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Assessment of genetic relationships among Mint species

Dima Sabboura*, Roula Yacoub and Salam Lawand

Dep. Cropfield, Agril. Fac. Damascus University, Damascus, Syria

Abstract: This search aimed to determine 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 study 46 ISSR primers were used,38 of them gave polymorphic ISSR products with the studied samples. The Studied samples were moderately close to each others, the relationship ranged from (0.07) and (0.53), amplified 130 clear and reproducible bands, of which 124 bands were polymorphic and the percentage of polymorphism was 94.123 %.The average of total bands 3.42 band/primer and the average of polymorphic bands 3.26 . The average of Polymorphic information content (PIC) was 0.432.The Dendrogram of the studied samples separated into two main clusters, the first one contained only " *Mentha viridis* ", which is Cultivated , 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, Al-Swaida which are geographically closed to each other. So ISSR markers could be realistically used to evaluate the genetic diversity and differentiation among *Mentha* species.
Keyword : *Mentha* ,PIC , ISSR, varieties.

Introduction:

Mint (*Mentha Sp.*) (Lamiaceae) is one of the most important medical and aromatic plants, which spread geographically[1] all over the world and it is native to Europe and Middle East[2] , The genus *Mentha* consists of about 25 species: *M. australis, M.asiatica*, *M.arvensis, M.longifolia*, *M.viridis, M.piperita, M.crispata, M.aquatica, M.spicata,* and *M.japonica* .Those species have morphological differences between the varieties and the different types of mint that appeared in the last 200 years[3] .

The species of genus *Mentha* typically have chromosomes number 2n=2x=12, but the other species vary widely, with *M. spicata* and *M. longifolia* have 2n=2x=48 and 2n=2x=24, respectively[4] the spearmint*, M. spicata*, is a hybrid of *M. longifolia* and *M. rotundifolia*, morphological, cytological and biochemical data have shown that the tetraploid species of *M. spicata* (2n=48) originated by chromosomal doubling of hybrids between the two closely related and inter-fertile diploids, *M. longifolia* and *M.suaveolens*.[5]

The spearmint, *M. spicata*, is a hybrid of *M. longifolia* and *M. rotundifolia*, mrphological, cytological and biochemical data have shown that the tetraploid species of *M. spicata* (2n=48)[6]

Inter Simple Sequence Repeat (ISSR) markers, developed by[7] based on the amplification of a single primer containing a microsatellite 'core' sequence anchored at the 5' or 3' end by a set of 2-4 purine or pyrimidine residues, offers a high degree of reproducibility with the detection of the rich level of polymorphism in a relatively simple procedure. Hence, it has been widely used in assessment of genetic diversity and cultivar identification[8]. ISSR has been successfully used to estimate the extent of the genetic diversity at inter and intra

specific level in a wide range of crop species which include rice, wheat, fingermillet, Vigna, sweet potato and Plantago [9]

ISSR primers 5`-3, can amplify the flanking regions of the target microsatellite, unlike Simple Sequence Repeat (SSR), previous information of the genome are not required to design ISSR primers, these primers generate high number of polymorphisms, because of their long sequence and high annealing temperature. ISSR primers are dependable in genetic identifications studies and inter cultivars and intracultivars studies.[10] they can yield reliable and reproducible bands, and the cost of the analysis is relatively lower than Amplified Fragment Length Polymorphism (AFLP) [11]

Ninety bands were generated for 47 individual shoots with 8 ISSR primers. Cluster analysis grouped together all the samples of *M. aquatica* into a single cluster with Jackard similarity of 0.65 and high bootstrap support of 98.[12]

ISSR technique were used in identification of 4 species of mint : *Mentha aquatic L. ,peppermint , Mentha spicata var crispa L.* , and avariety of *Mentha saveolens*. The results of the analysis confirmed genetic differences between compared mint species out of 20 primers used for the ISSR reaction distinct amplicons were generated in the reactions from 8 ones on the whole in the reactions carried out 134 ISSR products were amplified , of which 78 (52.8%) were monomorphic and 56 (41.8%) polymorphic. [13]

Genetic differences were studied to evaluate the level and distribution of diversity in twelve populations of the Portuguese endangered medicinal plant, *Mentha cervina* L., In this study, Deoxyribonucleic acid DNA extraction and amplification of the 121 individuals (corresponding to the 12 populations) with 10 ISSRs markers, genetic variation. Populations exhibited a relatively low genetic diversity (PPB ¼ 14.3–64.6%, He ¼ 0.051–0.222, I ¼ 0.076–0.332), with high structuring between them (GST ¼ 0.51). However, the genetic diversity at species level was relatively high (PPB ¼ 97.7%; He ¼ 0.320). [14]

Material and Methods

1. Plant Materials

Five Mint species were examined in this study (*M. viridis, M. piperita*), and three wild samples collected from Damascus, Latakia and Al-Swaida.

2. DNA extraction

DNA of studied Mint samples were extracted by using Sodium dodecyl sulfate SDS protocol previously [15] expressed with some modifications , 1g of fresh leaves was ground in pestle and mortar with liquid nitrogen. Then the powder was transferred to 1.5 ml eppendorf tubes with 500 µl of (CTAB) buffer, 01(w/v)% Polyvinyl polypyrrolidone (PVP) and 0.1 % (0.5µl) ß-mercaptoethanol. These tubes were Incubated 35 minutes at 65^{0}C. Cooled at room temperature then one volume of Chloroform Isoamylalcohol was added to each tube and mix gently for 1-2 minute. The tubes were centrifuged at 6000 rpm for 15 minutes, then the supernatant was transfered to another tube, and added 0.5 volumes of 5M NaCl. Adding one volumes of ice cold pure ethanol and kept at 6 – 7 °C for 24 hour. Centrifuged at 3000 rpm for 3 minutes and then Centrifuged, at 8000 rpm for 5 minutes Discard the supernatant and wash the pellet with 70% ethanol. Dry the pellet and dissolve it in 15µl TE buffer. Keep at 6 – 7 °C for 1 hour or overnight to dissolve the DNA completely.

3. PCR of the ISSR Technique

46 ISSR primers Table(1) were tested by using the polymerase chain reaction (PCR) in a final volume of 25 µL containing 1.0 µL (25ng) DNA diluted, 0.5 µL (2.5U) Taq polymerase, 2.5 µL 10X buffer, 2.5 µL (2mM/µl) dNTPs, 1.5 µL (50 mM) MgCl2, 2.0 µL (10 pmol/ µl) ISSR primer and 15 µL ddH2O. The amplifications were performed in a thermal cycler following the program: 94°C for 5 min, 40 cycles (1 min at 94°C, 1 min at 43°C and 1 min at 72°C) and final elongation of 7 min at 72°C.[16]

Table(1) codes, sequences and annealing temperature of used primers:

Annealing temperature°c	neclutide sequences	Primer code
52	(AG)8T	ISSR -1
52	(CA)8G	ISSR -2
56	(GA)8CG	ISSR -3
54	(TC)8GA	ISSR -4
54	(TC)8AG	ISSR -6
56	(AC)8GG	ISSR -7
48	(GGAGA)3	ISSR -8
56	CCAG(GT)7	ISSR -9
56	C(CT)4(GT)4G	ISSR -10
56	(CA)11	ISSR -11
56	(GA)10	ISSR -12
52	(AC)8TT	ISSR -13
56	(AC)8GC	ISSR -14
52	(TG)8AA	ISSR -15
50	(GA)8T	ISSR -16
50	(AG)8TC	ISSR -17
50	(AC)8T	ISSR -18
52	(CT)8G	ISSR-20
52	(TC)8AA	ISSR -21
52	(TG)8G	ISSR -22
52	(CA)8AT	ISSR -23
52	(AC)8G	ISSR -24
54	(CA)8AC	ISSR -25
54	(CA)8GT	ISSR -26
54	(AG)6TC	ISSR-27
56	(AC)7GG	ISSR -28
56	GGTC(CA)7C	ISSR -30
56	CGT(CA)7C	ISSR -41
56	CAG(CA)7C	ISSR -42
56	CACC(TC)7	ISSR -45
56	(GA)8CTT	ISSR -230/2
56	(AC)8CTT	ISSR -230/6
56	(AC)8CC	ISSR -230/14
58	(CA)9C	ISSR -230/15
58	(CT)9G	20/230 ISSR-
58	(AG)8CTC	22/230 ISSR-
58	(GA)8AGC	25/230 ISSR-
58	(CA)8AGG	28/230 ISSR-
60	(CTT)8AGC	40/230 ISSR-
60	(AG)8C	41/230 ISSR-
60	(CA)6ACAG	42/230 ISSR-
54	(GT)4(GA)5	43/230 ISSR-
52	(TC)8C	44/230 ISSR-
54	(CTT)6	ISSR -230/45

4. DNA Documentation

In order to load the samples on the 2% agarose gel stained with (2µl (10mg/ml)) Ethidium Bromide, 20 µl of PCR product of each sample were mixed with 2 µl of loading dye and loaded. 8 µl of 1 Kbp DNA ladder

was used. Electrophoresis was done at 80 Volts. The separated fragments were visualized with an ultraviolet (UV) Trans illuminator.

5. ISSR Data analysis:

The numbers of polymorphic bands were determined for each primer. Genotypes were scored (1) for present band, and (0) for absent band and then entered into a data matrix[17]. Percentage of polymorphism was calculated as the following equation: (polymorphic bands/total number of bands x 100). The tree diagram was produced by clustering the similarity data with the UPGMA method using POPGENE software V1.31[18]. A PIC (polymorphic information content) value was calculated according to the equation:

PIC =[Σ2(PI(1- PI)2]

Whereas: PI bands frequency resulting from using the primers in all samples[19].

Results and Discussion

The concentrations of the extracted DNA were between 0.73-1.53 µg/µl, and the purity of the samples was between 1.931- 2.080. Electrophoresis was applied on 0.8% agarose gel to determine the quantity of DNA.

ISSR technique was applied by using 44 primers for amplification of the 5 samples of Mint, 38 primers proved their ability in giving polymorphic ISSR products from the studied samples, while 8 primers didn't give any PCR products. ISSR primers were able to distinguish between studied mint samples, which corresponded with[20].

The studied varieties, (table 2) showed that the primers gave PCR products of about 130 bands, 124 of them were polymorphic, with a polymorphism percentage %94.123. The bands of each primer rated between 1 for primers (11,18), to 10 bands for primers (45), with an average of 3.42 bands/ primer. Polymorphism percentage was 0% for primer (11), 50% for primers (17,21), 66.66 % for primer (24), 80% for primers (15,26) and 100% for all other primers. Values (PIC) rated between 0 for the primers (11), so this primer is not efficient in determining, and 0.5 for the primer (22), and the average PIC value was 0.432.This result showed that the used ISSR markers were able to determine studied Mint varieties. (Figure 1)

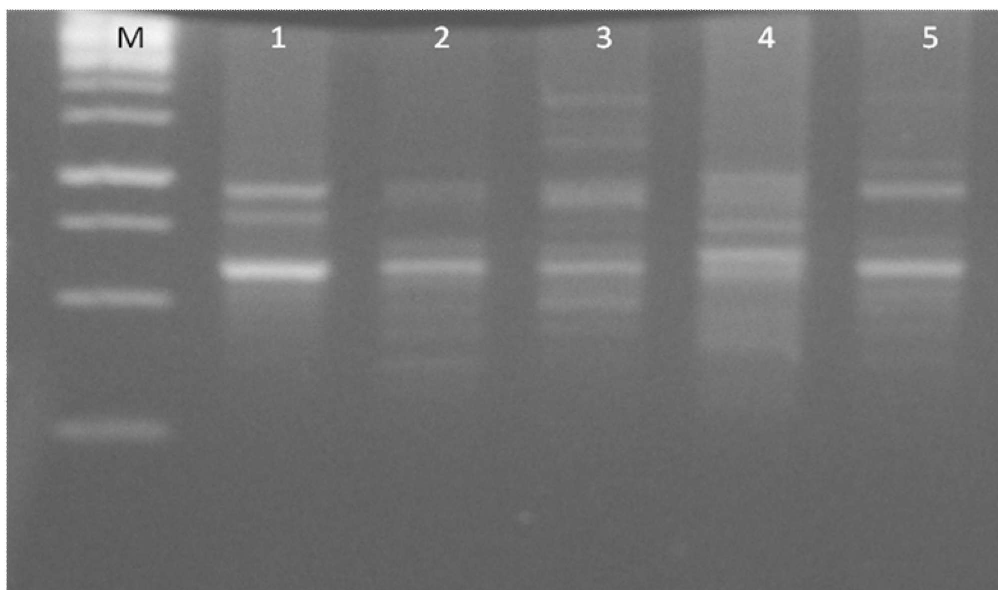

Figure 1. ISSR patterns obtained from the 5 species of Mint by using primer ISSR30.
M: 1000 pb molecular weight marker. (1- *M. viridis*, 2- *M. piperita*, 3- Latakia, 4- Damascus, 5- Al-Swaida)

Table (2). Total bands number from used primers, the percentage of polymorphic bands and PIC values

PIC value	Polymorphism %	Polymorphic Bands No.	Total Band No.	Primer name
0.48	100	2	2	ISSR -1
0.5	100	4	4	ISSR -2
0.42	100	4	4	ISSR -3
0.48	100	3	3	ISSR -4
0.32	100	5	5	ISSR -6
0.42	100	4	4	ISSR -7
0.48	100	2	2	ISSR -8
0.4982	100	3	3	ISSR -9
0.42	100	2	2	ISSR -10
0	0	0	1	ISSR -11
0.32	100	3	3	ISSR -12
0.3942	100	3	3	ISSR -13
0.48	100	3	3	ISSR -14
0.42	100	2	2	ISSR -15
0.48	100	4	4	ISSR -16
0.48	50	1	2	ISSR -17
0.3648	100	1	1	ISSR -18
0.42	50	1	2	ISSR -21
0.5	100	2	2	ISSR -22
0.4982	100	3	3	ISSR -23
0.4422	66.66	2	3	ISSR -24
0.4992	100	5	5	ISSR -25
0.4608	80	4	5	ISSR -26
0.42	100	4	4	ISSR -28
0.48	100	8	8	ISSR -30
0.4982	100	3	3	ISSR -41
0.4422	100	3	3	ISSR -42
0.3848	100	10	10	ISSR -45
0.49	100	3	3	ISSR -230/2
0.48	100	2	2	ISSR -230/6
0.442	100	3	3	ISSR -230/14
0.32	80	4	5	ISSR -230/15
0.4992	100	5	5	ISSR -230/22
0.42	100	2	2	ISSR -230/25
0.48	100	2	2	ISSR -230/28
0.49	100	3	3	ISSR -230/40
0.32	100	5	5	ISSR -230/41
0.48	100	4	4	ISSR -230/43
16.424		124	130	Total
0.432	94.123	3.26	3.42	Average

The Percentage of Disagreement Values matrix (PDV) for ISSR markers among the ten studied samples ranged from (0.47) between species collected from Damascus and Al-Swaida, which are the closest to each other and (0.93): between *M. viridis*, and the wild species collected from Latakia , which were the furthest from each other, and the average of PDV, was 0.72. The lowest PAV value was 0.40 between *M. viridis*, and the wild species collected from Latakia and the highest PAV value was 0,63 (Table 3)

Table (3). Percentage of Agreement Values Matrix (PDV)resulted from studying genetic similarity of studied species

	M. piperita	***Latakia***	***M. viridis***	***Damascus***	***Al-Swaida***
M.piperita	**0.00**				
Latakia	**0.52**	**0.00**			
M. viridis	**0.74**	**0.93**	**0.00**		
Damascus	**0.53**	**0.55**	**0.63**	**0.00**	
. Al-Swaida	**0.62**	**0.74**	**0.74**	**0.47**	**0.00**

The Dendrogram of the studied samples separated into two main clusters (38.09), the first one contained only " *M. viridis* ", which is Cultivated, The other cluster separated into two sub-clusters (30.73), the first sub-cluster contained, *M. piperita*, and the wild species collected from Latakia ' (26.39), and the other one contained the two wild species collected from Damascus, Al-Swaida (23.43) are Geographically close to each other ,Damascus and Al-Swaida are geographically close to each otherso we can conclude that the ISSR markers could be realistically used to evaluate the genetic diversity and differentiation among *Mentha varieties* (Figure 2).

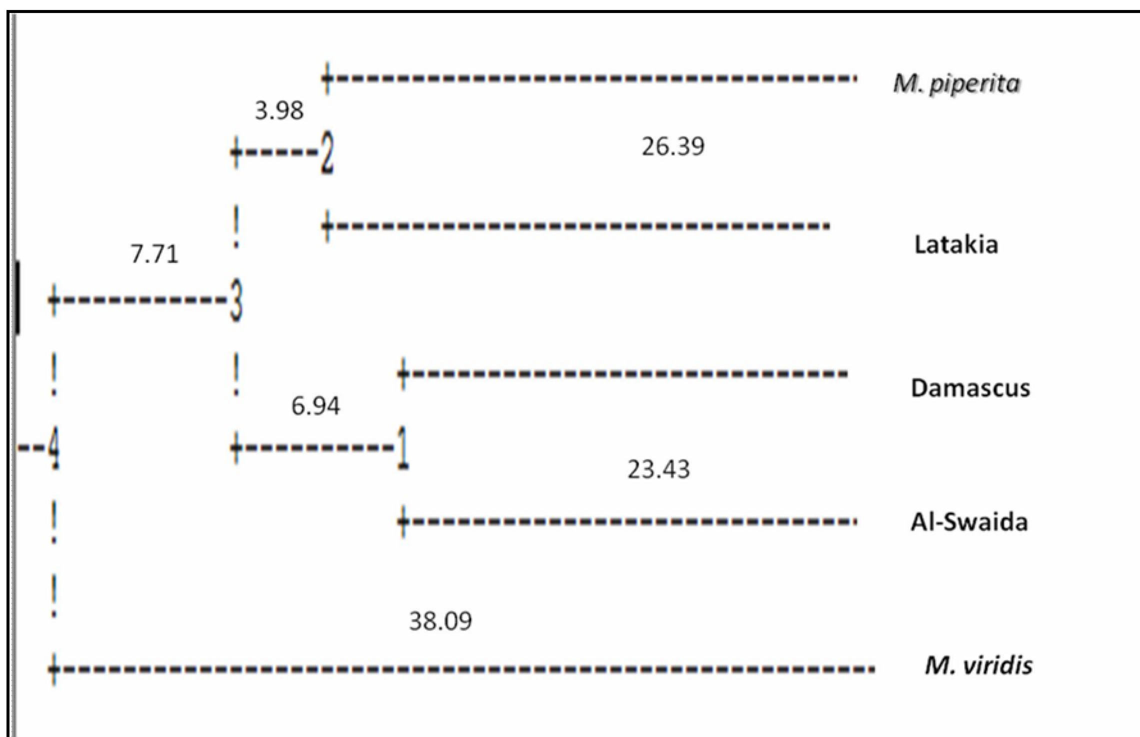

Figure 2: UPGMA based cluster tree of 5 mint species with 38 ISSR markers

Conclusions:

1. The ISSR technique proved its efficiency in differentiating the examined Mint varieties which were grouped according to their geographical distribution.
2. *Mentha piperita* was the closest variety to the wild variety collected from Latakia
3. There is a high Genetic relationship between the two wild species collected from Damascus and Al-Swaida, and the species collected from Latakia

4. Cultivated "*Mentha viridis*", showed genetic diversity from the rest of the wild varieties and hybrid *Mentha piperita*

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