermal morphology has been characterized, little reports have been studied to evaluate the diagnostic characters in the comparative study among *Mentha* species. In this work, upper and lower surfaces showed different and similar morphological characteristics among the four selected mint species indicating their taxonomical relationship.

The anatomical study

a) Lamina

The epidermal cell was composed in a single layer; the upper epidermal cells are elongated and bigger than the lower ones. Collenchymatous cells occurred below the upper and lower epidermis in the midrib region in all studied species. Glandular and non-glandular trichomes are present on both leaf surfaces. Mesophyll tissues differentiated into palisade and spongy tissues. The palisade was found below the upper epidermis in one layer of all studied species except *M. longifolia* subsp. *schimperi* and *M. piperita,* where the palisade consists of two layers and the spongy tissue has loosely arranged cells. Palisade tissue characterized by compact arrangement with relatively narrow intercellular spaces in *M. longifolia* subsp. *typhoides*, *M. longifolia* subsp. *schimperi* and *M. spicata*. While in *M.sativa* and *M. spicata*, the palisade tissue has loose cell with wide intercellular spaces. Multi layers of spongy parenchyma below palisade tissue are irregularly shaped with relatively wide intercellular spaces. The increment in lamina thickness is due to increase in spongy tissue more than in palisade tissue. Midrib vascular bundle is solitary and arc shaped in all species under studied except in *M. sativa* and *M. spicata* which was characterized by T-shaped. The vascular bundles are collateral with xylem oriented towards the upper side and the phloem towards the lower one. The increase in midrib thickness is related to increment in vascular bundle thickness; xylem more than phloem (Table 2 and Fig. 3). Rita and Animesh (2011)

Table 2: Anatomical measurements (µm) and counts of different tissues of leaf lamina and leaf petiole of the studied species of *Mentha* (average of 5 samples, 8 weeks old).

Characters	*M. longifolia* subsp. *typhoides*	*M. longifolia* subsp. *schimperi*	*M. sativa*	*M. spicata*	*M. piperita*
a-Lamina					
Upper epidermis thick.	30	35	55	55	65
Lower epidermis thick.	25	27	50	45	45
Upper collenchymasthick.	90	87	90	133	75
Lower collenchymas thick.	35	45	85	77	60
Lamina thick.	193	300	343	360	722
Palisade tissues thick.	98	100	150	100	330
Spongy tissues thick.	115	155	166	225	350
Main vein thick.	770	795	755	925	915
Vascular bundle dimension					
Length	230	225	240	262	255
width	860	745	450	620	345
Xylem thick.	120	130	170	145	147
Phloem thick.	90	65	65	115	77
b-Petiole					
Epidermis thick.	....	....	30	45	40
Cortex thick.	....	....	590	1040	630
Vascular bundle number	....	....	1	5	5
Xylem thick.	....	....	135	195	110
Phloem thick.	....	....	75	140	80
Cross section diameter	....	....	1385	1985	1645

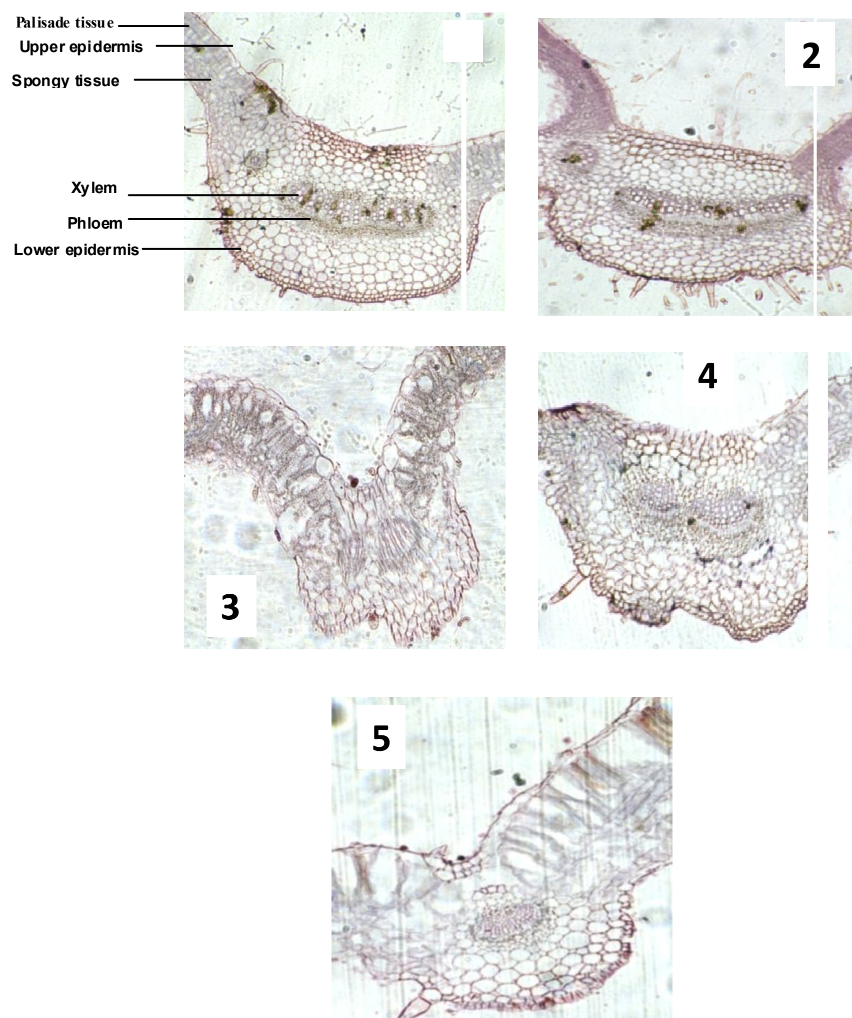

Fig. 3: Transverse section on the middle portion of leaves in *Mentha* species. Key: 1- *M. longifolia* subsp. *typhoides* 2- *M. longifolia* subsp. *schimperi* 3- *M. sativa* 4- *M. spicata* 5- *M. piperita.*

studied leaf structure of *Mentha piperita* and found that upper epidermis composed of large, clear epidermal cells with sinuous, vertical walls and possessing few or no stomata, few glandular trichomes present; palisade parenchyma, comprising a layer of columnar cells rich in chloroplasts; spongy parenchyma, of 4-6 layers of irregularly shaped chloroplastid-containing cells and intercellular air spaces. Lower epidermis of small epidermal cells with sinuous, vertical walls and numerous diacytic stomata; in the region of veins and midrib, exhibits non-glandular and glandular trichomes as outgrowths; non-glandular trichomes uniseriate, papillose, 1-8 celled; glandular trichomes have 1-2 celled stalk and 1-8 celled glandular head containing the essential oil.

b) The leaf petiole

The leaves of *M. longifolia* subsp. *typhoides* and subsp. *schimperi* are sessile, meanwhile, *M. sativa*, *M. spicata* and *M.piperita* are petiolate. The outline shape of petiole in cross section also vary; crescent in *M. sativa* and *M. piperita,* triangular in *M. spicata*. Epidermal cells are single layered, small and rectangular cells. Epidermal cells are covered with non-glandular trichomes in *M. spicata* and *M. piperita*, followed by multi layered parenchymatous cortex. The petiole vascular bundle consists of one large median arc shaped and four small lateral cortical ones except in *M.sativa,* which have one central large vascular bundle. Xylem vessels are present towards the upper side and phloem towards the lower one. The increasing petiole leaf diameter is related to increasing most tissues mainly cortex and vascular bundle (Table 2 and Fig. 4).

c) The stem

The transverse section of the stem was quadrangular in outline in all studied species. Epidermal cells were small with thick cuticle; 17 μ in *M. piperita* and 11 μ in *M. longifolia* subsp. *schimperi*. Moreover, glandular and non-glandular trichomes were observed in epidermal layer (Table 3 and Fig. 5). Cortex consist of some parenchyma layers; the highest number (10 rows) detected in *M. piperita,* while in *M. longifolia* subsp. *schimperi* exhibited 5 rows, followed by 4 main groups of cortex collenchymatous tissue opposite

Fig. 4: Transverse section on the leaf petiole of *Mentha* species. Key: 1- *M. sativa* 2- *M. spicata* 3- *M. piperita.*

Table 3: Anatomical measurements (μ) and counts of different tissues of median internode of the main stem of the studied species of *Mentha* (average of 5 samples, 8 weeks old).

Characters	*M. longifolias* subsp. *s typhoide*	*M. longifolia* subsp. *schimperi*	*M. sativa*	*M. spicata*	*M. piperita*
Epidermis thick.	12	11	15	15	17
Number of cortical parenchyma rows	6	5	7	7	10
Collenchymas thick.	230	330	190	260	260
Average cortex thick.	140	125	170	190	340
Sclernchyma thick.	55	70	35	75	45
Xylem tissue thick.	160	240	105	140	100
Phloem tissue thick.	70	50	30	35	40
Average stem diameter	2330	2480	1410	3050	2615
Pith diameter	1860	1810	1165	2420	1525
Vascular bundle thick.					
Length	830	730	530	950	705
Width					

to stem corners. Cortical collenchyma presented in all species with different thickness; *M. longifolia* subsp. *schimperi* was the highest (330μ) while the lowest (70μ) was in *M. longifolia* subsp. *schimperi*, recorded the thinnest all cortex (125 μ), whereas *M. piperita* exhibited the highest values (350 and 340 μ), respectively. The cortical fibrous is formed continually ring in all studied species and the thickness was about 35 μ in *M. sativa*. According to the xylem and phloem thickness, *M. sativa* showed the lowest; 105 and 30μ, respectively. Diameter of stem and pith were high in *M. spicata*, 3050 and 2420μ, respectively. Likewise, the highest vascular bundle width of 830μ was recorded in *M. longifolia* subsp. *typhoides* and the length of 300 μrecorded in *M. longifolia* subsp.

Table 4: Volatile oil components (%) of the *Mentha* species.

Components (%)	***Mentha* species**				
	M. longifolia* subsp. *Typhoides	***M. longifolia subsp. schimperi***	***M. sativa***	***M. spicata***	***M. piperita***
Alpha- pinene	1.96	1.46	1.77	1.64	1.05
Sabinene	2.00	1.68	1.47	1.04	0.87
Beta-pinene	2.21	2.40	2.00	1.53	1.51
Beta-myrcene	1.70	0.66	1.67	1.44	0.47
Octanol	0.72	-------	-------	-------	-------
D-limonene	7.56	-------	14.57	17.59	4.68
Encalyptol	3.37	17.96	3.23	-------	4.73
L-menthone	3.77	5.31	0.62	0.90	18.60
Menthofuran	4.35	3.58	1.25	2.15	14.58
Menthol	1.93	-------	-------	0.50	8.77
Iso-pulegone	-------	2.17	0.76	-------	0.51
Alpha-terpineol	0.74	1.81	-------	-------	0.44
Cis-D-dihydrocarveol	-------	-------	-------	0.90	0.20
Cis-carveol	-------	0.82	2.60	5.98	0.44
Pulegone	3.54	41.03	6.09	1.50	17.74
(-)-carvone	-------	5.26	52.95	41.16	10.66
Cis-piperitone oxide	11.51	1.40	0.78	1.90	-------
Iso-menthyl acetate	3.30	-------	-------	0.80	7.56
2 cyclohexen-1-3-methyl	-------	1.87	-------	-------	0.44
Piperitenone oxide	38.22	8.66	13.61	4.58	3.02
(-)-Beta-bourbonene	-------	-------	-------	1.21	0.22
Trans-caryophyllene	1.78	0.47	1.26	0.72	0.87
Germacrene-D	2.79	0.46	2.19	2.13	1.00
Tau-cadinol	1.56	0.45	0.49	-------	0.19
Phthalic acid, isobutyl ester	2.95	1.35	-------	0.85	0.57
1,2-Benzene dicarboxylic acid	-------	-------	2.33	-------	-------
Unidentified	4.04	1.20	1.10	0.75	0.88

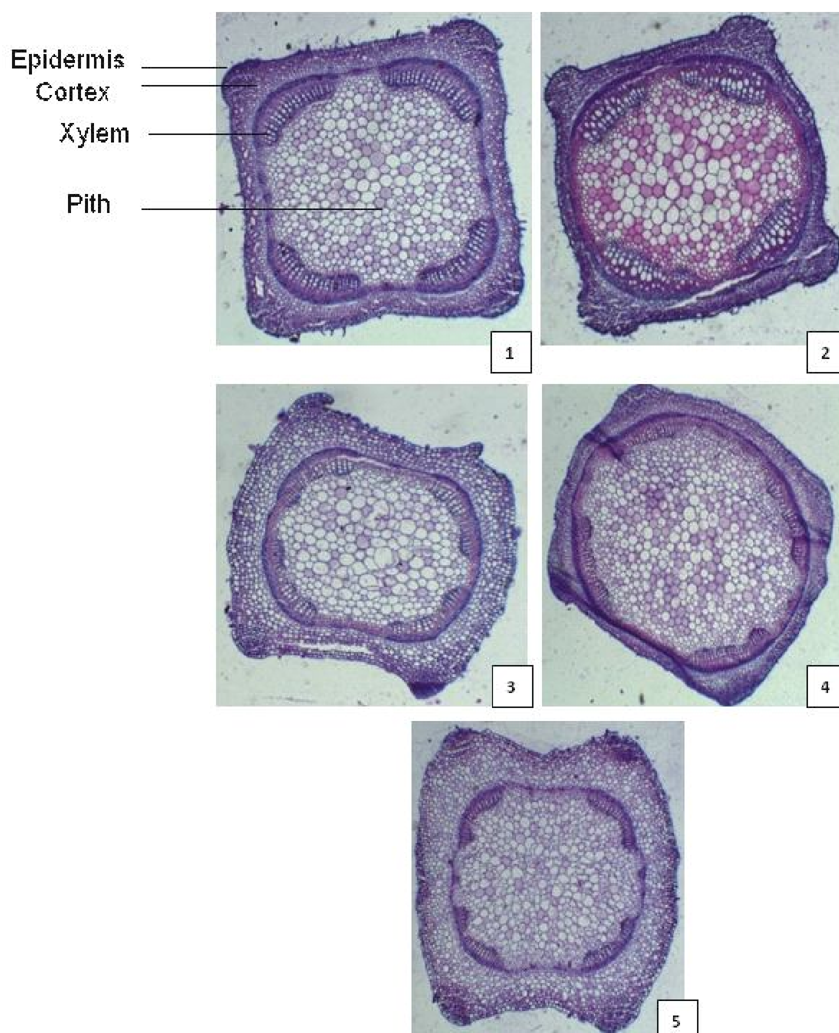

Fig. 5: Transverse section of the stem of *Mentha* species Key: 1- *M. longifolia* subsp. *typhoides* 2- *M. longifolia* subsp. *schimperi* 3- *M. sativa* 4- *M. spicata* 5- *M. piperita.*

Table 5: The sequences of SCOT primers.

Primer Nmber	Primer Name	Primer Sequence (5'-3')	Nucleotide Number	%GC
1	MPST11	GGTGTTGATGGCGACCT	17	59
2	MPST16	GATTTGAAATGGCTACCA	18	39
3	MPST30	CCATGGCTACCACCGCAC	18	67
4	MPST2	ACCACAAAATGGCGACCTA	19	47
5	MPST17	ATGGCTACCCTTAGCATG	18	50
6	MPST12	TTTGTTGATGGCGACCG	17	53
7	MPST13	GACAGCATGGCTACCAT	17	53
8	MPST14	ATGAGCATGGCTACCGA	17	53
9	MPST27	CAACAATGGCTACCACCC	18	56
10	MPST18	TTAGCATGCATGGCTACC	18	50

schimperi, while the lowest values; 530 and 105µ were in *M. sativa*.

Analysis of volatile oil

The volatile oil at flowering stage was obtained by means of water-steam distillation. It yielded 0.48, 0.98, 0.64, 0.37 and 0.46% for *M. longifolia* subsp. *typhoides*, *M. longifolia* subsp. *schimperi, M. spicata, M. sativa* and *M. peperita,* respectively. Analysis of volatile oil Using GC-MS technique proved the presence of 28 components of which 19 components are identified and the other rest 9 components were not identified in *M. longifolia* subsp. *typhoides*. While in *M. longifolia* subsp. *schimperi and M. spicata* recorded 21 components of which 19 components were identified and the other two components were not identified. In *M. sativa*, it was revealed the presence of 19 components of which 18 components are identified. Finally, 26 components of which 23 components were identified and the other three components were not identified recorded in *M. peperita.*

Data presented in Table 4 reveal that the major constituents present in the volatile oil of *M. longifolia* subsp. *typhoides* are piperitenone oxide (38.22%), Cispipertone oxide (11.51%) and D-limonene (7.56%) which they comprised to 57.29%. Worthy to mention that the other 9 components which were not identified were detected at the percentage from 0.34 to 0.70%. In *M. longifolia* subsp. *schimperi*, the first main component of volatile oil is pulegone (41.03%) whereas the second main component is eucalyptal (17.96%). The two non-identified components comprised 1.20%. *Cis*-piperitone epoxide, piperitenone oxide, carvone, limonene, and menthone have been identified as the main chemical constituents in *M. longifolia*by many reports (Farukh *et al.,* 2012; Huseyin *et al.,* 2013). On the other hand, Nikšiæ *et al.,* (2014) reported that the main constituents of the essential were oxygened monoterpenes piperitone oxide (1.9-63.6%) and 1, 8-cineole (5-12%), and sesquiterpenes trans-caryophyllene (3-9.3%) and germacrene D (0.16-10%). In our results, piperitenone oxide was the main chemical component in *M. longifoila* subsp. *typhoide* whereas in *M. lnogifoila* subsp. *schemperi*, the major constituent was pulegone. Also,

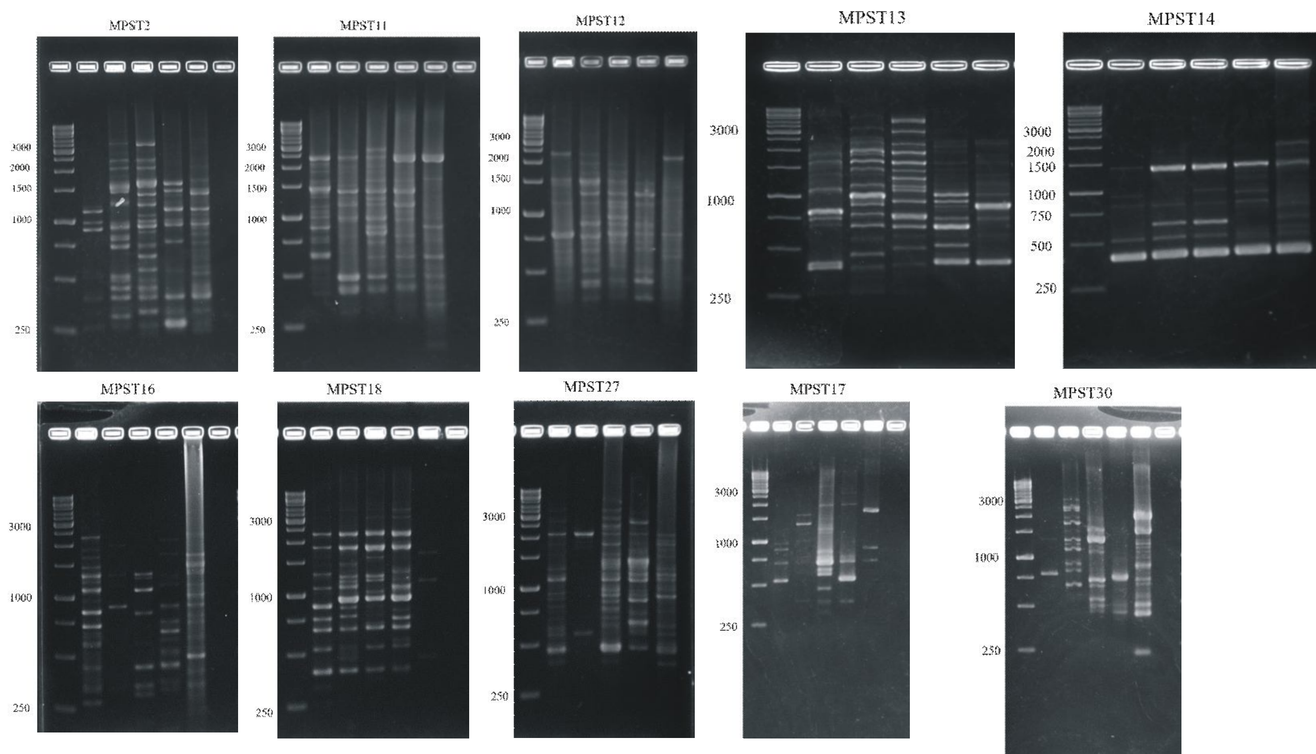

Fig. 6: SCOT markers profiles in the deferent *Mentha* species using MPST primers. Lane from left, 1: 1 kbp DNA ladder; 2-*M. sativa*; 3-*M. longifolia* subsp. *schimperi*; 4-*M. longifolia* subsp. *typhoides*; 5-*M. spicata*; 6-*M. piperita*. The circles illustrate some unique patterns in some species using MPST2, MPST16, MPST30 primers.

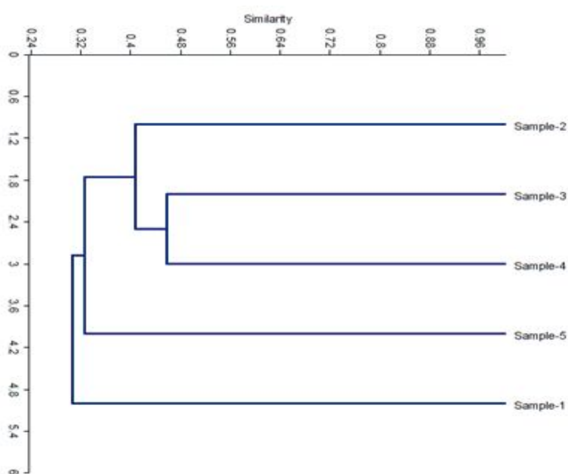

Fig. 7: Dendrogram depicting the genetic relationship among different species of *Mentha* based on SCOT data analysis. 1-*M. sativa*, 2-*M. longifolia* subsp. *schimperi*, 3-*M. longifolia* subsp. *typhoides*, 4-*M. spicata* and 5-*M. piperita*.

differences between these two subspecies in the percentages of the essential oil components were observed indicating that the chemical constituent level varies among the same species depending on the subspecies, cultivar, and/or variety.

Results given in Table 4 showed that the major components found in the volatile oil of *M. spicata* are (-)-carvone (41.16%), D-limonene (17.59%), piperitenone oxid (13.61%) and Cis-carveol (5.98%). Clearly, the previous four main components constitute are 78.34% and three compounds were detected at percentages ranged from 2 to 3%. The other 12 identified compounds were found at the rate of less than 2.0%. Such 12 components comprised 13.89% of the volatile oil. The residues two unidentified components constitute were 1.1%. Similarly, it has been documented that carvone is the major chemical composition in *M. spicata* leaves whereas limonene was present as a second component (Boukhebti *et al.,* 2011; Znini *et al.,* 2011; Nidaf khan *et al.,* 2017). Moreover, it was noticed that the first main components of the volatile oil in *M. sativa* Table 4 was (-) -carvone (52.95%) while he second main component was D-limonene (14.57%) followed by pulegone (6.09%). Such these three main components comprised 73.61%, the other 10 identified components were found at the rate of less than 2.0%. Results in Table 4 indicate that the major constitutes present in the volatile oil of *M. piperita* are L-menthone (18.60%), pulegone (17.74%), menthofuran (14.58%), (-)-carvone (10.66%), menthol (8.77%) and iso-menthyl acetate (7.56%). These six main components comprised 77.91% while the other 14 identified components were less than 2.00% and comprised 8.78% in *M. piperita*. Previously, it has been

reported that *M. piperita* containes menthol as a major constituents followed by menthyl acetate and menthone (Rita and Animesh 2011; Nidaf Khan *et al.,* 2017).

SCOT-PCR amplification

Ten SCOT primers were used in this study for analysis of genetic diversity among the four *Mentha* species (*M. sativa*, *M. longifolia* Subsp. *schimperi* and Subsp. *typhoides*, *M. spicata* and *M. piperita*) grown in Egypt Table 5. A total of 190 amplified products were scored of which 108 were polymorphic exhibiting 56.8% polymorphism, and 14 were monomorphic exhibiting 7.4% monomorphism. The molecular sizes of obtained fragment sranged between 250 to 3000bp (Fig.6). MPST27 primer exhibited 78.95% polymorphism while, the percentage of polymorphism with primers MPST 13,12, 30, 2, and 18 ranged from 61 to 70%. Also, there were 14 monomorphic patterns obtained by MPST2 at 250, 400, 950, and 1000 bp, MPST11 at 2500 bp, MPST12 at 650 and 750 bp, MPST13 at 400 and 750 bp, MPST14 at 450, 500, 850, and 1500 bp and MPST27 at 2100 bp. Table 6 and Fig. (6) showed that, certain SCOT primers revealed specific patterns. One unique band sized 800 bp in *M. Sativa,* two unique bands sized 1800 and 2500 bp in *M. Longifolia* subsp. *schimperi* and one unique band sized 250 bp in *M. piperita* were produced using MPST30. One unique band sized 500 bp was produced by MPST16 in *M. piperita*. One unique band sized 700 bp was produced in *M. Longifolia* subsp. *typhoides* using MPST2. These results revealed significant differences among the studied *Mentha* species and proved the potential of SCOT markers to identify/characterize some *Mentha* species and subspecies.

Genetic relationship and Cluster analysis

The genetic similarity percentages generated by the similarity matrix for Jaccard's coefficient of the four studied species of *Mentha* is presented in Table 7 to illustrate the genetic distance values among them. The similarity ranged from 25.7% to 45.5% of which, the highest similarity revealed by SCOT-PCR analysis was 45.5% between *M. longifolia* subsp. *typhoides* and *M. spicata.* While, the least similarity was 25.7% between *M. sativa* and *M. longifolia* subsp. *schimperi*. Also, Ibrahim (2017) reported that, the highest similarity was 44% between *M. longifolia* subsp. *typhoides* and *M. spicata* and the least similarity was 38% between *M. sativa* and *M. longifolia* subsp. *Schimperi* and subsp. *typhoides* by using RAPD markers. In this study, the similarity between *M. longifolia* subsp. *typhoides* and subsp. *schimperi* was 42.4% which close to the similarity between *M. longifolia* subsp. *typhoides* and *M. spicata* using SCOT markers.

Thus, *M. longifolia* subsp. *typhoides* and subsp. *schimperi* close to each other in both studies and also close to *M. spicata*. Also the two subspecies *M. longifolia* subsp. *typhoides* and subsp. *schimperi* fall in different subgroups with small difference in the genetic distance. In this study, *M. spicata* falls between the two *M. longifolia* subspp. on the same group and *M. sativa* and *M. piperita* fall on another separate groups. While, Ibrahim (2017) reported that, *M. spicata*, and *M. longifolia* subsp. *typhoides* fall on one group and *M. Sativa* and *M. longifolia* subsp. *schimperi* fall on another group. While, *M. piperita* is much less similar to them. Whereas the relationships between *M. Sativa* and *M. piperita* is almost the same in both studies, Thus, SCOT markers clarified the distance between *M. spicata* on one hand and *M. sativa* and *M. piperita* on the other hand. These results indicate that, using SCOT markers to detect the genetic diversity and identification of *Mentha* species is more accurate than RAPD markers because of the unique patterns profile detected in SCOT markers by the use of long primer which were designed following the short conserved region flanking the ATG initial codon (Collard and Mackill, 2009). Primers in range 18 to 24 nucleotides are preferable for generating reproducible

Table 6: SCOT markers specific to *Mentha.*

Species names	SCOT Primer specific band size [bp]	Species specific or genotype
M. sativa	MPST30	800 bp
M. longifolia subsp. *schimperi*	MPST 30	1800 bp and 2500 bp
M. longifolia subsp. *typhoides*	MPST2	700 bp
M. spicata	MPST 30	250 bp
M. piperita	MPST 16	500 bp

Table 7: Genetic identity and genetic distance values among the different species of *Mentha*.

Population ID	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Sample 1	100 %				
Sample 2	25.7 %	100 %			
Sample 3	31.5 %	42.4 %	100 %		
Sample 4	38.5 %	38.8 %	45.5 %	100 %	
Sample 5	26.4 %	27.1 %	31.0 %	39.1 %	100 %

Sample 1:*M. sativa*; 2:*M. longifolia* subsp. *schimperi*; 3:*M. longifolia* subsp. *typhoides*; 4:*M. spicata* and 5: *M. piperita*.

markers (Gillings and Holey 1997). In which, the optimal length of primer used in Target Region Amplification Polymorphism (TRAP) technique was 18 nucleotides (Hu and Vic 2003).

The dendrogram generated by the cluster analysis using the un weighted pair group method of the arithmetic averages (UPGMA) for the obtained SCOT dataset based on the genetic similarity among the *Mentha* species. Fig. 7 revealed that, the studied *Mentha* species were divided into three distinct groups. *M. sativa* was in the first group, *M. piperita* in the second group, and the third group subdivided into two subgroups, one of them consisted of *M. spicata*, *M. longifolia* subsp. *typhoides* and the other subgroup consisted of *M. longifolia* subsp. *schimperi*.

Start codon targeted (SCOT) markers technique have also been validated in the model rice species *Oryza sativa* to fingerprint a small diverse set of rice genotypes and considered as a useful tool for identifying population of the same species on a wide range of plants (Collard and Mackill, 2009). This DNA marker technique can be considered as a useful tool for identifying population of the same species on a wide range of plants (Collard and Mackill 2009). Genetic diversity of *Dendrobium nobile* Lindl*.,* an endangered medicinal orchid species, was also analyzed using SCOT markers system. The percentage of polymorphic loci generated by using this accurate marker system ranged from 25 to 56.82% and cluster analysis revealed high genetic variation among the genotypes (Bhattacharyya *et al.,* 2013). Genetic variability in *Mentha* was assessed by using RAPD molecular markers (Khanuja *et al.,* 2000 and Ibrahim, 2017). Also, DNA profiling revealed best results in *M. piperita* as compared to other *Mentha* species using SCOT marker analysis for correlating their similarity and distances between species (Nidaf Khan *et al.,* 2017). In the present study, the DNA fingerprinting using SCOT markers and the phylogenetic relationships were established in four *Mentha* plants in Egypt. These could be useful for designing breeding programs strategies. Also, taxonomy of *Mentha* species requires further investigation at the molecular level to clear up the phylogeny using several molecular marker systems between *Mentha* species, especially that these studies examined few number of species (only four species).

Conclusion

In this study, morphological and anatomical studies, as well as essential oil analyses, presented more knowledge in studying the taxonomical relationship among *Mentha* species. The recognition of unique bands by some SCOT primers indicates that SCOT markers can be useful in *Mentha* species identification. Also, DNA finger printing using SCOT markers established in *Mentha* species can provide more accurate assessments of genetic variation.

In the future, correlation between the plant structures and their demography in Egypt is needed to study. Also, studying the localization of the chemical constituents 'biosynthesis in lamina might present more details in the taxonomic studies of genus *Mentha*. Furthermore, the molecular markers used are important to find out more unique sequences in *Mentha* species which may be coding for specific traits and achieve more accurate phylogenetic relationships among different genotypes of *Mentha* species which should be useful for genetic improvement programs of *Mentha*. Taxonomy of *Mentha* species requires further investigation at the molecular level to clear up the phylogeny using several molecular marker systems between *Mentha* species, especially that these studies examined few number of species (only four species).

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