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Original Article

Genetic and Morphological Diversity of Wild Mint "*Mentha longifolia* (L.) Hudson subsp. *noeana* (Briq.) Briq." in South and Southeastern Iran

Zeinab Panjeshahin, Gholam-Reza Sharifi-Sirchi* and Davood Samsampour

Department of Agriculture, Faculty of Agriculture and Natural Sources, University of Hormozgan, Bandar Abbas, Iran

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Abstract

The wild mint "*Mentha longifolia* (L.) Hudson subsp. *noeana* (Briq.) Briq." is an important medicinal plant which contains several worthy components like: menthone, pulegone, pipertenon, menthol, thymol, and carvone in its essential oil. This plant grows extensively in center of Asia including Iran. Because of so worthy components and its big role in traditional and modern medicine, the wild mint diversity was considered based on morphological and phytochemical variables and DNA markers (RAPD and ISSR) in South and Southeastern Iran. A total of 64 genotypes of *M. longifolia* subsp. *noeana* genotypes were collected from 8 distribution centers. In total, the 15 morphological and pigments variables were measured and calculated. The cluster analysis based on morphological and phytochemical variables divided populations in the 4 main groups. The fifteen RAPD primers with reproducible and score able amplifications characters were selected. Cluster analysis based on RAPD markers revealed seven main clusters in genetic distance 0.22. The nine ISSR primers with score able amplifications characters were selected. Cluster analysis based on ISSR markers revealed five main clusters in genetic distance 0.23. The results of this research showed enough genetic diversity among and inside of the studied wild mint populations which could be applied in following breeding and gene bank conservation programs.

Keywords: Cluster analysis, ISSR, Morphological variable, RAPD, Wild mint

Introduction

The wild mint "*Mentha longifolia* (L.) Hudsonsubsp. *noeana* (Briq.) Briq.", family *Lamiaceae* grows extensively in center of Asia (as: Iran, Turkey, Tajikistan, Afghanistan, Nepal, and China). The plant is a variable perennial with a peppermint-scented aroma. It has a creeping rhizome along an underground rootstock with straight stems 40-120 cm in height. The leaves are oblong-elliptical to lanceolate, thinly to densely tomentose, green to greyish-green above and white below opposite each other along the square-shaped stem. The flowers are 3-5 mm long, lilac, purplish, or white, produced in dense clusters on tall, branched, and tapering spikes. The essential oil of *M. longifolia* subsp. *noeana* is rich in monoterpenes including: oxygenated monoterpenes, pulegone, menthone, isopulegole, isopulegone, and 1, 8-cineole. The percentages of sesquiterpenes in the essential oil of *M. longifolia* subsp. noeana is low, and including: caryophyllene oxide. Cis Piperitone epoxide, pulegone, piperitenone oxide, menthone, and isomenthone [1-3]. The other important *M. longifolia* subsp. *noeana* essential oil compounds are menthol, thymol, and carvone [4]. In pharmacological research and traditional folk research, there are plenty of documents for different biological effects of *M. longifolia* subsp. *noeana* organs (leaves, flower, stem, bark, and seeds) and components in medication and therapy including: gastrointestinal disorders (to stop diarrhea), antimicrobial

*Corresponding author: Department of Agriculture, Faculty of Agriculture and Natural Sources, University of Hormozgan, Bandar Abbas, Iran
Email Address: sharifisirchi@yahoo.com

(against *Escherichia coli*, *Salmonella typhimurium*, *Listeria monocytogenes*, *Aspergillus flavus*, *Botrytis cinerea*, *Fusarium oxysporum*, *Pseudomonas aeruginosa*, *Aspergillus niger*, *Trichophyton longifusus*, *Microsporm canis*, and *Mucor ramamnianus*), carminative, stimulant, and antispasmodic [5].

The plant genetic diversity represents heritable morphological, phytochemical and structure variation intra and inter plant species. Appropriate knowledge of mating system, floral morphology and mode of reproduction along with geographical diversity pattern of a species are necessary and basic information for screening and improving plant species with interested traits [6]. In recent years, there has been a significant increase in the application of morphological and molecular markers for assessing plant phylogeny, evolution and selecting appropriate conservation program for plant genetic resources. RAPD and ISSR techniques are quick, dominant, and easy PCR-based molecular markers for diversity study of unknown sequence plant species. Inter-simple sequence repeats (ISSRs) are genome regions which flanked by microsatellite sequences. PCR amplification of ISSRs using a single primer yields multiple amplification products .RAPD marker does not require prior DNA base sequence information and detects nucleotide sequence polymorphism using a single primer of arbitrary nucleotide sequence [7].

Wild mint plant is distributed widely through Iran and extensively medicated in Iranian traditional medicine. Because of the high medicinal value, loss, degradation and fragmentation of its natural habitats and risk of genetic eradication because of climate changes, deep analysis on wild mint traits is necessary to have proper view on cultivation, breeding, active genetic conservation of this medicinal, ecological and economical useful plant. The objective of this research was to analyze the morphological, and genetic diversity of 64 *M. longifolia* subsp. *noeana* genotypes from 8 important distribution centers of Iran for having proper programs on conservation and breeding of Iranian wild mint.

Material and Methods

Plant Material

A total of 64 genotypes of *M. longifolia* subsp. *noeana* genotypes were collected from 8 distribution centers of South and South East of Iran. The samples were collected at full bloom stage during July in 2015 and 2016. The sampled regions include: 3 regions in Kerman province (including: Sirch, Bardsir, and Jiroft), 2 regions from Fars province (including: Darab and Sepidan), 2 regions from Hormozgan province (including: Sikhuran and Segh), and Anarestan region from Bushehr province. The all distribution centers information including: Latitude, Longitude, and Altitude are registered in Table 1. Some information such as: leaf length, leaf width, petiole length scored in situ. All of the samples were kept up separately in -20 °C for other laboratory observations.

Morphological and Pigment Studies

In total, the 15 morphological and pigments variables including: the leaf length, width, and length/width rate (mm), plant height (cm), main stem length (cm), ear length and diagonal (mm), stem diagonal (mm), number of internodes on main stem, length of internodes on main stem (mm), number of brunches on main stem, chlorophyll a (mg g^{-1}), chlorophyll b (mg g^{-1}), total chlorophyll (mg g^{-1}), and carotenoid (mg g^{-1}) were measured.

Table 1 Geographic distribution and continental information of collected *Mentha longifolia* (L.) Hudson subsp. *noeana* (Briq.) Briq. genotypes.

No.	Population	Province	Genotype number	Longitude(E)	Latitude(N)	Altitude(m)
1	Jirft	Kerman	8	57° 44'	28° 40'	720
2	Sirch	Kerman	8	57° 32'	30° 11'	416
3	Bardsir	Kerman	8	56° 34'	29° 56'	2040
4	Sepidan	Fars	8	52° 16'	30° 02'	2300
5	Darab	Fars	8	54° 33'	28° 45'	1180
6	Sikhuran	Hormozgan	8	56° 28'	27° 50'	920
7	Segh	Hormozgan	8	56° 19'	27° 45'	720
8	Anarstan	Busheher	8	52° 21'	27° 47'	700

Pigment Studies

Extraction was performed with 80% aceton based on Mohammadi *et al*. 2017 [8]. Chlorophyll and Carotenoid contents were colorimetrically determined using the following formula:

Chl_a (mg g^{-1})=[(12.7×A_{663})-(2.6×A_{645})]×ml acetone / mg leaf tissue

Chl_b (mg g^{-1})=[(22.9×A_{645})-(4.68×A_{663})]×ml acetone / mg leaf tissue

Chl_T (mg g^{-1})=[(20.2×A_{645})+(8.02×A_{663})]×ml acetone / mg leaf tissue

C_{x+c}=1000A_{470}-1.90Chl_a-63.14Chl_b/214,

(x=xanthophylls and carotenes).

Were A is absorption value, V is ultimate volume of extract and W is leaf fresh weight.

Statistical Analysis

Multivariable analysis was performed on the measured fifteen variables. Cluster analysis was made using Xlstat 2016 statistical software by ward method. Analysis of variance (ANOVA) was performed with SAS software (SAS Institute, version 9.1.3 Cary, NC). The statistical significance was determined at the $p \leq 0.05$, and 0.01.

Genetic Study

DNA Extraction

Genomic DNA was extracted from young leaves of genotypes of *M. longifolia* subsp. *noeana* using the modified CTAB method. The CTAB extraction buffer consists of 2.0 g cetyl trimethylammonium bromide, 1.0 g polyvinyl pyrrolidone, 10.0 ml Tris-base1 M (pH 8), 28.0 ml NaCl5 M, 4.0 ml EDTA0.5 M (pH 8), and 40 ml H2O, which 100 µl 2-mercaptoethanol added to each 20 ml of CTAB extraction buffer. The young leaves (500 mg) from each sample were cryogenically ground in a mortar and pestle after chilling in liquid nitrogen. Pre heated-CTAB extraction buffer (1.5 ml) at 65°C was added to the fine homogenized leaf powder. The samples were then incubated at 65°C for 1 h in a water bath with slow shaking every 10 min. Subsequently, the mixture centrifuged for 15 minutes at 12600 rpm. Then, supernatant transferred to clean microfuge tube and an equal volume of chloroform: isoamyl alcohol (24:1) was added to solution and then, the solution was mixed by inversion. The mixture centrifuged for 5 min at 12600 rpm. The upper aqueous phase only transferred to a clean microfuge tube. To each tube added 500 µl of ice cold absolute ethanol and 50 µl of 7.5 M sodium acetate. After incubation at - 20 °C overnight, the mixture centrifuged for 30 min at 12600 rpm and then, the supernatant removed and allowed the DNA pellet to dry. Finally re-suspended the DNA pellet in 50 µl sterile water and stored at – 20 °C until using in PCR. The quality of the DNA was estimated on an agarose gel (1%).

RAPD and ISSR-PCR

Thirty RAPD primers were chosen for preliminary amplification tests. In primer screening, DNA amplifications were repeated at least twice for each primer. Fifteen RAPD primers (Table 2) with reproducible and score able amplifications characters were selected for next investigations.

PCR reactions were performed in a Thermal Cycler (from BIO-RAD) with a total volume of 25 µL containing 1×PCR reaction buffer, 1.5 mM MgCl2, 0.2 mM dNTPs, 20 pmol primer, 10 ng template DNA and 1U of *Taq* DNA polymerase. PCR condition was set at 94 °C for 5 min, following 35 cycles of 94 °C for 1 min, 30-34 °C (depend on Tm of each primer) for 45 sec, and 72 °C for 2 min, which followed by a final extension of 5 min at 72 °C.

The nine ISSR primers (Table 3) with score able amplifications characters were selected for next investigations. PCR condition for ISSR fragments amplification was set 94 °C for 5 min, following 40 cycles of 94 °C for 1 min, appropriate Ta depend on Tm of each primer for 45 sec, and 72 °C for 2 min, which followed by a final extension of 5 min at 72 °C.

The samples were run in 1.5% agarose gel in 1× Tris-borate-EDTA (TBE, pH 8.3) buffer. The gels were run at voltage 90 for 120 min and photographed under UV light using gel documentation system. The nucleic acid markers 100 bp-3 kb (Pishgam Company, Tehran, Iran) were used to compare the amplification product sizes.

Molecular Analysis

The RAPD and ISSR bands were scored for their presence (1) or absence (0) and then, transformed into a binary matrix. Polymorphic information content (PIC) was calculated, using the formula described by Powell *et al*. 1996 [9]. Cluster analysis based on genetic similarities was performed by using NTSYS software version 2.02 [10].

Table 2 The RAPD primers which used for genetic diversity study of eight populations of *Mentha longifolia* (L.) Hudson subsp. *noeana* (Briq.) Briq. in Iran.

Primer name	Sequences of primers	GC %	Melt temp °C
BA16	5'-CCACGCATCA-3'	60	32
BD13	5'-CCTGGAACGG-3'	70	32
BD17	5'-GTTCGCTCCC-3'	70	32
BE06	5'-AAGCGGCCCT-3'	70	32
D16	5'-AGGGCGTAAG-3'	60	32
J3	5'-TGCTAGCCTC-3'	60	32
J5	5'-CTCCATGGGG-3'	70	32
MAP04	5'-TGCGCGATCG-3'	70	32
MAP10	5'-GCGAATTCCG-3'	60	32
MAP20	5'-AGCCTGACGC-3'	70	32
MAP14	5'-AGGATACGTG-3'	70	30
OPA-01	5'-CAGGCCCTTC-3'	70	32
OPA-08	5'-GTGACGTAGG-3'	60	32
OPA-09	5'-GGGTAACGCC-3'	70	32
U532	5'-TTGAGACAGG-3'	50	30

Table 3 The ISSR primers which used for genetic diversity study of eight populations of *Mentha longifolia* (L.) Hudson subsp. *noeana* (Briq.) Briq. in Iran.

Primer name	Sequences of primers	GC%	Melt temp
UBC817	CACACACACACACACAA	47.1	50
UBC823	TGTGTGTGTGTGTGTGC	52.9	52.4
UBC824	TCTCTCTCTCTCTCTCG	52.9	52.4
UBC825	ACACACACACACACACT	52.9	50
UBC809	AGAGAGAGAGAGAGAGG	47.4	52.4
UBC810	GAGAGAGAGAGAGAGAT	47.1	50
UBC816	CACACACACACACACAT	47.1	50
UBC808	AGAGAGAGAGAGAGAGC	52.9	52.4
UBC811	GAGAGAGAGAGAGAGAC	52.9	52.4

Table 4 Variance analysis and heredity of studied variables in *Mentha longifolia* (L.) Hudson subsp. *noeana* (Briq.) Briq. genotypes.

Morphological and phytochemical variables	Variance analysis		heredity
	genotypic	phenotypic	
Shoot length	68730.71	59714.48	86.88
Main stem length	25579.25	15352.58	60.01
Leaf length	181.64	76.85	42.30
Leaf width	54.42	38.81	71.31
Length/Width rate	66.94	36.43	54.42
Ear length	752.89	577.01	76.63
Ear diagonal	1.73	0.53	30.63
Stem diagonal	0.414	0.026	6.28
Number of internodes in main stem	11492.68	3323.1	28.91
Length of Internodes	183.68	19.96	10.86
Number of main branches	19914.44	10385.65	52.15
Chlorophyll a	0.0214	0.02	93.45
Chlorophyll b	0.0218	0.019	87.15
Total Chlorophyll	0.0277	0.025	90.25
Carotenoid	0.476	0.02	4.20

Results

Morphological and Pigments Study

The analysis of variance and heredity showed that the traits of chlorophyll a, chlorophyll b, total chlorophyll, shoot length, ear length, and main stem length had heredity higher than 60% (Table 4). The traits of carotenoid, stem diagonal, and length of internodes in main stem had heredity less than 20% (Table 4).

Clustering Analysis Based on Morphological and Phytochemical Variables

Cluster analysis based on morphological and phytochemical variables divided populations in the 4 main groups. The first main group consisted of 13 genotypes of Sikhuran 1-3, Jiroft 3, Segh 1-3, Bardsir 1-3, and Bushehr 1-3. The second main group (the largest) was comprised of 24 genotypes of Darab 1-8, Jiroft 1 and 2, Bardsir 4, 5, 7, and 8, Sepidan 1-8, Sikhuran 6, and Sirch 5 (Fig. 1). The third main group consisted of 7 genotypes of Sirch 1-4, and Sirch 6-8. The fourth main group comprised of 20 genotypes of Anarestan 4-8, Segh 4, 5, 7 and 8, Jiroft 4-8, Sikhuran 2, 4, 5, and 7, 8, Bardsir 6, (Fig. 1). According cluster analysis. The main clusters cleave to tributary clusters which close genotypes were in them.

PCA Analysis

Principle component analysis (PCA) was evaluated and the first and second principal components which were a result of the linear combination of the 15 studied variables, were indicated. These two principal components explained 29.182% and 19.038% of the variance, respectively. The main four factors had 81.038% accumulated eigenvalue (Table 5).

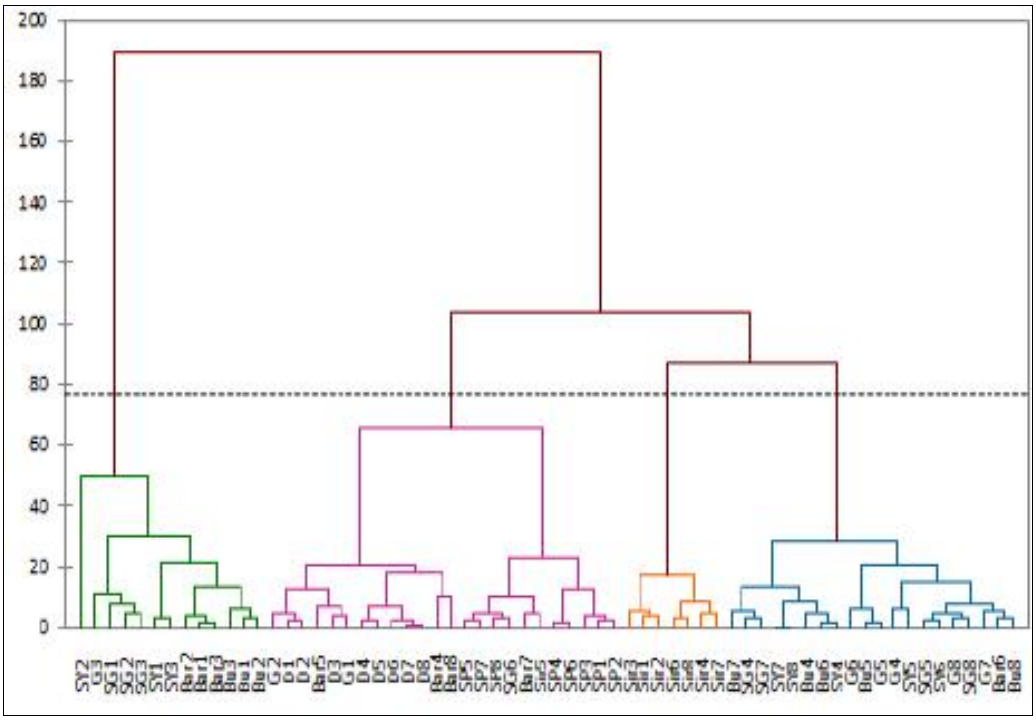

Fig. 1 The clustering of *Mentha longifolia* (L.) Hudson subsp. *noeana* (Briq.) Briq. genotypes by morphological and phytochemical variables (Dissmilarity). D; Darab; Sp; Sepidan; Sir: Sirch; Bar: Bardsir; G: Jiroft; Bu: Anarestan; SY: Sikhuran; SG: Segh

Table 5 Eigenvalue and accumulated proportion of principal component analysis.

Factor	Eigenvalue	Variability (%)	Cumulative (%)
1	4.377	29.182	29.182
2	2.856	19.038	48.220
3	2.428	16.186	64.406
4	1.313	8.756	73.162
5	1.181	7.876	81.038

Table 6 Polymorphism characteristics of each RAPD maker in genetics diversity study of the *M Mentha longifolia* (L.) Hudson subsp. *noeana* (Briq.) Briq. populations.

Primer	N	P	PIC	H	I	H_s	G_{ST}	N_m
BA16	17	17	0.18	0.32	0.49	0.25	0.07	2.39
OPA08	13	13	0.19	0.35	0.52	0.28	0.08	2.75
MAP20	17	16	0.45	0.39	0.59	0.22	0.19	0.77
BE06	11	11	0.23	0.32	0.48	0.24	0.09	2.92
OPA01	14	14	0.28	0.46	0.47	0.22	0.1	1.55
OPA09	13	13	0.29	0.29	0.51	0.21	0.1	2.45
BD13	16	16	0.20	0.28	0.4	0.21	0.08	3.66
BD17	17	17	0.18	0.32	0.47	0.25	0.08	3.66
U532	12	12	0.19	0.28	0.46	0.22	0.07	3.03
J5	11	11	0.33	0.39	0.57	0.26	0.15	1.31
MAP04	13	13	0.26	0.35	0.53	0.26	0.1	1.97
D16	14	14	0.19	0.26	0.48	0.23	0.05	2.79
J3	12	12	0.23	0.32	0.49	0.24	0.09	2.37
MAP14	14	13	0.13	0.28	0.49	0.27	0.05	4.15
MAP10	14	14	0.21	0.38	0.57	0.3	0.09	2.97
Total	207	206	-	-	-	-	-	-
Average	13/86	-	0.23	0.33	0.50	0.24	0.09	2.85

N: total number of bands; P: number of polymorphic bands; H: Nei's gene diversity; I: Shannon's information index; H_S: Genetic diversity index within populations; G_{ST}: Genetic differentiation coefficient between populations; Nm: Gene flow.

Genetic Studies

RAPD Polymorphism

The fifteen RAPD primers generated 208 bands, which 206 of them were polymorphic (99.03%). The number of band varied from 11 (primer J5) to 17 (primers of BA16, MAP20, and BD17) (Table 6). The polymorphic level, calculated as the number of polymorphic band per total number of bands, and it was 100% for the 13 applied primers. Just, MAP14 and MAP20 RAPD primers showed lower polymorphic rate with amplifying one monomorphic band, each (Table 6).

Population genetic diversity, differentiation (GST), and gene flow (Nm)

Between applied RAPD markers, the polymorphism information content (PIC) varied from 0.13 (primer MAP14) to 0.45 (primer MAP20), with an average of 0.23 (Table 6). The Nei's gene diversity index (H) varied from 0.26 (D16) to 0.46 (OPA01), with an average of 0.33. The Shannon's information index (I) ranged from 0.4 (BD 13) to 0.59 (MAP20), with an average of 0.50. The genetic diversity index within populations (HS) varied from 0.21 (OPA09 and BD13) to 0.3 (MAP10), with an average of 0.24. The genetic differentiation coefficient between populations (G_{ST}) ranged between 0.05 (D16) and 0.19 (MAP20), with an average of 0.09. The gene flow (Nm) ranged between 0.77 (MAP20) to 4.15 (MAP14), with an average of 2.85, which indicated that there was a medium gene exchange between *M. longifolia* subsp. populations (Table 6). The Nei's gene diversity (H) among populations ranged from 0.21 (Sikhuran) to 0.27 (Sirch and Darab). The Nei's gene diversity (H) and the Shannon's information index (I) were highest in the Darab population (H=0.41; =0.27) followed by Sirch population (H=0.40; I=0.27). Segh population had the lowest Shannon's information index (I = 0.18; H=0.25).

Clustering Analysis of 64 *M. longifolia* subsp. *noeana* Genotypes Based on RAPD Marker

Cluster analysis revealed seven main clusters in genetic distance 0.22. Almost, genotypes from the same population were clustered into the same group (Fig. 2). The main group 1 comprised mainly of Bushehr and Bardsir genotypes and Sirch genotypes were placed in second main group (Fig. 2). The main group 3 consist of four genotypes of Darab. Sikhuran, Jiroft, Sepidan genotypes and one genotype from Segh were consisted the biggest main group as fourth. The main group 5 comprised of six genotypes of Segh region. The smallest group, main group 6, consisted of genotype 2 from Segh region. The final group, main group 7, comprised of Darab 7 and Darab 8 gentotypes (Fig. 2). The cophenetic correlation coefficient indicated high correlation between the similarity matrix and the cophenetic matrix obtained from the UPGMA

dendrogram (r=0.83), indicating a good representation of the molecular relationships among genotypes.

AMOVA and PCoA Analysis

Analysis of molecular variance (AMOVA) showed that variance within the populations (77%) was higher than variance among the populations (23%). Principle coordinates analysis (PCoA) was calculated to display genetic relationships among forty the *M. longifolia* subsp. *noeana* genotypes. The first and second principle coordinates had 28.13% and 16.16% of the variances, respectively. The two-dimensional plot also confirmed the cluster analysis results (Fig. 3).

ISSR Polymorphism

The nine ISSR primers generated 49 bands, which all were polymorphic (100%). The number of band varied from 3 (primers of UBC809 and UBC817) to 8 (primers of UBC808 and UBC811) (Table 7). The polymorphic level, calculated as the number of polymorphic band per total number of bands, and it was 100% for all applied primers. (Table 7).

Population Genetic Diversity, Differentiation (GST), and Gene Flow (Nm)

Between applied ISSR markers, the polymorphism information content (PIC) varied from 0.12 (primer UBC816 and UBC824) to 0.58 (primer UBC825), with an average of 0.28 (Table 7). The Nei's gene diversity index (H) varied from 0.1 (primers of UBC808 and UBC816) to 0.25 (primers of UBC809, UBC811, and UBC817), with an average of 0.16.The Shannon's information index (I) ranged from 0.07 (primer of UBC808) to 0.8 (UBC824 primer), with an average of 0.25. The genetic diversity index within populations (HS) varied from 0.05 (primers of UBC808 and UBC825) to 0.15 (UBC824 primer), with an average of 0.09.

The genetic differentiation coefficient between populations (G_{ST}) ranged between 0.22 (UBC824 primer) and 0.66 (UBC817 primer), with an average of 0.46. The gene flow (Nm) ranged between 0.09 (UBC825 primer) to 1.99 (UBC824 primer), with an average of 0.625 (Table 6).

AMOVA and PCoA Analysis

Analysis of molecular variance (AMOVA) based on ISSR markers showed that variance within the populations (59%) was higher than variance among the populations (41%). Principle coordinates analysis (PCoA) was calculated to display genetic relationships among forty the *M. longifolia* subsp. *noeana* genotypes. The first and second principle coordinates had 61.84% and 10.68% of the variances, respectively. The two-dimensional plot also confirmed the cluster analysis results (Fig. 4).

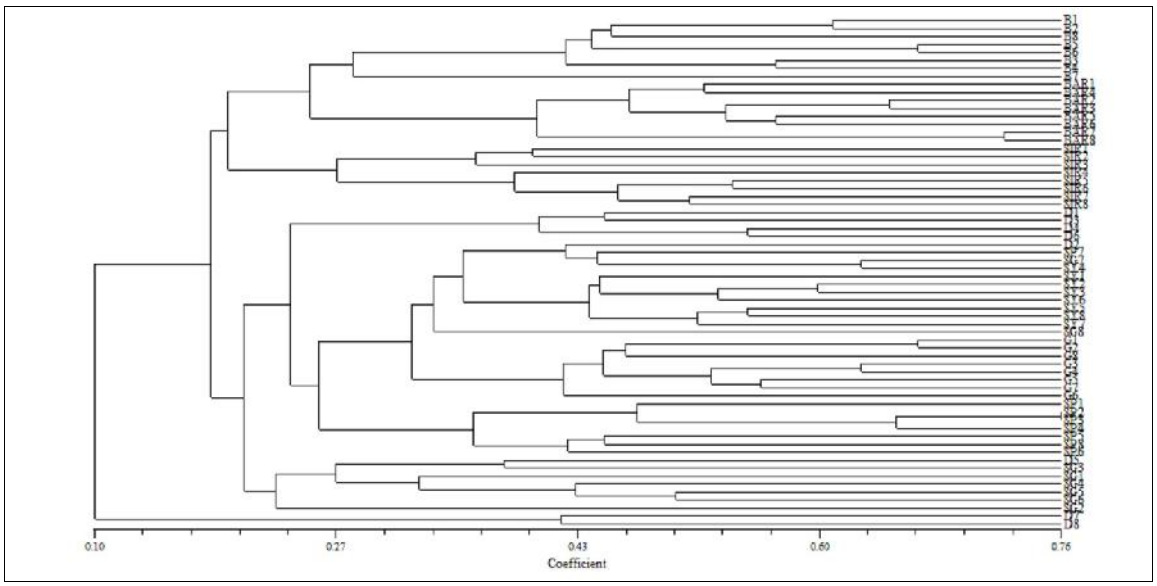

Fig. 2 Dendrogram of 64 genotypes of *Mentha longifolia* (L.) Hudson subsp. *noeana* (Briq.) Briq. by RAPD analysis based on unweighted pair-group method with arithmetic average (UPGMA).

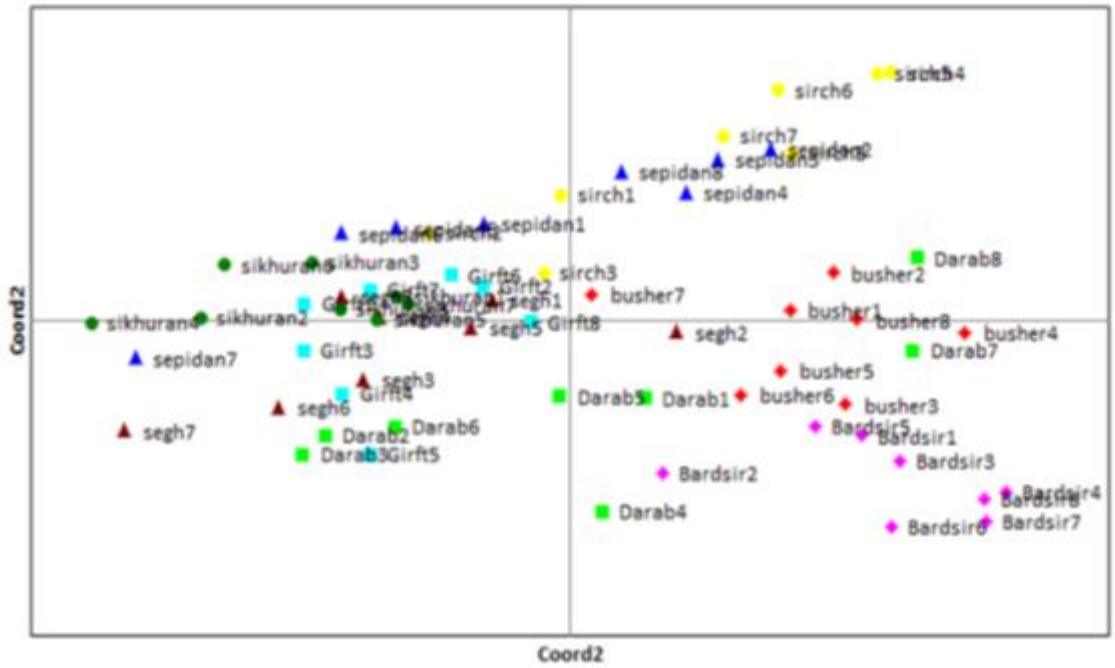

Fig. 3 Two-dimensional scatter diagram of five the *Mentha longifolia* (L.) Hudson subsp. *noeana* (Briq.) Briq. populations based on principal coordinate analysis performed by GenAlex 6.5 software (RAPD marker).

Table 6 Polymorphism characteristics of each ISSR maker in genetics diversity study of the *Mentha longifolia* (L.) Hudson subsp. *noeana* (Briq.) Briq. genotyps.

Primer	N	P	PIC	H	I	H_s	G_{ST}	N_m
UBC810	4	4	0.15	0.12	0.15	0.14	0.31	0.63
UBC816	5	5	0.12	0.1	0.18	0.09	0.35	1.03
UBC817	3	3	0.35	0.25	0.2	0.1	0.66	0.14
UBC825	6	6	0.58	0.12	0.08	0.05	0.71	0.09
UBC808	8	8	0.35	0.1	0.07	0.05	0.39	0.24
UBC809	3	3	0.48	0.25	0.15	0.07	0.64	0.46
UBC811	8	8	0.14	0.25	0.12	0.08	0.37	1.08
UBC824	7	7	0.12	0.2	0.8	0.15	0.22	1.99
UBC823	5	5	0.25	0.11	0.56	0.08	0.54	0.2
Total	49	49	-	-	-	-	-	-
Average	5.4	-	0.28	0.16	0.25	0.09	0.46	0.65

The Nei's gene diversity (H) among populations ranged from 0.06 (Segh and Jiroft populations) to 0.25 (Sirch). The Nei's gene diversity (H) and the Shannon's information index (I) were highest in the Srich population (H = 0.25; I = 0.24) followed by Sepidan population (H = 0.15; I = 0.14). Segh and Jiroft populations had the lowest Shannon's information index (I = 0.05) (Table 7).

Table 7 Genetic variation and polymorphic (marker ISSR) features estimated in the populations of *Mentha longifolia* (L.) Hudson subsp. *noeana* (Briq.) Briq.

Population	Monomorphic bands	Polymorphic bands	Polymorphism (%)	H	I
Anarestan	37	12	24.49	0.12	0.15
Darab	38	11	22.45	0.12	0.13
Sepidan	37	12	24.49	0.15	0.14
Sirch	28	21	42.86	0.25	0.24
Bardsir	37	12	24.49	0.12	0.15
Jiroft	44	5	10.20	0.06	0.05
Segh	44	5	10.20	0.06	0.05
Sikhuran	46	3	6.12	0.14	0.3

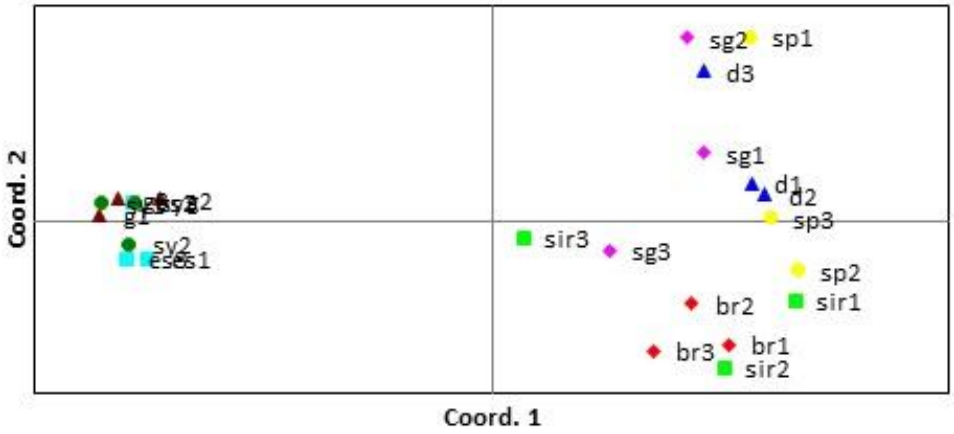

Fig. 4 Two-dimensional scatter diagram of five the *M. longifolia* subsp. *noeana* populations based on principal coordinate analysis performed by GenAlex 6.5 software (ISSR markers). Sir: Sirch; br: Bardsir; sp: Sepidan; sg: Segh; d: Darab; es; Anarestan; sy: Sikhuran; g: jiroft. Three genotypes were analyzed in each population.

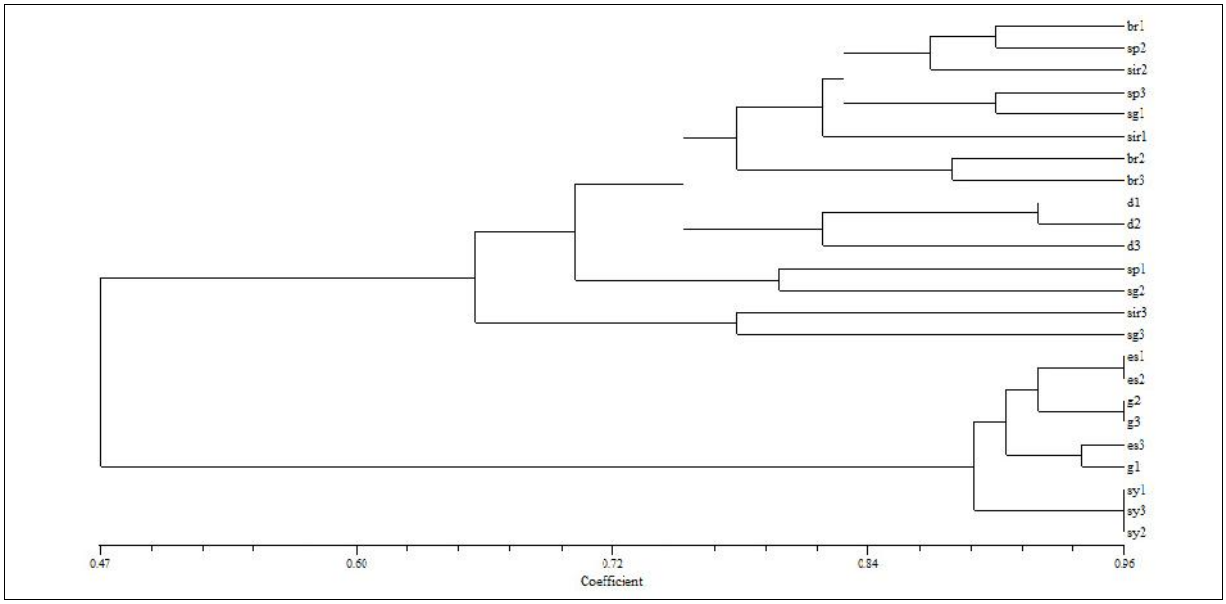

Fig. 5 Dendrogram of 24 genotypes of *Mentha longifolia* (L.) Hudson subsp. *noeana* (Briq.) Briq. by ISSR analysis based on unweighted pair-group method with arithmetic average (UPGMA). Sir: Sirch; br: Bardsir; sp: Sepidan; sg: Segh; d: Darab; es; Anarestan; sy: Sikhuran; g: jiroft. Three genotypes were analyzed in each population.

Clustering Analysis of 24 *M. longifolia* subsp. *noeana* Genotypes Based on ISSR Marker

Cluster analysis revealed five main clusters in genetic distance 0.23. Almost, genotypes from the same population were clustered into the same group (Fig. 5). The main group 1 comprised of entire Bardsir genotypes, Sirch 1 and 2, Spidan 2 and 3, and Segh 1 genotypes. Daraab genotypes were placed in second main group (Fig. 5). The main group 3 consist of genotypes of Spidan 1 and Segh 2. Sich 3 and Segh 3 genotypes formed main group 4. The biggest main group as fifth comprised of entire genotypes of Jiroft, Anarestan, and Sikhuran (Fig. 5), The cophenetic correlation coefficient indicated high correlation between the similarity matrix and the cophenetic matrix obtained from the UPGMA dendrogram (r= 0.94), indicating a good representation of the molecular relationships among genotypes.

Discussion

Genetic diversity of 8 *M. longifolia* subsp. *noeana* populations from South and Southeastern Iran indicated that they were clustered to 4 main groups based on morphological and phytochemical variables, 7 main groups based on RAPD marker,

and 5 main groups based on ISSR marker. In entire genetic diversity studies which we had performed on wild mint genotypes, it was remarked that genotypes from one region almost placed in the same cluster. Although, some genotypes placed in other cluster. It illustrated that wild mint genotypes from each region developed and adapted concomitant and synchronous. In some cases, genotypes from almost equal geographic characteristics and atmosphere with close distance, placed in the same cluster (some genotypes from Segh and Sikhuran) which can be because of wild type plants adaptation in a special environment during long-term natural selection pressure. In other case, some genotypes despite unequal geographic characteristics and atmosphere with long distance placed the same cluster (some genotypes from Anarestan and Darab) which can be associated with migration or genetic exchanges current. The clustering results based on three different variables (morphological and phytochemical variables, RAPD marker, and ISSR marker) were not completely equal. It could be due to different characteristics of each marker and different distribution of them on the wild mint genome.

Zeinali *et al*. [13] studied morphological and essential oil content diversity of twelve Iranian mints (*Mentha spp*). Their results showed the significant differences among landraces for all tested traits with the exception of the number of nodes per lateral branch. The leaf length justified 53 percent of the total variation and was accounted as the most important component of essential oil content. The cluster analysis divided the landraces into 3 groups, each of which having 5, 3 and 4 clones [13]. In other research, genetic diversity and relationships of five *Mentha* species using RAPD marker were considered. The six decamer arbitrary primers amplified 114 bands with the size of 100-3000 bp. The similarities between the species ranged 0.28-1.00. The cluster analysis based on unweighted pair group method of arithmetic means (UPGMA) divided the 5 *mentha* species into three main clusters [14]. Kabir *et al*. [15] studied genetic diversity of mint gene pool in genetic resources institute of Islamabad, Pakistan. Genetic diversity were considered among 20 accessions using 10 primers. The primers amplified 60 bands with the size of 250-4500 bp. The similarities between the accessions ranged 0-78%. The cluster analysis based on unweighted pair group method of arithmetic means (UPGMA) divided the 20 *mentha* accessions into three main clusters [15]. Sabboura *et al* [16] considered the genetic relationship between two mint species (*Mentha viridis*, *Mentha piperita*), and three wild species which had been collected from Damascus ,Latakia and, Al-Swaida by ISSR molecular markers. In this research, 46 ISSR primers were used, 38 of them gave polymorphic ISSR products. The similarities between the species were moderately and ranged from 0.07 to 0.53. The ISSR primers amplified 130 clear and reproducible bands, of which 124 bands were polymorphic and the percentage of polymorphism was 94.123%. The Dendrogram of the studied samples separated into two main clusters, the first one contained only *Mentha viridis*. The other cluster separated into two sub-clusters, the first sub-cluster contained *Mentha piperita*, and the wild species collected from Latakia, and the other one contained the two wild species collected from Damascus. They concluded that the ISSR markers could be realistically used to evaluate the genetic diversity and differentiation among Mentha varieties as they differentiated based on their geographically distribution and distance [16].

The increasing human population, current and expected effects of climate changes have all led to raised awareness of the need for more attention to be paid to global and national conservation, domestication and breeding of medicinal plants. In conclusion, it should be mentioned that this research denoted high genetic and phytochemical diversity among studied *M. longifolia* subsp. *noeana* Populations. These high genetic diversity could be applied in following breeding and gene bank conservation programs to improve their appropriate characteristics which are used in industry and medicine. .

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References

1. Gulluce M, Sahin F, Sokmen M, Ozer H, Daferera D, Sokmen A, Polissiou M, Adiguzel A, Ozkan H. Antimicrobial and antioxidant properties of the essential oils and methanol extract from *Mentha longifolia* L. ssp. *Longifolia*. Food Chem. 2007;103:1449-1456.

2. Iscan G, Kirimer N, Kurkcuoglu M, Baser KH, Demirci F. Antimicrobial screening of *Mentha piperita* essential oils. J Agric. Food Chem. 2002;50:3943-46.
3. Ertas A, Gören AC, Hasimi N, Tolan V, Kolak U. Evaluation of Antioxidant, Cholinesterase Inhibitory and Antimicrobial Properties of *Mentha longifolia* subsp. *noeana* and Its Secondary Metabolites. Rec Nat Prod. 2015;9:105-115.
4. Sharopov FS, Sulaimonova VA, Setzer WN. Essential oil composition of *Mentha longifolia* from wild populations growing in Tajikistan. JMAP. 2012;1:76-84.
5. Mikaili P, Mojaverrostami S, Moloudizargari M, Aghajanshakeri S. Pharmacological and therapeutic of *Mentha Longifolia* L. and its main constituent, menthol. Anc Sci Life. 2013;33:131-138.
6. Loveless MD, Hamrick JL. Ecological determinants of genetic structure in plant populations. Annu Rev Ecol Syst. 1984;15:65-95.
7. Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 1990;18:6531-6535.
8. Mohammadi F, Sharifi-Sirchi GR, Samsampour D. Morphological, genetic and pigment diversity of *Nerium indicum* Mill in Iran. Cell Mol Biol. 2017;63:64-71.
9. Powell W, .Morgante M, Andre C, Vogel J, Tingey S, Rafalski A. The comparison of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis. Mol Breed. 1996;2:225-238.
10. Rohlf FJ. On applications of geometric morphometrics to studies of ontogeny and phylogeny. Syst Biol. 1998;47:147-158.
11. Peakall R, Smouse PE. GeneAlex 6.5: genetic analysis in Excel. Population genetic software for teaching and research-an update. Bioinformatics. 2012;28::2537-2539.
12. Yeh FC, Yang R, Boyle TJ, Ye Z, Xiyan JM. PopGene32, Microsoft Windows-based freeware for population genetic analysis, version 1.32. Molecular Biology and Biotechnology Center. University of Alberta, Canada. 2000.
13. Zeinali H, Arzani A, Razmjo K. Morphological and essential oil content diversity of Iranian mints (Mentha spp.). IJST. 2004;28:1-9.
14. Ibrahim HMM. Assessment of genetic diversity and relationships of five mentha species using RAPD marker. Curr Sci Int. 2017;6:271-277.
15. Kabir S, Tahira R, Fareed Khan M, Naeemullah M, Jamal A. Molecular characterization of genetic diversity of mint gene pool through RAPD Markers. IJAIR. 2014;2:576-580.
16. Sabboura D, Yacoub R, Lawand S. Assessment of genetic relationships among mint species. Int J Chem Tech Res. 2016;9:462-468.