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Molecular Characterization of Genetic Diversity of Mint Gene Pool through RAPD Markers

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Abstract **– Present study was conducted to investigate the genetic relatedness among mint gene pool conserved at Plant Genetic Resources Institute, Islamabad. Genetic diversity was explored among 20 *Mentha* accessions through randomly amplified polymorphic DNA (RAPD) markers. All the used markers produced 60 band, on average 6 bands per primer. Size of the amplified products ranged from 250bp to 4500bp. Highest size band was amplified by OPA-12 primer while, OPA-11, OPA-16, and OPB-3 produced smallest band. Similarity between the accessions ranged from 0-78%, maximum (100%) difference was observed among seven pairs of accessions. Dendrogram based on unweighted pair group method of arithamtic means (UPGMA) divided 20 mint accessions into three main clusters and catnip and jangli podina as two distinct accessions. This information could be useful in proper management and expansion of mint germplasm.**

Keywords **– Mint, Genetic Diversity, RAPD, Molecular Markers, Medicinal Plants.**

I. INTRODUCTION

Herbs and plants make up a large portion of the ingredients used by people throughout the ages to help combat disease and illness. Mint is one of those medicinal plants being used world wide which are of great importance in folk medicine and food industry.

The genus *Mentha* belongs to the family Lamiaceae consisting of about 25 to 30 species and additional 11 hybrids mainly found in temperate and sub-temperate regions of Eurasia, Australia, South Africa and North America [1]. Classification of genus *Mentha* is complicated due to elevated incidence of polyploidy, wide range in the morphology and numeral variation in chromosomes, recurrent inter-specific hybridization and vegetative spread. Natural inter-specific hybridization occurs with high frequency both in cultivated and wild population of *Mentha.* Several cytological, morphological, chemical and molecular markers have been reported to reveal relationship among *Mentha* species [1].

The most common species of the genus *Mentha* found in Pakistan are *M. pulegium*, *M. arvensis, M. spicata*, *M. longifolia*, *M. piperita* and *M. rolyana* [2, 3]. Although, some cultivars of *Mentha* have been domesticated but no attempt has been made to analyze variability among different populations of this plant. Information on genetic variation of the available germplasm is fundamental to its domestication, improvement and management. This could also provide information on the evolving process and distribution of the germplasm in different isolated regions [4]. An understanding of the extent and organization of genetic diversity among *Mentha* species could be useful for both its genetic improvement and conservation. It is difficult to distinguish phenotypically similar cultivars using morphological and physiological methods or isozyme analyses [5]. The limitation of these analyses is that they are phenotypic based and affected by environment. The development of Polymerase Chain Reaction (PCR) technique has revolutionized the field of molecular biology [6, 7]. Molecular markers offer the best estimate of genetic diversity since they are independent of the confounding effects of environmental features.

The DNA fingerprinting technique of Randomly Amplified Polymorphic DNA (RAPD) provides an unlimited number of markers which can be used for various purposes [8]. RAPD markers can be generated using short arbitrary primers to amplify genomic DNA, giving a genotype-specific pattern of bands. RAPD markers are the most widely used molecular technique for DNA fingerprinting. The RAPD technique has become an increasingly popular tool in genetic studies [9]. *Mentha* species have been assessed for genetic relationship [10] and cultivar identification [11] by using RAPD markers.

Present study was undertaken to have an estimate of genetic diversity among local and exotic germplasm of mint collected and maintained at Plant Genetic Resources Institute (PGRI), National Agriculture Research Center (NARC), Islamabad. RAPD markers were employed to figure out the relatedness of germplasm.

II. MATERIALS AND METHODS

A. Collection of Plant Material

Fresh and young leaves of 20 mint accessions (Table 1) were collected from mint clonal repository, PGRI, NARC, Islamabad.

B. Genomic DNA Extraction

Total genomic DNA was isolated from young leaves by CTAB method following Tahira et al [12]. Leaf tissue was ground in liquid nitrogen in a mortar and pestle. To the ground tissue, added 2-3ml of 2X CTAB buffer with 1% Merceptoethanol and incubated in a water bath at 60^0C for 30 minutes. The contents were then brought to room

temperature and to each sample 0.75ml of Chloroform-Isoamyl alcohol (24:1) was added. Samples were agitated gently by inverting the tube 4-5 times and centrifuged at 10,000 rpm for 10 minutes. The supernatant (600µl) was then shifted carefully to a new tube and 480µl ice chilled iso-propanol was added while the debris was discarded. For fine precipitation of DNA the tube was swirled gently and samples were incubated at ice for 30 minutes. Samples were then centrifuged for 5 minutes at 5000rpm to collect precipitated nucleic acid and pellet was washed with 200µl 70% ethanol, centrifuged for 10 minutes at 12000rpm and supernatant was removed carefully. DNA pellet was dried, resuspended in 50µl of 0.5 M Tris-EDTA buffer and treated with RNAse for 30 minutes. Chloroform-isoamyl alcohol step was repeated and genomic DNA was precipitated. Methanol washed DNA pellet was dried and resuspended in double distilled deionized water.

Table 1 Mint Accessions Included in the Study

Sr. No.	Accession	Source	Sr. No.	Accession	Source
1.	Field mint	Local	11.	Lavender mint	Local
2.	Purple flower	Local	12.	*Mentha spicata*	Canada
3.	White flower	Local	13.	Mentha rolyana	AJK
4.	Nana Maghrabi	Saudi Arabia	14.	*Mentha* sp.(Mansehra)	Manshera
5.	Catnip	Local	15.	*Mentha acquatica*	Local
6.	Nana Asavi	Saudi Arabia	16.	Jungli Podina	Local
7.	Mint camphor (Rawlakot)	AJK*	17.	Mint camphor (Islamabad)	Local
8.	*Mentha piperita*	Local	18.	Lemon mint	Local
9.	White mint	AJK	19.	Pepper mint	Local
10.	Black mint	Local	20.	*Mentha* sp.(Kila Bharwala)	Local

*Azad Jammu & Kashmirs

C. DNA Quantification and Dilution

Genomic DNA was quantified through Nanodrop- 1000 3.3.1 Spectrphotometer and checked for quality on 0.8% agarose gel. DNA was diluted (15 ng/µl) by using the following formula: $N_1V_1=N_2V_2$

Where N_1= known DNA concentration

N_2= Required concentration

V_1= Required volume V_2= Final volume make up

D. RAPD PCR

Twenty RAPD primers from series OPA and OPB presented in (Table 2) were obtained from Operon technologies Inc. Alamedos, USA. PCR reactions were performed using Fermentas kit in total volumes of 25µl containing 2.5µl 10X PCR buffer,2 µl (25mM) $MgSO_4$, 0.4 µl (10mM) DNTP's (dATP, dCTP, dGTP, dTTP), 2 µl primer, 1 µl of DNA template, 0.2 µl of Taq Polymerase and 16.9 µl ddH2O. DNA amplification was achieved in a thermo cycler (Master Cycler Gradient, Eppendorf, Germany) programmed for 35 cycles of denaturing for 30 sec at 94°C, annealing for 30 sec at 36°C and final extension for 1 min at 72°C. The PCR tubes were kept at 72°C for 10 min and then held at 4°C until the tubes were removed. After the completion of PCR, the products were resolved on 1% agaorose gel and visualized with UVI Doc. Gel Documentation system (JICA, JAPAN).

E. Statistical Analysis

The data was scored as "1" for the presence of the band and "0" for absence of the band and the fragment size was determined by 1kb DNA ladder. The data was analyzed for genetic diversity with the help of NTSYS PC 20 and genetic distances were calculated as Nie and Li coefficients [13]. The coefficient of similarity matrix was subjected to UPGMA (Unweighted Pair wise Methods with Arithmetic averages) cluster analysis to construct a dendrogram.

Table 2: RAPD Primers Used in the Present Study

Sr. No.	Primer	Primer Sequence	Sr. No	Primer	Primer Sequence
1	OPB-01	GTTTCGCTCC	11	OPA-12	TCGGCGATAG
2	OPB-03	CATCCCCCTG	12	OPA-17	GACCGCTTGT
3	OPA-05	AGGGGCTTTG	13	OPA-11	CAATCGCCGT
4	OPA-09	GGGTAACGCC	14	OPA-16	AGCCAGCGAA
5	OPA-10	GTGATCGCAG	15	OPA-20	GTTGCGATCC
6	OPA-14	TCTGTGCTGG	16	OPB-08	GTCCACACGG
7	OPA-19	CAAACGTCGG	17	OPA-04	AATCGGGCTC
8	OPB-10	CTGCTGGGAC	18	OPB-16	TTTGCCCGGA
9	OPA-03	AGTCAGCCAC	19	OPB-05	TGCGCCCTTC
10	OPA-18	AGGTGACCGT	20	OPB-19	ACCCCCGAAG

III. Results and Discussion

Estimation of DNA polymorphism is of great importance especially in plant breeding as they show the tendency to have a deeper insight into genetic diversity [11]. Present studies were carried out for assessment of molecular diversity among *Mentha* species present in clonal repository of medicinal plants at PGRI. RAPD is an effective technique used for estimation of genetic association within the genus *Mentha* [10, 11]. According to these investigations the quantity of shared bands assisted to calculate the genetic distances within the targeted genotypes. Polymorphic bands produced by RAPD primers could be used to understand the extent of genetic relatedness. The characteristic profiles generated by RAPD primers for twenty mint accessions revealed that RAPD could be used in mint to distinguish between the genotypes.

Initially three randomly selected accessions were used to screen all the twenty primers out of which 9 primers producing clear and score-able bands were selected and applied to all genotypes under study. Total number of bands amplified by all selected primers was 60, on average; six bands were produced by each primer. OPA -1 and OPB-08 (Fig. 1) primers produced maximum number (6) of bands. Khanuja et al [10] reported 10.5 bands per primer while assessing genetic dissimilarity among *Mentha* accessions. This difference may be due to different *Mentha* accessions and RAPD primers used.

The highest band size of 4500bp was amplified by primer OPA-12, while the lowest band size was 250bp amplified by three primers, OPA-11, OPA-16, and OPB-3. This variation might be the influence of varying factors such as primer sequence, template quantity and less number of annealing sites in the genome [14].

Maximum (100%) polymorphism was observed among seven pairs of accessions, catnip and field mint, *Mentha* piperita and catnip, *Mentha roylana* and catnip, *Mentha* sp.(Mansehra) and catnip, Jungli podina and catnip, mint camphor (Islamabad) and catnip, Jungli podina and lavender mint. This variation could be the result of their strong geographical differences, out-breeding and the extensive seeds and pollen scattering [15, 16]. Increased stage of genetic variation within population is indicative of genotype affluence and gene flow [17, 18]. Two out of twenty mint cultivars were considered as out groups which showed maximum genetic difference (100%) between each other as well as from all twenty genotypes. The level of polymorphism observed in the present study was in close similarity to the value reported by Khanuja et al [10] 2000) whose amplification profiles generated by 60 RAPD primers on Mentha species gave 630 bands as a whole from which 41 were monomorphic while 589 were polymorphic thus showed 93.5% polymorphism. Shinwari et al [2], also reported 93.6% and 100% RAPD polymorphism in *Mentha spicata* and *Mentha rolyana* populations respectively.

The results of similarity matrix presented in Table 3, showed 78% resemblance between two mint accessions i.e., Nana Asavi and Nana Maghrabi which reveals that both accessions are genetically least diverse from each other. The reason of maximum similarity between nana Asavi and Nana maghrabi in this study could be their similar background and ecological zone as [19] reported that Nana Maghrabi and Nana Asavi were two accessions of genus *Mentha* belonging to Saudi Arabia. Nana Maghrabi and White flower showed 60% and 57% similarity with white flower and purple flower respectively. The maximum Jaccard's similarity value in this study was 0.78 which is different from values reported by Al-Rawashdeh et al. and Soheila et al. [20, 21], 0.68 and 0.69 respectively but is very close to 0.79 reported by Kazemi et al. [17]. The history of the species, the reproductive system and ecology was also reported as some major factors on which the genetic similarity values could be heavily dependent [16]. Estimation of genetic diversity at the molecular level can be applied to identify and collect genetically exclusive germplasm. Estimation of germplasm diversity helps both plant breeding and germplasm collection [17] because genetic uniformity is one of the most important fundamentals for the flourishing of micro-propagation of several crop species.

The similarity matrix was used to create a dendrogram using UPGMA shown in Fig. 2. The dendrogram divided twenty mint accessions into 3 main clusters, cluster 1, cluster 2 and cluster 3.

Cluster I was the largest group in the dendrogram comprising of nine out of twenty mint accessions and was divided into sub-cluster 1A and sub-cluster 1B. Sub-cluster 1A contained three accessions, field mint, purple flower and white flower. This grouping may be due to the reason that these three accessions belonged to *M. arvensis.* The sub-cluster 1B consisted of nana maghrabi, nana asavi, white mint, *Mentha piperita,* mint camphor (Rawlakot) and black mint. Two accessions, Nana Asavi and Nana Maghrabi lie on the same position in this cluster. These two accessions showed minimum diversity between each other and were placed on same genetic linkage in the cluster and considered as another outlier of this cluster. This could be the result of same geographical positions or the common ancestors of the accessions.

Cluster 2 was the second largest cluster of the dendrogram which comprised seven mint accessions and was further divided into two sub-clusters, sub-cluster 2A and sub-cluster 2B. Two accessions, lavender mint and *Mentha spicata* fell into sub-cluster 2A while sub-cluster 2B comprised of five accessions, *Mentha* sp. (Mansehra), *Mentha acquatica,* Pepper mint, *Mentha* sp. (Kila Bharwala) and lemon mint.

Cluster 3 in the dendrogram comprised of only two mint accessions, *Mentha rolyana* and mint camphor (Islamabad).

Jungli podina and catnip were two distinct accessions in the dendrogram which neither resemble genetically with each other nor with any other member of all the clusters and showed maximum dissimilarity among twenty accessions of mint used in this study.

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IV. Conclusion

It was concluded that *Mentha* species maintained at clonal repository of PGRI possessed high rank of genetic polymorphism. This genetic distinction within accessions illustrated the genotype wealth and gene flow and would be helpful in generating a distinct germplasm of *Mentha* which can be further exploited using different molecular techniques and breeding methods. Further, RAPD markers could be used successfully to demonstrate the level of genetic relatedness among diverse collection of germplasm.

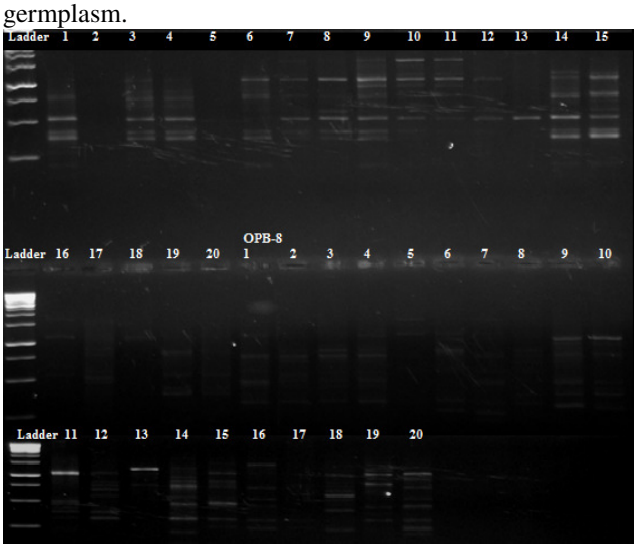

Fig. 1 Twenty *Mentha* accessions with primer OPA- 1& OPB-8

1kb DNA ladder, Lane 1- 15 = field mint, purple flower, white flower, nana Maghrabi, catnip, nana Asavi, white mint, *Mentha Piperita*, mint camphor (rawlakot), black mint, lavender mint, *Mentha spicata*, *Mentha rolyana*, *Mentha* sp.(mint Mansehra) and *Mentha acquatica* , Lane 1 kb Ladder lane 16- 20, jangli podina, mint camphor (Islamabad), Lemon mint, pepper mint and *Mentha* sp.(kila Bharwala) with primer OPA-1.

1kb DNA ladder, Lane 1- 15 = field mint, purple flower, white flower, nana Maghrabi, catnip, nana Asavi, white mint, *Mentha Piperita*, mint camphor (rawlakot), black mint, lavender mint, *Mentha spicata*, *Mentha rolyana*, *Mentha* sp.(mint Mansehra) and *Mentha acquatica* , Lane 1 kb Ladder lane 16- 20, jangli podina, mint camphor (Islamabad), Lemon mint, pepper mint and *Mentha* sp.(kila Bharwala) with primer OPB-8.

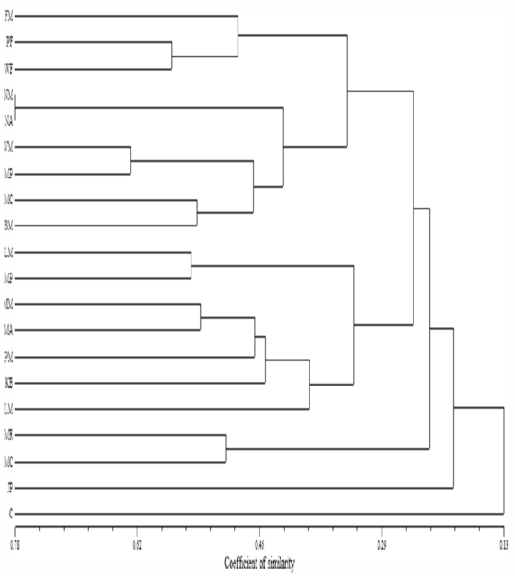

Fig.2. Dendrogram representing the genetic relationships among all *Mentha* accessions.

(FM= field mint, PF= purple flower, WF=white flower, NM= nana Maghrabi, NA= nana Asavi ,WM= white mint, MP= *Mentha* piperita, MC1= mint camphor (rawlakot), BM= black mint, LM1= Lavender mint, MS= *Mentha* spicata, MM= *Mentha* sp.(mint Mansehra) , MA= *mentha* acquatica, PM= pepper mint, KB= *Mentha* sp. (kila Bharwala), LM2= lemon mint, MR= *Mentha* rolyana, MC2= mint camphor (Islamabad), JP= Jungli Podina, C= catnip.

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Table 3: Similarity Matrix for Jaccard's Coefficient of 20 Mentha Accessions

C	FM	PF	WF	NM	C	NA	WM	MP	MC1	BM	LM1	MP	MR	MM	MA	JP	MC2	LM2	PM	KB
FM	1.00																			
PF	0.42	1.00																		
WF	0.55	0.57	1.00																	
NM	0.42	0.32	0.60	1.00																
C	0.00	0.11	0.17	0.21	1.00															
NA	0.40	0.30	0.50	0.78	0.20	1.00														
WM	0.30	0.16	0.27	0.35	0.21	0.44	1.00													
MP	0.32	0.26	0.43	0.44	0.15	0.41	0.63	1.00												
MC1	0.40	0.12	0.45	0.46	0.10	0.43	0.38	0.50	1.00											
BM	0.36	0.24	0.27	0.47	0.29	0.39	0.47	0.50	0.54	1.00										
LM1	0.13	0.09	0.14	0.25	0.46	0.24	0.31	0.20	0.25	0.50	1.00									
MS	0.09	0.13	0.20	0.25	0.00	0.23	0.33	0.27	0.25	0.33	0.55	1.00								
MR	0.17	0.27	0.20	0.33	0.00	0.31	0.17	0.27	0.13	0.25	0.27	0.43	1.00							
MM	0.27	0.18	0.37	0.32	0.00	0.78	0.39	0.48	0.35	0.32	0.28	0.48	0.48	1.00						
MA	0.31	0.08	0.34	0.30	0.22	0.29	0.42	0.32	0.32	0.42	0.39	0.26	0.17	0.53	1.00					
JP	0.17	0.13	0.10	0.16	0.00	0.22	0.40	0.17	0.12	0.16	0.00	0.13	0.13	0.36	0.25	1.00				
MC2	0.16	0.12	0.09	0.23	0.00	0.21	0.38	0.25	0.11	0.23	0.17	0.25	0.50	0.26	0.24	0.24	1.00			
LM2	0.15	0.11	0.08	0.29	0.18	0.20	0.43	0.15	0.10	0.29	0.31	0.33	0.11	0.40	0.44	0.32	0.30	1.00		
PM	0.07	0.11	0.25	0.21	0.09	0.13	0.36	0.38	0.20	0.36	0.31	0.44	0.22	0.48	0.44	0.21	0.30	0.27	1.00	
KB	0.31	0.08	0.21	0.30	0.15	0.17	0.30	0.13	0.16	0.42	0.26	0.26	0.17	0.40	0.50	0.33	0.16	0.44	0.44	1.00

FM= Field Mint, PF= Purple Flower, WF=White Flower, NM= Nana Maghrabi, C= Catnip, NA= Nana Asavi, WM= White Mint, MP= *Mentha piperita*, MC1= Mint Camphor (rawlakot), BM= Black Mint, LM1= Lavender Mint, MS= *Mentha spicata*, MR= *Mentha rolyana*, MM= *Mentha* sp.(mint Mansehra), MA= M*entha A*cquatica, JP= Jungli Podina, MC2= Mint Camphor(Islamabad), LM2= Lemon Mint, PM= Pepper Mint, KB= *Mentha* sp. (kila Bharwala)