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Comparative Study for Some *Mentha* Species and Varieties under Sandy Soil Conditions

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ABSTRACT

The present study was conducted with the objective of finding out the concordance between growth and yield production, biochemical (the volatile oil) and molecular genetic (RAPD and ISSR-PCR) characteristics of three species of genus *Mentha* (*Mentha viridis*; *Mentha piperita*; *Mentha aquatica*) and two subspecies (*Mentha spicata* var. *Morocana*; *Mentha spicata* var. *Longofolia*). *Mentha* species and subspecies were collected from the Experimental Farm of the El-Kasaseen Research Station, Horticulture Research Institute, A.R.C. The highest oil percentage of fresh and dry *Mentha* herb obtained of *Mentha aquatica* (0.46, 2.11), respectively and *Mentha piperita* (0.40, 2.09), respectively in the second season. While the production yield of herb/fed. *Mentha piperita* recorded (3.148, 3.047 t) in the first and second season, respectively while *Mentha aquatica* recorded (1.513, 1.418 t) in the first and second season, respectively. GC analysis of the volatile oil prepared by hydrodistillation from aerial parts of spearmint, the major constituents of the oils in *Mentha viridis* were limonene (15.73%) and carvone (75%) while the major ones in *Mentha piperita* oil were limonene (10.57%), 1, 8-cineol (8%), menthone (14.74%), isomenthone (8.34%), menthol (13%) and β-caryophyllene (28.85%) which was the main one in *Mentha spicata* var. *Longofolia* oil by (46.75%) and (70.3%) in *Mentha spicata* var. *Morocana*. Menthyl acetate (86.11%) was the major constituents of *Mentha aquatica* oil. For molecular study Random Amplified Polymorphic DNA (RAPD) was performed which was efficient in detecting polymorphism and genetic variation within and between *Mentha* species and varieties. In RAPD analysis, 5 selected primers displayed a total of 71 amplified fragments, in which 55 (77.46%) were polymorphic fragments. The number of total amplified fragments scored per primer ranged from 7 (primer OP-E19) to 26 (primer OP-Ax06). Thirty-three out of 71 RAPD-PCR fragments were found to be useful as cultivar specific markers. The largest number of RAPD-PCR markers was scored for *Mentha viridis* (9 markers) while the lowest (5 markers) was scored for *Mentha aquatica* and *Mentha spicata* var. *Morocana*. In the meantime, the largest number of RAPD-PCR cultivar-specific markers was generated by primer OPAX-6 (12 markers) while the lowest number of RAPD-PCR specific markers (2 markers) was generated by primer OP-E19. In ISSR analysis, 5 of the tested ISSR primers generated variable banding patterns. A total of 50 out of 64 ISSR fragments were polymorphic. Twenty-six DNA amplified fragments were considered as cultivar-specific markers. Genetic similarities among the *Mentha* species/varieties were estimated according to the RAPD and ISSR data. Results of the combination of the banding patterns of the two techniques (RAPD and ISSR) data exhibited that the most two closely related cultivars were *Mentha piperita* and *Mentha spicata* var. *Morocana* with the highest similarity index (1.00). On the other hand, the two most distantly related cultivars were *Mentha aquatica* and *Mentha spicata* var. *Longofolia* with low similarity index (0.00). In conclusion, RAPD and ISSR polymorphisms could be used as efficient tools for the detection of similarities and phylogenetic relationships of the studied genotypes which could be useful in the breeding programs.

Key words: *Mentha* species, essential oil composition, GC/MS, DNA fingerprinting, genetic relationship, molecular markers

INTRODUCTION

Family Labiatae comprises of about 210 genera and some 3,500 species (Davidson, 1999). The family name Labiatae refers to the flowers that have typical petals fused into an upper lip and a lower lip. Although this is still considered an acceptable alternate name, most botanists now use the name "Lamiaceae" in referring to this family. The genus *Mentha* belongs to the family Lamiaceae consisting of about 25-30 species mainly found in temperate regions of Eurasia, Australia, South Africa and North America (Brickell and Zuk, 1997). All mints prefer and thrive in cool, moist spots in partial shade (Bradley and Ellis, 1992). Mints grow 10-120 cm tall and can spread over an indeterminate sized area. In botany, mint is the common name for any of the various herbaceous plants and perennial aromatic herbs that are cultivated for their essential oils and culinary purposes.

The genus *Mentha* L. (Lamiaceae) produces secondary metabolites such as alkaloids, flavanoids, phenols, gummy polysaccharides. Terpens and quinines are used in food and pharmaceutical, cosmetics and pesticide industries (Khanuja *et al*., 2000). Some members of this genus are also used as herbal teas and condiments both in fresh and dried form due to their distinct aroma (Baser, 1995). The species of *Mentha* have a medicinal and economic importance due to the volatile oils in their vegetative parts (Simpson and Conner-Ogorzaly, 1986). *Mentha* is represented in the Egyptian flora by three species; *M. spicata*, *M. pulegium* and *M. longifolia*. The latter species is widely distributed and includes two subspecies; *M. longifolia* subsp., *Typhoides* and *M. longifolia* subsp., *Schemprei* (Mohammed, 1986). *M. spicata* (spearmint) is cultivated for its volatile oils and is used in food flavoring. *M. piperita* (peppermint) was added to this study due to its wide cultivation in Egypt for its economic uses. The active constituents in peppermint oil which is prepared through distillation of the ground parts of the peppermint plant, include menthol, menthone, cineol and several other volatile oils (Blumenthal, 2000). In addition to morphological traits and other cellular biochemical, molecular evidences have been used effectively to assess genetic variation and phylogenetic relationships within and among populations of the same species and also between closely related species (Gottlieb, 1977; Crawford, 1985; Hamrick and Godt, 1997).

Random Amplified Polymorphism DNA (RAPD) markers are a modification of Polymerase Chain Reaction (PCR) used in the late 1980 (Williams *et al*., 1990). Among PCR based molecular markers RAPD is a widely used technique in different plants (Nazar and Mahmood, 2011; Mahmood *et al*., 2010a, b, 2011). PCR technique is one of the best available DNA-based tools for scoring variations between cultivars within species (Lakshmikumaran and Bhatia, 1998). One probable disadvantage is the degree of reproducibility of these markers, sometimes which can be low (Muralidharan and Wakeland, 1993; Ellsworth *et al*., 1993; Skroch and Nienhuis, 1995) particularly between laboratories (Penner *et al*., 1993; Jones *et al*., 1997). This is due to sensitivity of RAPD banding patterns to reaction conditions. The technique is being successfully used widely for the estimation of genetic variability as well as the cultivar identification/differentiation in various plant species, including rice (Mackill, 1995), broccoli and cauliflower (Hu and Quiros, 1991), banana (Howell *et al*., 1994), *Brassica* (Jain *et al*., 1994), *Triticum* (Joshi and Nguyen, 1993), *Medicago* (Yu and Pauls, 1993), *Coffea* (Orozco-Castillo *et al*., 1994) and *Lycopersicon* (Williams and St. Clair, 1993) etc.

Genetic variation and/or taxonomic relationships in the genus *Mentha* were previously investigated using morphological, chemical, cytological and molecular traits (Gobert *et al*., 2002; Ikoda *et al*., 1991; Shasany *et al*., 2001). In the present study, we describe the similarity and diversity in terms of RAPD and ISSR profiles of three mint species and two subspecies and investigate genetic diversity among them.

MATERIALS AND METHODS

Plant materials: Three species of genus *Mentha* (*Mentha viridis*; *Mentha aquatica*; *Mentha piperita*) and two subspecies (*Mentha spicata* var. *Morocana*; *Mentha spicata* var. *Longofolia*) were used in the present study. Table 1 showed the physical and chemical analysis of the experimental soil and Table 2 showed the chemical analysis of the irrigation water.

In March 2012, at the Experimental Farm of the El-Kasaseen Research Station the seedlings of the three species and two subspecies of genus *Mentha* were obtained from the Medicinal and Aromatic Plants Section at El-Kanater El-Khairia, Kalubia Governorate, Horticulture Research Institute, A.R.C. The seedlings were 10-15 cm in length, with 10-12 leaves and were planted in a randomized complete block

Table 1: Physical and chemical analysis of the experimental soil during 2011 and 2012 seasons

Properties	First season	Second season
Physical analyses (%)		
Saturation (capacity)	25	25
Field capacity	11	11
Wilting coefficient (point)	6	6
Available water	5	5
Mechanical analyses (%)		
Sand	87.13	87.02
Silt	7.24	7.42
Clay	5.63	5.56
Soil texture	Sandy	Sandy
Chemical properties		
Salt analysis		
EC (dSm^{-1})	1.6	1.5
pH	7.08	7.09
Cations (meq L^{-1})		
Ca^{2+}	5.7	5.4
Mg^{2+}	2.6	2.6
Na^{+}	7.0	7.02
K^{+}	0.8	0.7
Anions (meq L^{-1})		
Cl^{-}	7.6	7.4
CO_3^{2-}	0	0
HCO_3	2.8	2.9
SO_4^{2-}	5.6	5.1
Available elements (mg kg^{-1})		
Nitrogen	7.1	7.3
Phosphorus	2.1	2.8
Potassium	13.4	13.9
Organic matter (OM) (%)	0.01	0.03

Table 2: Chemical analysis of the used irrigation water

Characters	Values
EC (dSm^{-1})	0.387
pH	8.350
Cations (meq L^{-1})	
Ca^{2+}	1.800
Mg^{2+}	1.000
Na^{+}	0.860
K^{+}	0.170
Anions (meq L^{-1})	
CO_3^{2-}	0.000
HCO_3^{-}	2.700
Cl^{-}	1.000
SO_4^{2-}	0.140

Table 3: List of the primer names and their nucleotide sequences used in the study for RAPD procedure and ISSR procedure

No.	Primer code	Sequence
RAPD		
1	OP-AX6	5′ AGG CAT CGT G3′
2	OP-C02	5′GTGAGGCGTC 3′
3	OP-D07	5′TTGGCACGGG 3`
4	OP-Q015	5` GGGTAACGTG 3`
5	OP-E09	5`ACGGCGTATG 3`
ISSR		
1	44A	5'CTC TCT CTC TCT CTC TAC 3'
2	49A	5'CAC ACA CAC ACA AG 3'
3	44B	5'CTC TCT CTC TCT CTC TGA 3'
4	98B	5' CAC ACA CAC ACA GT3'
5	49B	5' CAC ACA CAC ACA GG 3'

design with three replicates. The herb was cut at three times (the first half of June, September and November), the data were calculated on the basis of three cutting means. In March 2013, the seedlings from each species and subspecies cultivar were planted by same previous method. Data of 2012 and 2013 seasons were recorded on the follow traits:

- Plant height (m) (P.H)
- Fresh weight/plant (g) (F.W/P)
- Dry weight/plant (g) (D.W/P)
- Fresh yield of herb/plant (g) (F.y/p)
- Dry yield of herb/plant (g) (D.y/p)
- Dry yield of herb/fed (ton) (D.y/fed.)
- Volatile oil percentage in fresh herb (%) (V.O.P)
- Volatile oil percentage in dry herb (%) (V.O.P)
- Volatile oil yield in dry herb/plant (mL) (V.O.Y./P)
- Volatile oil yield in dry herb/Fed. (L.) (V.O.Y./fed.)
- Analysis of the essential oil by GC/MS
- Molecular genetics identification using RAPD and ISSR methods

Determination of essential oil content and composition: Essential oil percentage was determined in fresh weight according to the method described in the General Medical Council (1963). Oil yield per plant was calculated by multiplying oil percentage by herb yield/plant and expressed as mL/plant. Samples taken for the essential oil obtained in the second season were analyzed using DsChrom 6200 Gas Liquid Chromatography equipped with a flame ionization detector for separation of volatile oil constituents. The analysis conditions were as follows.

The chromatograph apparatus was fitted with capillary column BPX-5, 5% phenyle (equiv.) polysillphenylene-siloxane 30×0.25 mm ID X 0.25 μm film. Temperature program ramp increase with a rate of 10°C min^{-1} from 70-200°C. Flow rates of gases were nitrogen at 1 mL min^{-1}, hydrogen at 30 and 330 mL min^{-1} for air. Detector and injector temperatures were 300 and 250°C, respectively. The obtained chromatogram and report of GC analysis for each sample were analyzed to calculate the percentage of main components of volatile oil.

RAPD analysis Polymerase Chain Reaction (PCR): In order to obtain clear reproducible amplification products, different preliminary experiments were carried out in which a number of factors were optimized. These factors included PCR temperature cycle profile and concentration of each of the template DNA, primer, $MgCl_2$ and Taq polymerase. A total of twenty random DNA oligonucleotide primers were independently used according to Williams *et al.* (1990) in the PCR reaction. Only five primers succeeded to generate reproducible polymorphic DNA products. The PCR amplification was performed in a 25 μL reaction volume containing the following: 2.5 μL of dNTPs (2.5 mM), 1.5 μL of $MgCl_2$ (25 mM), 2.5 μL of 10x buffer, 2.0 μL of primer (2.5 μM), 2.0 μL of template DNA (50 ng μL^{-1}), 0.3 μL of Taq polymerase (5 U μL^{-1}) and 14.7 μL of sterile ddH_2O. The reaction mixtures were overlaid with a drop of light mineral oil per sample. Amplification was carried out in Techni TC-512 PCR System. The reaction was subjected to one cycle at 95°C for 5 min, followed by 35 cycles at 96°C for 30 sec, 37°C for 30 sec and 72°C for 30 sec, then a final cycle of 72°C for 5 min. The PCR products were run at 100 V for 1 h on 1.5% agarose gels to detect polymorphism between *Mentha* species and varieties strains under study. Only five primers succeeded to generate reproducible polymorphic DNA products. Table 3 lists the base sequences of these DNA primers that produced informative polymorphic bands. The PCR products were separated on a 1.5% agarose gels and fragments sizes were estimated with the 100 bp ladder marker (1500, 1000, 900, 800, 700, 600, 500, 400, 300, 200 and 100 bp).

ISSR analysis Polymerase Chain Reaction (PCR): ISSR-PCR reactions were conducted using five primers. Amplification was conducted in 25 μL reaction volume containing the following reagents: 2.5 μL of dNTPs (2.5 mM), 2.5 μL $MgCl_2$ (2.5 mM) and 2.5 μL of 10x buffer, 3.0 μL of Primer (10 pmol), 3.0 μL of template DNA (25 ng μL^{-1}), 1 μL of Taq polymerase (1 U μL^{-1}) and 12.5 μL of sterile dd H_2O. The PCRs were programmed for 1 cycle at 94°C for 4 min followed by 45 cycles of 1 min at 94°C, 1 min at 57°C and 2 min at 72°C the reaction was finally stored at 72°C for 10 min. The PCR products were separated on a 1.5% agarose gels and fragments sizes were estimated with the 100 bp ladder marker.

Only five primers succeeded to generate reproducible polymorphic DNA products. Table 3 lists the base sequences of these DNA primers that produced informative polymorphic bands.

Statistical analysis: A randomized complete block design was adopted for the present trial data and statistically analyzed by the factorial method according to Snedecor and Cochran (1990) where LSD test was used for comparison between means. The DNA bands generated by each primer were counted and their molecular sizes were compared with those of the DNA markers. The bands scored from DNA profiles generated by each primer were pooled together. Then the presence or absence of each DNA band was treated as a binary character in a data matrix (coded 1 and 0, respectively) to calculate genetic similarity and to construct dendrogram tree among the studied apricot strains. Calculation was achieved using Dice similarity coefficients (Dice, 1945) as implemented in the computer program SPSS-10.

RESULTS AND DISCUSSION

Results of growth parameters and yield of fresh and dry weights of herb *Mentha* species and subspecies in both seasons are shown in Table 4. It is clear that, the differences between *Mentha* plants in all studied parameters are significant. *Mentha piperita* and *Mentha viridis* were superior in plant height and also produced the highest values in fresh and dry weights either g plant^{-1} or ton/feddan, in the first and second seasons, respectively. While, *Mentha spicata* var. *Morocana* showed the lowest values in plant height and *Mentha aquatica* showed the lowest values in fresh and dry weights. It is clear that, the growth of different species/varieties of *Mentha* could be arranged in a descending order as follows; *Mentha piperita*; *Mentha viridis*; *Mentha spicata* var. *Morocana*; *Mentha spicata* var. *Longofolia* then *Mentha aquatica* from production point of view. Among the species and varieties of *Mentha* in this study, *Mentha piperita* was more suitable for sandy soil conditions than the others (Table 4).

Data in Table 5 indicated that, volatile oil percent on fresh weight bases significantly differed and ranged from 0.14-0.43% in the first season and from 0.16-0.46% in the second season for *Mentha spicata* var. *Longofolia* and *Mentha aquatica*, respectively in the fresh herb. While in the dry herb the volatile oil percent ranged from 1.25-2.07% in the first season and from 1.30-2.11% in the second season for *Mentha viridis* and *Mentha aquatica*, respectively. The volatile oil percent increased in the second season than the first one in all species/varieties of *Mentha*. This may be attributed to the differences in environmental factors i.e., temperature (air and soil), light levels and moisture conditions. The synthesis of secondary metabolites has been related to the capture of light energy (Ali *et al*., 1986; El-Ballal *et al*., 1983; Morales *et al*., 1993; Omer *et al*., 1994). The minimum oil yield was observed with *Mentha spicata* var. *Longofolia* in the first season and with *Mentha aquatica* in the second season while the maximum was observed with *Mentha piperita* in the both seasons (57.58, 58.11) (L.)/fed, respectively. In other words, *Mentha aquatica* was the highest in the essential oil percent and *Mentha piperita* was the highest in the oil yield. It related to the production yield of herb/fed which reflects on oil yield. *Mentha piperita* recorded (3.148, 3.047 t) for dry yield of herb/feddan in the first and second seasons, respectively while *Mentha aquatica* recorded (1.513, 1.418 t) in the first and second season, respectively.

The results of the GC/MS analysis of the volatile oils of the *Mentha* species/varieties in second season are shown in Table 6. The identified compounds are ranged from 94.84% in *Mentha aquatica* to 100% in *Mentha spicata* var. *Morocana*. The majority of compounds (10 oxygenated monoterpenes) ranged from 10.43% in *Mentha spicata* var. *Morocana* to 89.22% in *Mentha aquatica* while the monoterpene hydrocarbons are represented (6 compounds) and ranged from

Table 4: Growth and yield of different *Mentha* species/varieties cultivated under Egyptian sandy soil conditions in the two seasons of growth

	Plant height (cm)		F.W of herb (g)		D.W of herb (g)		Fresh yield of herb/plant (g)		Dry yield of herb/plant (g)		Dry yield of herb/fed (t)	
Species/varieties	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Mentha viridis	45.33	45.33	190.20	187.60	38.17	37.53	570.60	562.80	114.50	112.60	2.863	2.802
Mentha piperita	53.00	55.67	194.87	186.50	41.97	40.63	584.60	559.50	125.90	112.90	3.148	3.047
Mentha aquatic	38.67	35.00	100.17	94.53	20.17	18.90	300.53	283.60	60.50	56.70	1.513	1.418
Mentha spicata var. *Longofolia*	36.67	38.33	103.93	102.90	23.67	23.63	311.80	214.70	71.00	70.90	1.775	1.773
Mentha spicata var. *Morocana*	33.33	29.00	127.60	121.23	26.20	24.20	382.80	363.70	78.60	72.00	1.965	1.815
LSD at 5%	3.46	6.72	23.91	19.76	5.44	4.88	71.71	57.09	16.32	14.65	0.408	0.367

F.W: Fresh weight, D.W: Dry weight

Table 5: Volatile oil percentage and volatile oil yield of different *Mentha* species/varieties cultivated under Egyptian sandy soil conditions in the two seasons of growth

	Essential oil in fresh herb (%)		Essential oil in dry herb (%)		Essential oil yield in dry herb (mL plant^{-1})		Essential oil yield in dry herb (L.)/fed	
Species/varieties	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Mentha viridis	0.24	0.23	1.25	1.30	1.43	1.49	29.89	35.04
Mentha piperita	0.37	0.40	2.00	2.09	2.50	2.53	57.58	58.11
Mentha aquatica	0.43	0.46	2.07	2.11	1.25	1.21	26.72	27.75
Mentha spicata var. *Longofolia*	0.14	0.16	1.61	1.73	1.15	1.23	26.37	28.29
Mentha spicata var. *Morocana*	0.19	0.20	1.73	1.77	1.35	1.29	31.13	29.59
LSD at 5%	0.10	0.06	0.21	0.32	0.12	0.14	3.22	4.17

Table 6: Volatile oil composition (%) of different *Mentha* species/varieties cultivated under Egyptian sandy soil condition (second season)

Peak No.	Components	Retention time (min)	*Mentha viridis*	*Mentha piperita*	*Mentha aquatica*	*Mentha spicata* var. *Longofolia*	*Mentha spicata* var. *Morocana*
1	α-pinene	2.62	0.57	2.33	4.94	3.79	1.70
2	β-pinene	2.83	0.38	1.41	0.68	5.74	0.37
3	Phellandrene	2.87	0.58	2.08		5.14	0.67
4	Limonene	3.16	15.73	10.57		13.19	15.59
5	1, 8-cineol	3.21	5.17	8.01		9.95	6.07
6	γ-terpinene	3.48		1.40		0.74	0.94
7	P-cymene	3.67		0.47		0.79	
8	Menthone -trans	3.85		0.51		-	
9	Octanol	3.97		1.18		0.71	
10	Menthyl acetate	4.24		1.45	86.11	2.52	
11	Menthone	4.33		14.74	0.04	1.25	
12	Isomenthone	4.44		8.34	3.07	0.78	
13	Menthol	4.53		13.09		2.46	0.08
14	Isomenthol	4.59		1.01		1.38	
15	Neoisomenthol	4.66		0.43		-	
16	Linalool	4.81		0.73		1	4.28
17	β-caryophyllene	5.37		28.85		46.75	70.30
18	Carvone	5.38	75.09	-		-	
	Total identified		97.52	96.60	94.84	96.19	100.00
	Unknown		2.48	3.40	5.16	3.81	0.00
	Monoterpene hydrocarbons		17.26	18.26	5.62	29.39	19.27
	Oxygenated monoterpenes		80.26	48.31	89.22	19.34	10.43
	Sesquiterpene hydrocarbons			28.85		46.75	70.30
	Other compounds			1.18		0.71	

Values are means of three replicates

5.62% in *Mentha aquatica* to 29.39% in *Mentha spicata* var. *Longofolia*. The β-caryophyllene was the only Sesquiterpene hydrocarbons compound found and ranged from 28.85% in *Mentha piperita* to 70.30% in *Mentha spicata* var. *Morocana*, however it was absent in *Mentha viridis* and *Mentha aquatica*. *Mentha* varieties/species were differed in these contents of monoterpenes and sesquiterpenes, these plants were characterized by high contents of oxygenated monoterpenes which ranged from 10.43% in *Mentha spicata* var. *Morocana* to 89.22% in *Mentha aquatica*. The content of essential oils expressed in percentage were as follows: for *Mentha spicata* var. *Morocana* 100%, *Mentha viridis* 97.52%, *Mentha piperita* 96.6%, *Mentha spicata* var. *Longofolia* 96.19% and *Mentha aquatica* 94.84%.

Table 6 lists the chemical components of the volatile oils. The main constituents of *Mentha spicata* var. *Morocana* essential oil were β-caryophyllene (70.30%), limonene (15.59%),1, 8-cineol (6.07%) and linalool (4.28%). In the volatile oil of *Mentha viridis* major compounds were carvone (75.09%), limonene (15.73%) and1,8-cineol (5.17%), whereas, in the volatile oil of *Mentha piperita* β-caryophyllene (28.58%), menthone (14.74%), menthol (13.09%), limonene (10.57%) isomenthone (8.34%) and 1, 8-cineol (8.01%) were dominant. The most abundant components observed in *Mentha spicata* var. *Longofolia* were β-caryophyllene (46.75%), limonene (13.19%), 1, 8-cineol (9.95%), β-pinene (5.74%) and phellandrene (5.14%) while menthyl acetate (86.11%) was the major component in *Mentha aquatica* followed by α-pinene (4.94%) and isomenthone (3.07%). These results are in accordance of previous published data on volatile oils of different *Mentha* species (Mimica-Dukic *et al*., 1991; Mkaddem *et al*., 2009). This study revealed that the volatile oil of *Mentha viridis* has carvone chemotype plant because *Mentha viridis* is the only species content carvone (75.09%), whereas, β-caryophyllene was the major compound in *Mentha piperita* (28.58%), *Mentha spicata* var. *Longofolia* (46.75%) and *Mentha spicata* var. *Morocana* (70.30%) and was absent in *Mentha viridis* and *Mentha aquatica* which has menthyl acetate (86.11%) as a major compound. Other components occur in all studied species/varieties except one species/variety, such as, phellandrene, limonene and 1, 8-cineol are found in all studied species and varieties and absent in *Mentha aquatica*.

The important differences were determined by the presence or absence of carvone, β-caryophyllene and menthyl acetate, these three compounds were considered to identify possible chemotypes. *Mentha* volatie oil could be classified into three chemotypes: Carvone type, β-caryophyllene type and menthyl acetate type. Results of this study showed that volatile oil of *Mentha viridis* has carvone as the major component (75.09%) is in good agreement with other researches (Misra *et al*., 1989; Ozguven and Kirici, 1999; Telci *et al*., 2004). The obtained results are in accordance with Soheil *et al*. (2004) who stated that monoterpenes are the major essential components of the mint including peppermint.

Molecular genetic identification

Randomly Amplified Polymorphic DNA (RAPD) markers: The five 10-mer arbitrary primers succeeded in amplifying DNA fragments for the five genotypes of *Mentha* species/varieties as illustrated in Table 7 and Fig. 1. Polymorphism levels differed from one primer to another. OP-E19 primer exhibited low level of polymorphism (42.86%). On the other hand, OP-C02 (50%) primer exhibited

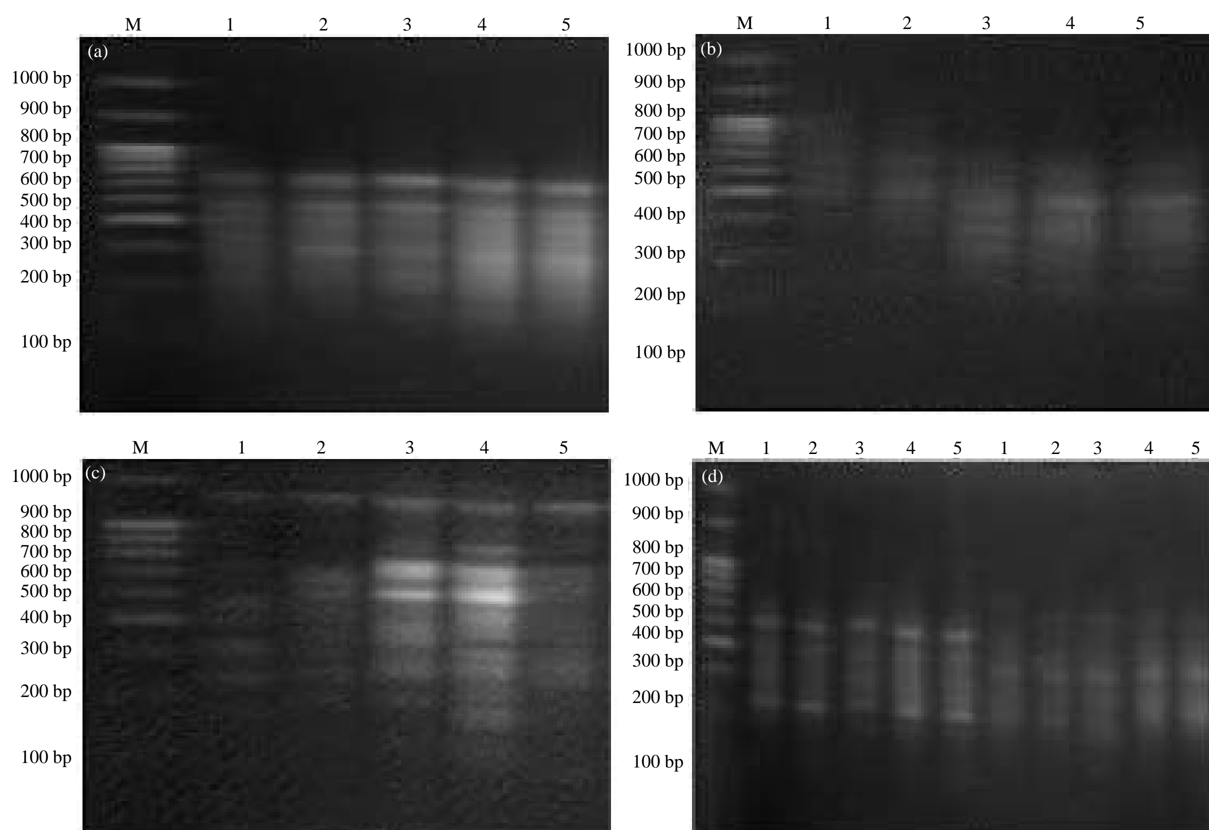

Fig. 1(a-d): RAPD-PCR analysis of different *Mentha* species/varieties cultivated under Egyptian sandy soil condition (second season), (a) OP-CO2, (b) OP-DO7, (c) OP-AXO5 and (d) OP-E29 and Q15: *Mentha viridis*, 2: *Mentha aquatic*, 3: *Mentha piperita* 4: *Mentha spicata* var. *Morocana* and 5: *Mentha spicata* var. *Longofolia*

Table 7: Species-specific RAPD and ISSR markers for *Mentha* species/varieties genotypes

Primers code	Range of M.S.	TAF	MF	PF	SM	Polymorphism (%)
RAPD primers						
OP-Ax06	113-1120	26	2	24	12 (613, 133, 113)-(573, 528, 115)-(331)-(148, 116)-(463, 207, 107) bp	92.30
OP-C02	145-527	10	5	5	3 (185)-(180)-(191) bp	50.00
OP-D07	140-767	16	2	14	10 (492, 379)-(385, 304, 252)-(390, 227)-(402, 193, 140) bp	87.50
OP-E19	204-698	7	4	3	2 (698, 204) bp	42.86
OP-Q15	166-508	12	3	9	6 (508, 404)-(307, 214)-(156)-(398) bp	75.00
Total RAPD primers		71	16	55	33	
ISSR primers						
44A	141-813	16	4	12	7 (284)-(197)-(141)-(162)-(522, 271, 152) bp	75.00
44B	199-779	13	3	10	6 (421, 190)-(332, 179, 147)-(779) bp	76.90
49A	154-659	16	3	13	6 (659, 410, 262, 183)-(548, 416) bp	81.25
49B	126-818	12	1	11	5 (162)-(140)-(173, 126)-(194) bp	91.67
98B	153-371	7	3	4	2 (368)-(341) bp	57.14
Total ISSR primers		64	14	50	26	
Total		135	30	105	59	

TAF: Total amplified fragments, MF: Monomporphic fragments, PF: Polymorphic fragments, SM: Specific markers

moderate levels of polymorphism. However, OP-Q15 (75%), OP-D07 (87.50%) and OP-AX6 (92.30%) primers exhibited high levels of polymorphism.

The number of Total Amplified Fragments (TAF), Polymorphic Fragments (PF), Monomorphic Fragments (MF) and Specific Markers (SM) for each sample using the five primers are shown in Table 7. OP-AX6 primer produced twenty six fragments with molecular size ranging from 113-1120 bp (Fig. 1). Twenty four fragments were polymorphic (92.3%) and twelve of them were species-specific

Table 8: Similarity value (Pairwise comparison) of *Mentha* species/varieties genotypes based on RAPD data

	Mentha viridis	*Mentha aquatic*	*Mentha piperita*	*Mentha spicata* var. *Morocana*
Mentha viridis				
Mentha aquatica	0.75			
Mentha piperita	0.08	0.50		
Mentha spicata var. *Morocana*	0.25	0.50	1.00	
Mentha spicata var. *Longofolia*	0.08	0.00	0.50	1.00

markers at 613, 133, 113 bp for *Mentha viridis* (573, 528, 115 bp) for *Mentha aquatica*, 331 bp for *Mentha piperita* (148, 116 bp) for *Mentha spicata* var. *Morocana* and (463, 207, 107 bp) for *Mentha spicata* var. *Longofolia* while the other two fragments were present in all genotypes which are considered as common fragments. OP-C02 primer resulted in ten DNA fragments with molecular size ranging from 145-527 bp, five fragments were polymorphic (50%) in which three of them were species-specific marker at 185 bp for *Mentha piperita*, 180 bp for *Mentha spicata* var. *Morocana* and 191 bp for *Mentha spicata* var. *Longofolia* and the other five fragments were present in all genotypes which are considered as common fragments. OP-D07 primer resulted in sixteen DNA fragments with molecular size ranging from 140-767 bp, fourteen fragments were polymorphic (87.50%) and ten of them were species-specific marker at 492, 379 bp for *Mentha viridis*, (385, 304, 252 bp) for *Mentha piperita*, (390, 227 bp) for *Mentha spicata* var. *Morocana* and (402, 193, 140 bp) for *Mentha spicata* var. *Longofolia* while the other two fragments were presented in all genotypes which are considered as common fragments.

OP-E19 primer resulted in seven DNA fragments with molecular size ranging from 204-698 bp, in which three fragments were polymorphic (42.86%) and two of them were species-specific markers at 698 and 204 bp for *Mentha viridis* and the other four fragments were present in all genotypes which are considered as common fragments. OP-Q15 primer resulted in twelve DNA fragments with molecular size ranging from 166-508 bp, nine fragments were polymorphic (75%) in which six of them were species-specific markers at 508 and 404 bp for *Mentha viridis* (307, 214 bp) for *Mentha aquatica*, 156 bp for *Mentha piperita* and 398 bp for *Mentha spicata* var. *Longofolia* while the other three fragments were presented in all genotypes which are considered as common fragments.

Genetic similarity and cluster analysis based on RAPD markers: The RAPD data were used to estimate the genetic similarity values among the five genotypes of *Mentha* species/varieties by using UPGMA computer analysis (Table 8, Fig. 1). The highest similarity value (1.0) was recorded between *Mentha piperita* and *Mentha spicata* var. *Morocana* genotypes and also between *Mentha spicata* var. *Morocana* and *Mentha spicata* var. *Longofolia* genotypes while the lowest similarity value (0.08) was detected between *Mentha viridis* and *Mentha spicata* var. *Longofolia* genotypes as well as between *Mentha viridis* and *Mentha piperita* genotypes. On the other hand there was no similarity between *Mentha aquatica* and *Mentha spicata* var. *Longofolia* genotype. A dendrogram for the genetic relationship among

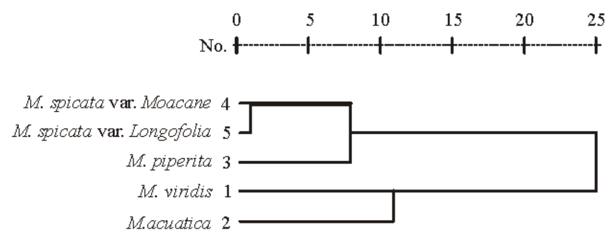

Fig. 2: Dendrogram illustrating the genetic distance for *Mentha* species/varieties genotypes based on RAPD data

the five genotypes of *Mentha* species/varieties genotypes is exhibited in Fig. 2 which separated them into two major groups. The first group included *Mentha viridis* and *Mentha aquatica* genotype while the second group included two subgroups, the first subgroup involved *Mentha piperita* only and the other subgroup included *Mentha spicata* var. *Morocana* and *Mentha spicata* var. *Longofolia* genotypes. The results of RAPD analysis are in harmony with those obtained by Hassan *et al.* (2002).

Inter Simple Sequence Repeats (ISSRs) markers: The five ISSR primers succeeded in amplifying DNA fragments for the five *Mentha* species/varieties genotypes (Fig. 3). Polymorphism levels differed from one primer to another, i.e., 44A, 44B, 49A and 49B primers exhibited high levels of polymorphism (75, 76.9, 81.25 and 91.67%), respectively while, 98B primer exhibited moderate level of polymorphism (57.14%) as exhibited in Table 7. The number of Total Amplified Fragments (TAF), Polymorphic Fragments (PF), Monomorphic Fragments (MF) and Specific Markers (SM) for each primer of the five primers are shown in Table 7. The 44A primer showed 16 DNA fragments with molecular size ranging from 141-813 bp (Fig. 3, Table 7), twelve fragments were polymorphic (75%) and seven of them were positive species-specific markers at (284, 197, 141 and 162 bp) for *Mentha viridis*, *Mentha aquatica*, *Mentha piperita* and *Mentha spicata* var. *Morocana* genotypes, respectively and the last three of them for *Mentha spicata* var. *Longofolia* genotype at (522, 271 and 152 bp).

The 44B primer showed thirteen DNA fragments with molecular sizes ranging from 199-779 bp, ten fragments were polymorphic (76.9%) and six of them were positive species- specific markers at 421 and 190 bp for *Mentha viridis* genotype, 332, 179 and 147 bp for *Mentha aquatica* genotype and 779 bp for *Mentha spicata* var. *Longofolia* genotype. The 49A primer showed sixteen DNA fragments with molecular size ranging from 154-659 bp, thirteen fragments were

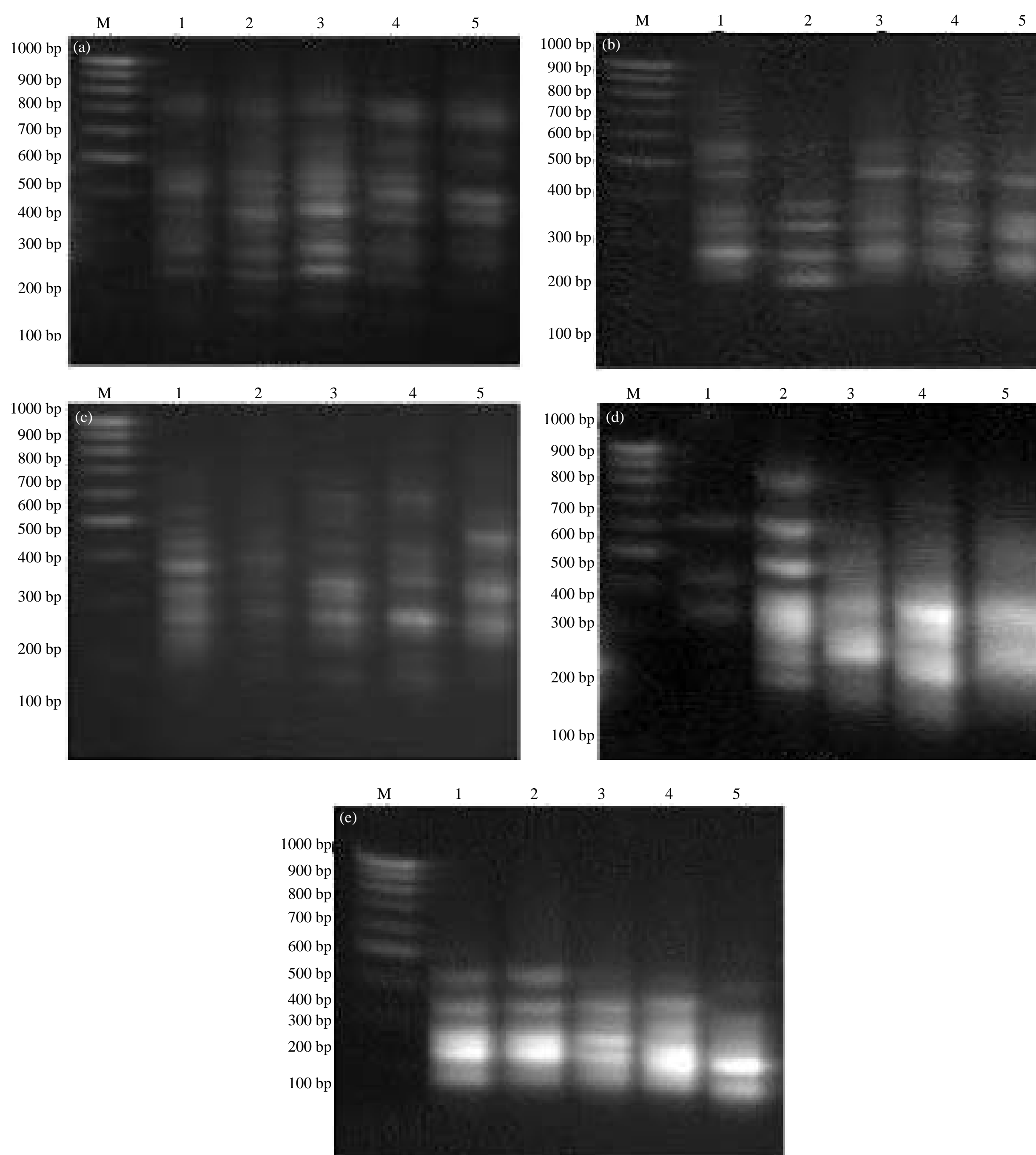

Fig. 3(a-e): ISSR-PCR analysis of different *Mentha* species/varieties cultivated under Egyptian sandy soil condition (second season) (a) 44A, (b) 44B, (c) 49A, (d) 49B and (e) 98B, 1: *Mentha viridis*, 2: *Mentha aquatic*, 3: *Mentha piperita*, 4: *Mentha spicata* var. *Morocana* and 5: *Mentha spicata* var. *Longofolia*

polymorphic (81.25%) and six of them were positive species-specific markers at 659, 410, 262 and 183 bp for *Mentha viridis* genotype and at 548 and 416 bp for *Mentha spicata* var. *Longofolia* genotype. The 49B primer showed twelve DNA fragments with molecular size ranging from 126-818 bp, eleven fragments of them were polymorphic (91.67%) and five of them were positive species-specific markers at 162 bp for *Mentha aquatica* genotype, 140 bp for *Mentha piperita* genotype, (173 and 126 bp) for *Mentha spicata* var. *Morocana* genotype and at 194 bp for

Table 9: Similarity value (Pairwise comparison) of *Mentha* species/varieties genotypes based on RAPD data

Species	*Mentha viridis*	*Mentha aquatica*	*Mentha piperita*	*Mentha spicata* var. *Morocana*
Mentha viridis				
Mentha aquatica	0.52			
Mentha piperita	0.38	0.61		
Mentha spicata var. *Morocana*	0.42	0.57	1.00	
Mentha spicata var. *Longofolia*	0.33	0.00	0.61	0.47

Table 10: Similarity value (Pairwise comparison) of *Mentha* species/varieties genotypes based on over-combination of RAPD and ISSR analysis

Species	*Mentha viridis*	*Mentha aquatica*	*Mentha piperita*	*Mentha spicata* var. *Morocana*
Mentha viridis				
Mentha aquatica	0.57			
Mentha piperita	0.24	0.57		
Mentha spicata var. *Morocana*	0.33	0.54	1.00	
Mentha spicata var. *Longofolia*	0.21	0.00	0.57	0.66

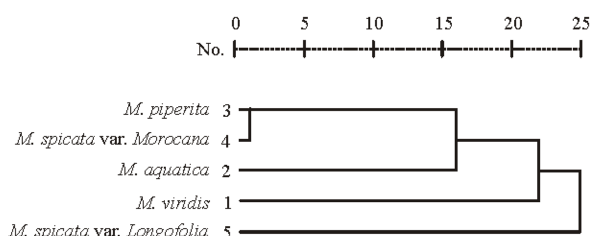

Fig. 4: Dendrogram illustrating the genetic distance for *Mentha* species/varieties genotypes based on ISSR data

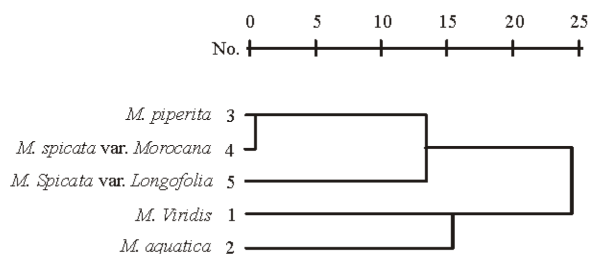

Fig. 5: Dendrogram illustrating the genetic distance for *Mentha* species/varieties genotypes based on over-combination of RAPD and ISSR analysis

Mentha spicata var. *Longofolia* genotype. The 98B primer showed seven DNA fragments with molecular size ranging from 153-371 bp, four fragments were polymorphic (57.14%) and two of them were positive species-specific markers at 368 and 341 bp for *Mentha spicata* var. *Morocana* and *Mentha spicata* var. *Longofolia* genotypes, respectively and the other three fragments were present in all genotypes which are considered as common fragments.

Genetic similarity and cluster analysis based on ISSR markers: The ISSR data were used to estimate the genetic similarity values among the five genotypes of *Mentha* species/varieties by using UPGMA computer analysis (Table 9, Fig. 3). The highest similarity values were recorded (1.0) between *Mentha piperita* and *Mentha spicata* var. *Morocana* genotypes, while the lowest similarity value (0.33) was recorded between *Mentha viridis* and *Mentha spicata* var. *Longofolia* genotypes and there was no similarity between *Mentha aquatica* and *Mentha spicata* var. *Longofolia* genotypes. A dendrogram for the genetic relationship among the five genotypes of *Mentha* species/varieties is illustrated in Fig. 4. As they were separated into two major groups. The first group included only *Mentha spicata* var. *Longofolia* genotype while the second group was divided into two subgroups. The first subgroup included each of *Mentha aquatica*, *Mentha spicata* var. *Morocana* and *Mentha piperita* genotypes and the other subgroup included only *Mentha viridis* genotype.

Combined identification based on RAPD and ISSR analyses: Genetic similarities and phylogenetic relationships among the five *Mentha* species/varieties genotypes based on a combined data of RAPD and ISSR-PCR markers (Table 10, Fig. 5) were determined using UPGMA computer program. The highest similarity values were recorded (1.0) between *Mentha piperita* and *Mentha spicata* var. *Morocana* genotypes,while the lowest similarity value (0.21) was recorded between *Mentha viridis* and *Mentha spicata* var. *Longofolia* genotypes and there was no similarity between *Mentha aquatica* and *Mentha spicata* var. *Longofolia* genotypes. The dendrogram based on RAPD and ISSR-PCR markers (Fig. 5) separated the five *Mentha* species/varieties genotypes into two major groups. The first group included *Mentha viridis* and *Mentha aquatica* genotypes while the second group is divided into two subgroups. The first subgroup included only *Mentha spicata* var. *Longofolia* genotype while the other subgroup included *Mentha piperita* and *Mentha spicata* var. *Morocana* genotypes. These results could be explained on the bases that, due to ecological variation, there is possibility that some genomic mutations could be took place. In this regard, authors have hypothesized that the absence of intraclonal RAPD polymorphism cannot guarantee genetic stability, because important variations like genomic mutations could be missed (Palombi and Damiano, 2002; Cuesta *et al*., 2010). Moreover, study on genetic diversity of *Mentha* species showed that the taxa maintained high levels of genetic polymorphism among species but not among populations. The polymorphism within populations depicted genotype richness, recombination and gene flow. Clustering of populations based on UPGMA cluster analysis showed some unresolved accessions which were not clustered together. Polymorphisms revealed through RAPD and ISSR

Fig. 6(a-e): Herbs of different *Mentha* species/varieties cultivated under Egyptian sand soil condition which used in this study, (a) *Mentha viridis*, (b) *Mentha aquatica*, (c) *Mentha piperita*, (d) *Mentha species* var. *Morocana* and (e) *Mentha species* var. *Longofolia*

techniques may be due to deletion, elimination of primer binding site, an insertion making a fragment too large for polymerization and nucleotide substitutions in the primer annealing site (Fritsch and Rieseberg, 1992). The analysis of genetic variation both within and among plant materials is of fundamental interest to plant breeders. The genetic diversity of *Mentha* species is imprecise and of heterozygote nature. This obscures the determination of genetic diversity patterns based on morphological and phonological observations (Campos-de-Quiroz and Ortega-Klose, 2001). Fewer studies have been made on this genus, so a need was felt to explore and study this economically important genus. Considerable morphological and genetic variation was observed among *Mentha* species also showed close affinities with each other which might be due to sharing of almost similar habitat and ecology (Fig. 6). Further studies need to be done on different aspects including more species ecology, medicinal importance and further molecular studies. However, present results were in accordance with the DNA-based studies by Gobert *et al*. (2006) which demonstrated that mint species can be in most cases genetically clearly distinguished.

CONCLUSION

It may be concluded that *Mentha* is more suitable to sandy soil, thus we may recommend its cultivation in sand soil, of Egypt. Among the species and varieties of *Mentha* in this study, *Mentha piperita* was more suitable for sandy soil conditions than the others. The essential oil of *Mentha* species are useful for commercial purpose as they possess a range of aroma chemicals used in perfumery, flavour, pharmaceutical and other allied industries. Moreover, the major/marker constituents in their essential oils may be utilized as an important tool in oil authentication. *Mentha viridis* is a very good source of carvone (75.09%) and *Mentha piperita*, *Mentha spicata* var. *Longofolia* and *Mentha spicata* var. *Morocana* are a good source of β-caryophyllene (28.85, 46.75, 70.3%), respectively. The essential oil of *Mentha aquatica* is an excellent source of menthyl acetate (86.11%). This study provides evidence that RAPD and ISSR polymorphisms could be used as efficient tools for the detection of similarities and phylogenetic relationships of the studied genotypes. The same conclusion was obtained by Alexander (2002), Abdel-Tawab *et al*. (2001) and Heikal *et al*. (2007). These results may explain the results of volatile oil which classified the five *Mentha* species/varieties into three chemotypes: carvone type (*Mentha viridis*), menthyl acetate type (*Mentha aquatica*) and β-caryophyllene type (*Mentha piperita*, *Mentha spicata* var. *Longofolia* and *Mentha spicata* var. *Morocana*).

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