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Genetic Relationships of Some Mint Species Using Seed Storage Protein Pattern

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ABSTRACT

Mints are herbaceous perennial plants with aromatic leaves that are cultivated for their essential oils. The essential oil of Mint used in pharmaceutical, perfumery and cosmetics applications. The aim of this study was to analyze the relationship between nine different genotypes of *Mentha spicata* and *M. piperita* collected from different regions of Iran according to the seed storage protein profile using the SDS-PAGE method. The electropherogram showed a total of 31 bands of proteins with the molecular weight ranging from 12 kDa to 103 kDa. Among the genotypes G1, G5 and G9 showed the maximum number (26) of protein bands while the minimum number (9) of bands was present in genotype G7. Cluster analysis based on the seed storage protein profile, using the Euclidean distance matrix and the UPGMA method, grouped the evaluated genotypes into three clusters. Five genotypes (G1, G4, G5, G8, and G9) were in group 1, group two contained genotype G3 and the rest of the genotypes were in the third group (G2, G6, G7). Cluster analysis showed that some of the genotypes of two different species of *M. piperita* and *M. spicata* were closely related to each other and were in the same group. With regard to *M. piperita* is a natural hybrid of *M. spicata* and *M.aquatica*, this result is expected. The protein variability analysis clearly showed that there was divergence among and within the studied genotypes. It was concluded that this study will be useful for the future breeding and conservation program of *Mentha* genotypes.

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Introduction

The *Mentha* genus (Lamiaceae family) contains 18 species and 11 hybrids (Šarić-Kundalić *et al*., 2009) that are distributed in temperate and sub-temperate regions of the world (Baht *et al*., 2002; El-Ghorab, 2006). *Mentha* species have been cultivated for thousands of years. Essential oils of *Mentha* species are used for food, flavor, cosmetic and pharmaceutical industries because of the presence of certain monoterpenes (Baht *et al*. 2002). Nowadays, *Mentha piperita* (Peppermint) is the most commonly cultivated species but in earlier times, *Mentha spicata* (spearmint) has been widely cultivated (Taghiloofar *et al*., 2014). Spearmint and peppermint are aromatic plants belonging to the Lamiaceae family that is cultivated commercially all over the world. The fresh and dried leaves of spearmint are used as a spice and herbal tea (Areias *et al*., 2001; Badr *et al*., 2003). Indeed, basic information about genetic diversity is required to have a variety of better traits of any crop (Shinwari *et al*., 2013). Knowledge of genetic variation is a useful tool for the selection of desirable genotypes, utilization of germplasm and gene bank management (Elham *et al*., 2010; Vishwanath *et al*., 2011).

The characterization of germplasm is necessary for the evaluation of genetic resources and using desirable genotypes in conservation and breeding programs (Hamrick *et al*., 1991; Crawford *et al*., 2001). In different plants, seed storage protein pattern has been used successfully to determine taxonomic and evolutionary processes (Khan *et*

al., 2010). The SDS-PAGE of seed storage proteins method is used to classify plant varieties according to protein pattern because these proteins are highly preserved (Iqbal *et al.*, 2005; Javaid *et al.*, 2004). Since plants have different types of proteins that are diverse from each other, so this property is used to identify diversity using the SDS-PAGE method (Rahman and Hirata, 2004; Shah *et al*., 2011). One of the methods to estimate genetic variation is the seed storage protein pattern using SDS-PAGE (Kakaei and Kahrizi, 2011). Because of usefulness to identify the genetic construction of crop germplasm, SDS-PAGE is commonly used as the biochemical techniques (Nisar *et al*., 2011). Also, the first step of improving programs is the evaluation of genetic diversity and classification of the plant (Kakaei and Kahrizi, 2011). Since storage proteins are highly independent of environmental changes and are the direct product of genes (Bompalli and Nallabilli, 2013), the SDS-PAGE of proteins is particularly considered as a reliable technology (Javaid *et al*., 2004; Iqbal *et al*., 2005). So this technique could be used to assay variation among genotypes (Stoynova *et al*., 1992). In addition, its banding pattern is very stable which is used for identification purposes in medicinal plants and to assess the genetic variation among the populations of the wild species (Elham *et al*., 2010; Vishwanath *et al*., 2011). Seed storage protein study on some populations of *Salvia* showed 22 bands which some of them were specific for the population (Jafari *et al*., 2009). The cluster analyses of protein electrophoretic criteria showed that environmental changes play a major role in genetic variations among the examined populations of *Mentha* in Egypt (Badr *et al*., 2003). Using seed storage protein, accessions of *Cuminum cyminum, Foeniculum vulgare, Falcaria vulgaris* were classified into three groups (Masoumi *et al*., 2012). Thus, the objective of this study was to investigate genetic diversity in *Mentha piperita and M. spicata* genotypes in Iran based on variations in their seed storage electrophoretic profiles.

Materials and Methods

For this research, overall nine genotypes of two species including *M. spicata* and *M. piperita* were supplied from Isfahan Pakan Bazr Company (Table 1). Then, the seeds were separately powdered. The protein extraction carried out with extraction buffer (Tris–HCl 0.05 M, pH 7.4; 2% SDS and 2.5% 2-mercaptoethanol), other steps were done according to Xi *et al.* (2006). The SDS-PAGE method in resolving gel with 12.5 % acrylamide and stacking gel with 5 % acrylamide was applied to assess extracted proteins in these genotypes.

Table 1. Species of the genus *Mentha* collected from different sites.

Code	Specie	Region
G1	*M. piperita*	Jahrom-Shiraz
G2	*M. piperita*	Isfahan
G3	*M. piperita*	Kashan
G4	*M. piperita*	Mashhad
G5	*M. piperita*	Nishabur
G6	*M. spicata*	Dezful
G7	*M. spicata*	Farhadi Village-Ardestan
G8	*M. spicata*	Qum
G9	*M. spicata*	Qumrod-Qum

After electrophoresis, the gel was stained with the coomassie brilliant blue R-250 and destained with methanol and acetic acid. The identification of each protein band was carried out according to the standard protein marker. Following SDS-PAGE bands scoring, the NTSYS-pc software version 2.02 was used for cluster analysis via the UPGMA method, principal coordinate analysis (PCoA) and calculating the cophenetic correlation coefficient between the similarity matrix and the cophenetic matrix derived from the dendrogram.

Results

As shown in Fig. 1, 31 protein bands with diverse molecular weights ranging from 12 to 103 kDa were identified in the studied genotypes. The genotypes showed considerable variation in protein band numbers ranging from 9-26. The least molecular weight protein (12.2 kDa) was present in all genotypes with an Rf value of 0.92 and the highest molecular weight protein (98.2 kDa) was found in G1, G4, G5, G8 and G9 genotypes with Rf value of 0.1 (Table 2). Bands number 8, 14, 15, 16, 21, 22, 23, 26, 30 and 31 were common in all studied genotypes. Variability in intensity was viewed in some protein bands that showed the number of protein peptides mounting up at a specific molecular

weight**.** Results of AMOVA analysis (Table 4) showed that the percentages of molecular variance within genotypes are 100%.

Table 2. Number, relative mobility and molecular weight of observed bands

Band	Rf	MW	Band	Rf	MW
1	0.10	103.59	17	0.55	31.94
2	0.12	98.20	18	0.57	30.83
3	0.14	93.08	19	0.61	27.21
4	0.166	86.68	20	0.62	26.73
5	0.172	85.15	21	0.66	24.45
6	0.19	80.71	22	0.68	23.18
7	0.23	72.53	23	0.71	21.20
8	0.26	68.75	24	0.72	20.46
9	0.29	62.89	25	0.75	19.05
10	0.31	59.61	26	0.79	17.43
11	0.34	54.53	27	0.80	16.82
12	0.37	51.69	28	0.81	16.52
13	0.41	46.45	29	0.83	15.38
14	0.45	41.74	30	0.91	12.64
15	0.46	40.27	31	0.92	12.20
16	0.50	36.84	-	-	-

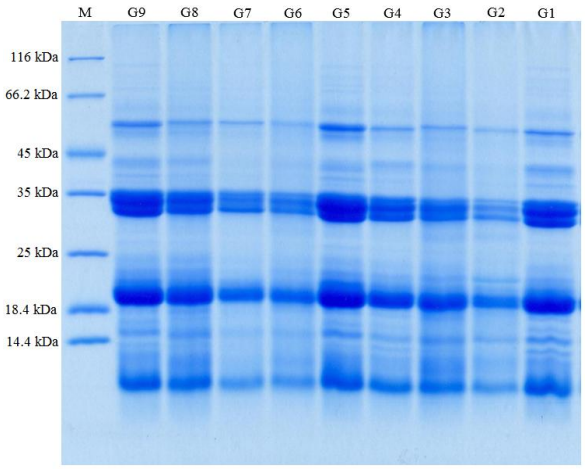

Fig. 1. Electrophoretic banding pattern produced by SDS-PAGE of total seed proteins of *Mentha* species; M= Molecular weight marker; G1= *M.piperita* (Jahrom); G2= *M.piperita* (Isfahan); G3= *M.piperita* (Kashan); G4= *M.piperita* (Mashhad); G5= *M.piperita* (Nishabur); G6= *M. spicata* (Dezful); G7= *M. spicata* (Ardestan); G8= *M. spicata* (Qum); G9= *M. spicata* (Qumrod).

Table 4. Analysis of molecular variance among and within species using SDS-PAGE pattern.

S.O.V	(df)	SS	MS	Est.Var	%Var
Between Populations	1	2.267	2.267	0	0
Within populations	7	32.4	4.629	4.629	100
Total	8	34.667		4.629	100

The Polymorphism percentage for *M. spicata* and *M. piperita* was 54.84 and 51.61 respectively. Among the genotypes G1, G5 and G9 showed the maximum number (26) of protein bands while the minimum number (9) of bands was present in genotype G7. The band number three was observed only in genotypes G1, G4, G5 and G9. Besides, this study indicated a low level of heterozygosity (*He*=0.221-0.243) in *M. piperita* and *M. spicata* respectively. Also, the effective number of alleles (Ne=1.46-1.38) and Shannon information index (I=0.34-0.32) were observed in *M. piperita* and *M. spicata* respectively (Table 3).

Table 3. Genetic diversity parameters determined in *M. piperita* and *M. spicata*.

Species	N	Na	Ne	I	He	uHe	%P
M.piperita	5	1.36	1.46	0.34	0.24	0.27	51.61
M.spicata	4	1.39	1.38	0.32	0.22	0.25	54.84

The matrix of eigenvectors and values of the principal components (PCs) resulting from electrophoretic data of the total seed proteins (Table 6) indicated that the first three components justified 88% of the total variation. The scatter of genotypes based on the first two components was in agreement with cluster analysis results (Fig. 3). Cluster analysis based on the seed storage protein profile in the evaluated genotypes, using the Euclidean distance matrix and UPGMA method indicated three groups, five genotypes (G1, G4, G5, G8, and G9) were in group 1. The group two consisted of genotype G3 and the rest of the genotypes were in the third groups: G2, G6, and G7 (Fig. 2)**.** Cluster 2 was the smallest cluster having one genotype. Cluster 1 was the largest cluster with the maximum number of 5 genotypes, that contained 3 sub-clusters, sub-cluster I, II and III with 3, 1 and 1 genotypes, respectively. Cluster 3 was further divided into 2 sub-clusters, sub-cluster I and II with 2 and 1 genotypes, respectively**.**

Table 6. Principal components analysis for seed storage pattern in *M. piperita* and *M. spicata*.

	Eigen values	Relative variance	Cumulative variance
PC1	1.95	67.99	67.99
PC2	0.378	13.14	81.14
PC3	0.199	6.93	88.07

Table 5. Jaccard similarity coefficients between genotypes of *Mentha*.

	G1	G2	G3	G4	G5	G6	G7	G8	G9
G1	1								
G2	0.423	1							
G3	0.653	0.647	1						
G4	0.923	0.400	0.640	1					
G5	1	0.423	0.653	0.923	1				
G6	0.423	0.833	0.647	0.458	0.423	1			
G7	0.346	0.818	0.529	0.375	0.346	0.818	1		
G8	0.923	0.400	0.640	0.920	0.923	0.458	0.375	1	
G9	1	0.423	0.653	0.923	1	0.423	0.346	0.923	1

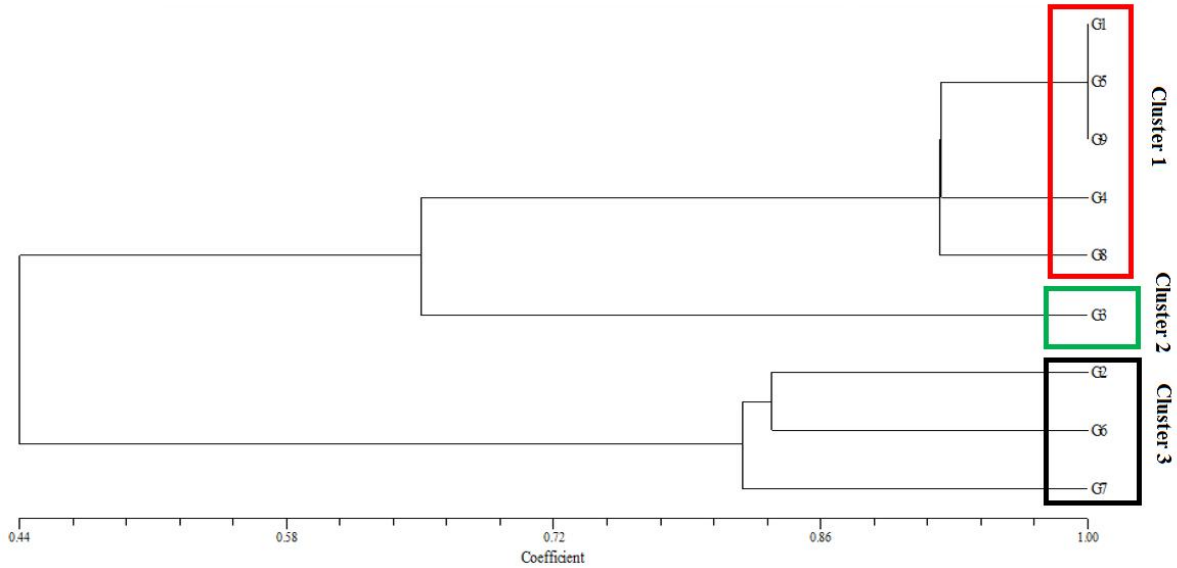

Fig. 2. UPGMA cluster analysis based on seed storage pattern.

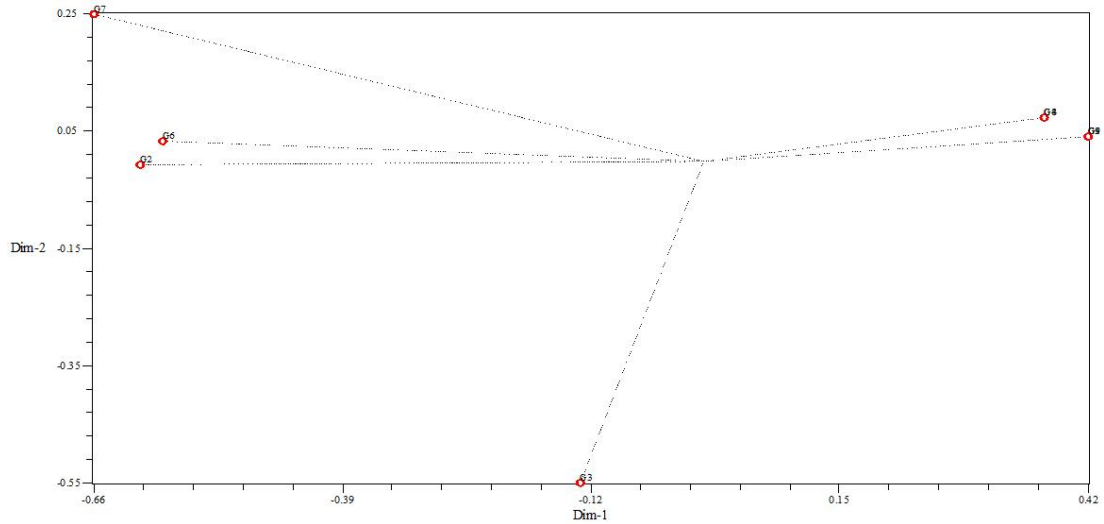

Fig. 3. The principal coordinate plot of Jacquard's genetic similarity for the first two principal components.

Jacquard's similarity coefficients were based on the data of SDS-PAGE profiles of the evaluated genotypes (Table 5). The similarity coefficients were calculated on the basis of the presence and absence of bands. The range of similarity coefficients was from 0.346 to 1.00.

Discussion

With respect to the necessity of genetic diversity determination in plant breeding programs, knowledge of genetic variability could be used for efficient sampling and selection of better genotypes that would be applied in improvement programs (Elham *et al*., 2010; Vishwanath *et al*., 2011). With respect to the high level of genetic polymorphism among the nine studied genotypes of two species including *M. piperita* and *M. spicata*, it can be concluded that seed protein pattern is a useful method for evaluation of these genotypes. These results were confirmed by (Kumar and Tata, 2010) and (Alwhibi, 2017) which examined *Chili peppers* and *Heliotropium digynum* respectively. Their results showed that seed storage protein analysis can be successfully applied to assess genetic diversity in germplasm. Plants have different types of proteins which they are diverse from each other and this could be a source of genetic diversity in plants (Shah *et al*., 2011). Ten bands with molecular weight 68.75, 41.74, 40.27, 36.84, 24.45, 23.14, 21.2, 17.43, 12.64 and 12.20 KDa were common in all studied genotypes. A low level of differences in major bands in different genotypes indicates that genes coding for these proteins is preserved in the species (Ali *et al*., 2007).

In the previous study, SDS-PAGE was utilized to determine the variability in different genotypes of *Ocimum sp* (Bompalli and Nallabilli, 2013), some population of *salvia* (Jafari *et al*., 2009) and *Mentha* populations in Egypt (Badr *et al*., 2003) which confirmed the results of this research. The polymorphism percentage for *M. spicata* and *M. piperita* was 54.84 and 51.61 respectively. Assessment of genetic diversity of some populations of *Thymus kotschyanus* (Khoshsokhan *et al*., 2014) and salvia (Safaei *et al*., 2016) showed high levels of diversity within populations than among populations that confirmed the result of this research. It is clear that the high genetic diversity may allow populations to adapt to environmental changes easily (Khoshsokhan *et al*., 2014). In addition, high genetic diversity within species may have occurred because of new mutations that gradually accumulate within populations of certain species (Safaei *et al*., 2016). With regard to AMOVA and heterozygosity, it can be concluded that the most observed variation was related to variability within genotypes. The variability within genotypes can reflect genotype richness, recombination and gene flow (Yousef and El-Leel, 2015) and could be due to out-breeding, the wide dispersal of pollen grains and seeds (Lawrence, 2007), cross-pollinated nature (Momeni *et al*., 2006), the differences in chromosomes numbers (Gobert *et al*., 2003; Lawrence 2007) and occurrence of the natural interspecific hybridization with high frequency in *Mentha* (Gobert *et al*., 2003). Shasany *et al.* (2005) evaluated genetic variation in *M. arvensis* and *M. spicata* using RAPDs and AFLPs markers which were showed the existence of a higher polymorphism in interspecific rather than intraspecific hybridizations. It might be suggested to use molecular markers for the determination of genetic variation among the presently studied genotypes. Based on cluster analysis, it was showed that some of the genotypes of two different species of *M. piperita* and *M. spicata* are closely related to each other and were in the same group but genotypes in the same species, grouped in distinct clusters. Since, *M. piperita* is a hybrid between *M. spicata* and *M. aquatica* (Murray *et al*., 1972), it is expected that these two species grouped in the same

cluster. As shown in figure 1, G1 and G5 genotypes of *Mentha piperita* that collected from Jahrom and Nishabur showed 100% genetic similarity. So, it was suggested that geographical origin could not be an appropriate index of genetic structure among genotypes (Shinwari *et al*., 2011). Yousef *et al*. (2015) showed the most two closely related cultivars of *M. piperita* and *M. spicata* with the highest similarity index (1) using RAPD and ISSR markers. It has been shown that *M. spicata* and *M. piperita* were closely related and clustered in the same group using RAPD markers (Momeni *et al*., 2006).

These studies are in agreement with the result of the present research. Cluster analysis based on the seed storage protein profile indicated three groups, five genotypes (G1, G4, G5, G8, and G9) were in group 1, group 2 contain genotype G3 and the rest of the genotypes were in the third group (G2, G6, G7) that showed intraspecific variation. The high similarity between species in Mint could be attributed to natural hybridization between these species during their evolution (Zinodini *et al*., 2013). This biochemical assessment provides the first information on the diversity of *M. spicata* and *M. piperita* genotypes in Iran based on the SDS-PAGE result.

The present research activity will be helpful to make a gene bank of genetic resources of diverse *M. piperita* and *M. spicata* genotypes in Iran. In previous studies, estimation of genetic distance using the number of shared bands showed that native spearmint is much more closely related to *M. gracilis* than to peppermint (Fenwick and Ward, 2001) and on the basis of RAPD markers *M. piperita* is closer to *M. aquatica* than *M. spicata* (Momeni *et al*., 2006). With regard mentioned studies, investigation of the relationship between *M. aquatica*, *M. piperita*, *M. gracilis,* and *M. spicata* is recommended. Moreover, the genotypes of nearby regions were in different clusters which showed that the genetic diversity and geographic distribution were independent of each other and no definite relationship existed between genetic diversity and geographic diversity.

Conclusion

In the study of genetic relationships of some mint species using seed storage protein pattern, it was shown more intra-species variation in comparison to inter-specific variation. However, the SDS-PAGE technique is sufficient for the discrimination of the *M. piperita* and *M. spicata* genotypes solely. So the use of molecular markers is suggested to investigate the genetic diversity and relationships of the studied genotypes.

Conflicts of interest

The authors have declared that no competing interests exist.

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