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GENETIC DIVERSITY ANALYSIS OF DIFFERENT VARIETIES OF *MENTHA PIPERITA* FROM CENTRAL AND SOUTH GUJARAT (INDIA) THROUGH RAPD, ISSR AND SSR MARKERS

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ABSTRACT

Generic diversity study was carried out among four different species of *Mentha piperita* (family lamiaceae) collected from central Gujarat (India) using RAPD primers, SSR primer and ISSR primers. In RAPD analysis total 133 bands were observed and all bands were polymorphic. It shows 100% polymorphism. In SSR analysis 75.75% polymorphism was observed and in ISSR analysis 80.95% polymorphism was observed. These all shows high level of generic variation. After this the dendogram was constructed using UPGMA method. The similarity index was also constructed by Jaccard's coefficient. This report provides the basic platform for the future crop improvement program and cross breeding program and in investigation of new mentha plant varieties.

Keywords: Genetic Diversity, RAPD, ISSR, SSR, *Mentha piperita*

Abbreviation: RAPD (Random Amplified Polymorphic DNA), SSR (Simple Sequence Repeats), UPGMA (Unwieghted Pair Group Method of Arithmetic Means), PCR (Polymerase Chain Reaction), PIC (Polymorphism Index Content)

INTRODUCTION

In traditional taxonomy, classification of plants is done on the basis of morphological characters of the plant. Mentha (also known as mint, from Greek mintha, linear B mi-ta) is

a genus of flowering plants in the family lamiaceae **[1]**. The total number of species varies from 13 to 18 **[2]**. Mostly distributed across Europe, Africa, Asia, Australia and North America. Species of mentha have very important value in crop improvement experiments. *Mentha piperita* (mentha x piperita) and also known as peppermint. It is a hybrid mint, a cross between water mint and spearmint.

Firstly found in Europe but now cultivated in all over the world. *Peppermint* was first described in 1753 by Carolus Linnaens. Leaves are dark green with reddish veins and acute apex. Generally grows best in shaded locations and expands by underground stolons. It has very large tradition of medicinal importants. It is having high mentha content, which is useful in tea, flavoured ice-cream, chewingum, toothpaste and other products. The oil contains menthone and methyl acetate. Oil possesses fresh sharp menthol odour & pungent taste followed by cooling sensation **[3]**. It is also useful in treating many diseases and syndromes related to respiratory tract and inflammation of the oral mucosa. Also useful for cosmetics in some shampoo, soaps and skin care products. Commonly useful in treating symptoms. Examples are nausea, vomiting, abdominal pain indigestion. So this has wide range of potential and therapeutic use. The toxicity studies of the plant have received controversial results. Some authors reported that plant may lead to hepatic disease **[4]**. While other found that it protects against liver damage caused by heavy metals **[5]**.

RAPD (Random amplified polymorphic DNA) **[6]**; popular and important technique because it is simple, fast & easy in performing and doesn't require DNA sequence information **[7-8]**. The technique has been useful in generic linkage mapping **[9]**, gene tagging **[10]**, study of generic diversity **[11]**. In other marker techniques inter simple sequence repeats (ISSRs) use repeat anchored primers to amplify DNA. SSR and ISSR are also important tools for genetic diversity studies. inter-simple sequence repeats (ISSRs) use repeat-anchored primers to amplify DNA sequences between two inverted SSRs **[12]**. It has high annealing temperature and longer sequence of ISSR primers, they can yield reliable and reproducible bands, and the cost of the analysis is relatively lower than AFLP.

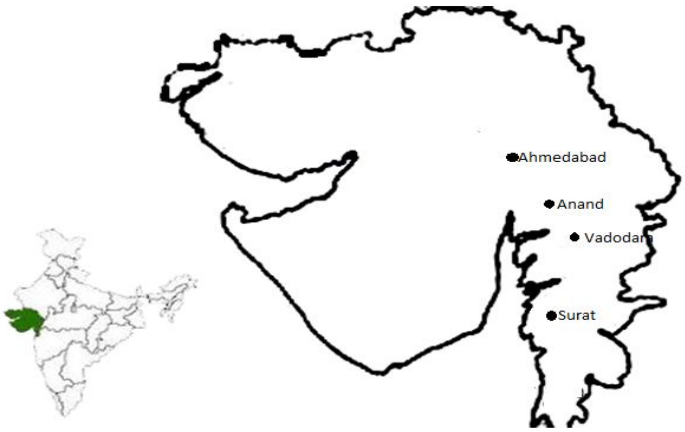
MATERIALS AND METHODS

Plants

The plant variety *Mentha piperita* from four different districts Surat, Vadodara, Ahmedabad and Anand were collected as shown in **Table 1**.

Table 1: *Mentha piperita* From Four Different Districts of Gujarat

SR NO	Name of the plant varieties	Leaves	Flower
1	***Mentha piperita*(Surat)**		
2	***Mentha piperita*(Anand)**		
3	***Mentha piperita*(Ahmedabad)**		
4	***Mentha piperita*(Vadodara)**		



Genomic DNA Isolation

The genomic DNA was extracted from young leaves by the method described by **[13]**, which is standard CTAB (Cetyl Trimethy Ammonium Bromide) method with some modification.

RAPD (Random Amplified Polymorphic DNA), SSR (Simple Sequence Repeats), ISSR (Inter simple sequence Repeats) Analysis

The RAPD analysis was done by method of **[6]**. Polymerase chain reaction were performed in 15 µ1 system containing 1.5 µ1 10 X assay buffer (10mM Tris - Cl , pH-9.0 , 1.5 mM Mgcl2, 50 mM Kcl and 0.01 % gelatin) 0.5 µl Tag DNA polymerase (Bangalore genei pvt. ltd., Bangaluru , India), 1.5 µ1 of each dNTPs (dATP , dTTP , dCTP , dGTP), ,0.5 µl of primer & 0.5 µ1 of template DNA. 10.5 µ1 PCR water .The reaction was carried out using thermo cycler (Corbett research gradient automatic, UK) reaction was carried out in three steps. The first step of initial denaturation was performed at 94ºC for 5 min. Following the initial denaturation step, PCR was carried out

for 45 cycles (in case of RAPD) and 40 cycles (in case of ISSR and SSR). Each cycle consisted of a denaturation step of 1 min at 94ºC, followed by primer annealing step for 45 seconds at 37 ºC (for RAPD) , annealing step for 1 min at 53 ºC (for ISSR), Annealing step of 1 min at 54 ºC (for SSR) and an extension step of min 1:30 min at 72ºC. The last cycle was followed by final extension step of 5 min (in case of RAPD) and 7 min (in case of ISSR and SSR) at 72 ºC. The holding temperature was 4ºC.

Agarose Gel Electrophoresis

The amplified products were checked on gel electrophoresis (1.5 percent gel for RAPD, 2 percent gel for SSR and ISSR). The electrophoresis was carried out at voltage of 100 volts and the bands were visualized under U.V transilluminator and photographs were taken. 100bp, 1 kb ladder was used to determine the size of the amplicons.

RESULT AND DISCUSSION

Figure 1 A- D and Figure 2 A –B show results of PCR amplification.

Scoring and Data Analysis

Each band repeated the genotype at a single locus because all RAPD markers are nearly dominant **[6]**. The RAPD patterns were scored on the basis of presence or absence of band. If the band is present than it is scored as '1'.and if the band is absent than it is scored as '0'.the data obtained from amplified products are used to estimate genetic similarity among different varieties on basis of amplified products **[14]**.

The Jaccard's coefficient **[15]** was used to construct the similarity matrix among all four varieties of *M. piperita*. The similarity coefficients were used to generate Dendogram by using UPGMA (unweighted pair group method of Arithmetic means) through the programme NTSYS-PC (Numerical taxonomy system, applied Biostatistics. Inc, New York, U.S.A., software version 2.02e **[16]**. The polymorphism information content (PIC) value was calculated as PIC=1-ΣP2i; Pi is the band frequency of the ith allele **[17]**. Principle coordinates analysis (PCA) was performed by using EIGEN value and Eigen vectors from a correlation matrix which was generated using a standardized data matrix 2-D and 3-D plots. "Maximum comp" option was used to find relationships among different varieties which are genetically diversified and for evaluation of grouping.

For RAPD analysis, four primers were used. They were OPA03, OPD8, RP16 and OPF1. These primers gave maximum number of amplification product in comparison with high intensity and minimal smearing. It has high resolution and could be seen as clear bands (**Figure 1- A, B, C, D**). Total 133

bands were observed after RAPD (1.5 percent get electrophoresis) and all are polymorphic bands. Thus, it indicates 100% polymorphism. Out of all four primers OPA03 gave maximum number of bands (53) and RPI6 gave minimum number of bands (24). The range of amplicons was found between 160-1210 bp. The highest similarity index was noted 0.9166667 and it was between M4 and M15 [**Figure 3(D)**]. Dendogram based on UPGMA method indicate segregation of varieties of *Mentha piperita*. Similar variation was observed in Dendrogram and in 2D [**Figure 3(A)**] and 3D [**Figure 3(B)**] plots. In Dendrogram [**Figure 3(C)**], cluster 1 and 2 were observed. In cluster 1 it includes M1, M2, M3, M4 (Using primer OPD8), M5, M6, M7, M8 (Using primer RP16), M9, M10, M11, M12 (Using primer OPA03), M13, M15, M16 (Using primer OPF1) and in cluster 2 it includes M14 (Using primer OPF1).Cluster 1 is divided into cluster A, cluster B and cluster C. In cluster A, it includes varieties M1 and M2.Cluster B was further divided into B1 and B2. B1 is further divided into B1a and B1b. B1a is further divided into B1ai and B1aii. B1ai id further divided into B1aia and B1aib.B1b is divided into B1bi and B1bii. B1bii is further divided into B1biia and B1biib.B2 is also divided into B2bi and B2bii. M1 (*M. piperita*, Surat) and M2 (*M. piperita*, Ahmedabad) are in cluster A. M3 (Ahmedabad), M4 (Vadodara), M15 (Ahmedabad) are in cluster B1aia, M7 (Ahmedabad) is in B1aib. M9 (Surat) is in cluster B1bi, M10 (Anand) and M11 (Ahmedabad) are in B1biia. M12 (Vadodara) is in cluster B1biib. M5 (Surat), M8 (Vadodara) is in B2bi and M13 (Surat) is in cluster B2bii. M6 (Anand) is in cluster C. M14 (Anand) is included in cluster 2. Here M1 and M2 were 100% similar M3, M4 and M15 were 100% similar. M10 and M11 were 100% similar. M5 and M8 were 100% similar.

The dendogram constructed by RAPD marker successfully differentiated all four varieties of different region and this result shows that this marker is powerful in studying closely related taxa. The highest PIC value was obtained by primer RPI6 (0.8888). The average PIC value of all four primers was 0.90260. Thus on the basis of RAPD, the study were similar to the observations of [18]. The good correlation “r value” was observed among all 4 markers which was 0.83161 and “t value” was 4.9620 **[19]** studied the genetic variation in cultivars of (*C. roseus* using RAPD markers).

Previously *Mentha*, a taxonomically complex section was studied by **[20]** by fingerprinting. They used RAPD primers to analyse 11 accessions from six taxa of *Mentha* developed

by CIMAP. The amplification profiles produced by 60 primers gave total of 630 bands, out of which only 41 were monomorphic. This showed 93.5% polymorphism. Maximum numbers of bands were produced by primer MAP-04 (19) and minimum numbers of bands were produced by primer OPT-11(1).

While in our study of diversity of *Mentha piperita*, four varieties were taken from different four districsts of central and south Gujarat region. In pooled RAPD analysis, all four primers, 134 total scorable bands were observed. All were polymorphic, which indicated 100% polymorphism. Maximum numbers of bands (53) were observed by primer OPA-03 and primer RPI-6 gave minimum number (24) bands. The PIC value was noted 0.9334595. Thus it gave higher polymorphism rate than previous study of *Mentha*.

In SSR analysis, the primer used was (GAA) 7. Total 33 bands were observed and out of those 25 bands were polymorphic, thus this indicated 75.75% polymorphism. The range of amplicons was from 890-1400 bp. In this also similarity matrix was plotted using Jaccard's coefficient (data not shown) and it range 0.81818 to 0.18181 and it gives PIC value of 0.9127640. Thus it indicates higher degree of genetic variation. In this also Dendogram was constructed using UPGMA method as well as 2D [**Figure 4(B)**] and **3D** [**Figure 4(C)**] plots were constructed. Dendogram [**Figure 4(A)**] includes cluster 1 and cluster 2. Cluster 1 divided in to MA (*M. piperita*, Anand), MB (*M. piperita*, Ahmedabad) and MC (*M. piperita* , Surat) and these three are 100% similar. While MD (*M. piperita*, vadodara) is included in cluster 2.

In ISSR analysis, the primer used was (GA) 9T and it gives comparatively higher number of bands than other primer. In ISSR analysis, total 21 bands were observed and out of those 17 bands are polymorphic and 4 bands are monomorphic (observed in 2% gel electrophoresis). Total polymorphism is 80.95% which is higher. The range of amplicons was from 230-920 bp. And similarity matrix was plotted using Jaccard's coefficient (data not shown) and it ranges from 0.555 to 0.166 by ISSR marker. The PIC value was observed about 0.714285.In our study, the ISSR analysis detected high degree of genetic diversity. Similar variation was observed in dendogram constructed using UPGMA method as well as in 2D [**Figure 5 (B)**] and 3D [**Figure 5 (C)**] plots.

In Dendogram [**Figure 5 (A)**], cluster 1 and cluster 2 was observed Cluster 1 includes ME (*M. piperita*, Anand). MF (*M. piperita*,

Ahmedabad), MH *(M. piperita*, Vadodara) these all three are 100% similla, and in cluster 2, it includes MG (*M. piperita*, Surat).

The present research work in mint species of DNA profiling shows that it is possible to analyze the RAPD patterns for correcting similarity and distance between species and accessions by which it can be predicted the origin of the species and cultivars to a great **[20]**. This would be useful tool in identifying and protecting the mint varieties from possible infringements in future and in further development of new *Mentha* varieties **[21]**.

CONCLUSION

From our overall study, it can be concluded that these all the three markers (RAPD, ISSR and SSR) can be used for designing strategy for maintaining the genetic diversity or enhance them. These data can be useful for crop improvement programs of *Mentha piperita* & for plant breeding. It is useful for Mentha breeders to develop and analyse novel hybrids for society.

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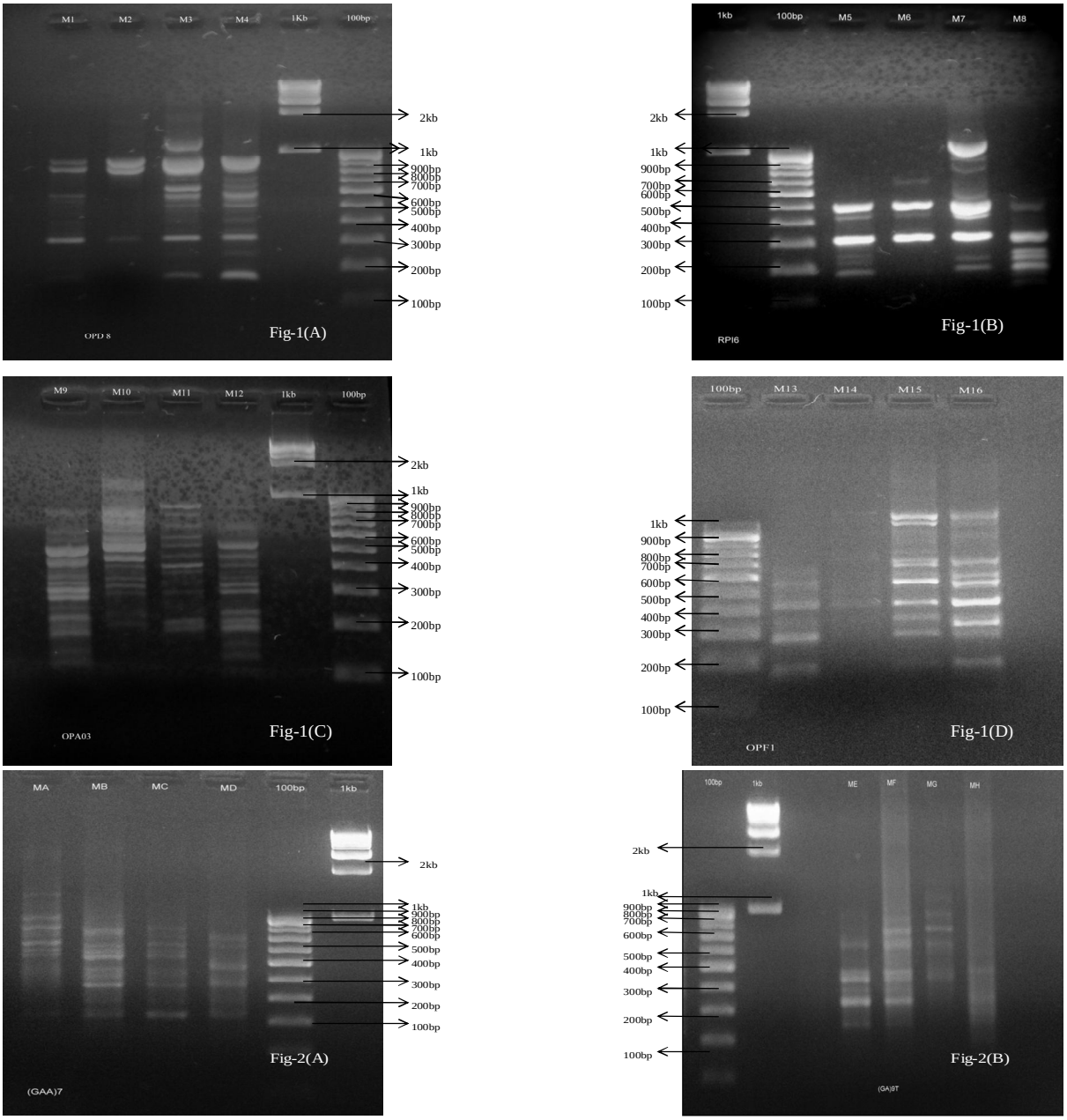


Figure 1 (A,B,C,D) shows PCR amplification by primers OPD8, RP16, OPA03, OPF1 respectively and RAPD banding pattern of four *Mentha piperita* varieties where [*M. piperita* (surat)-M1, M5, M9, M13] ; [*M. piperita* (Anand)-M2, M6, M10, M14] ; [*M. piperita* (Ahmedabad)-M3, M7, M11, M15] ; [*M. piperita* (vadodara)-M4, M8, M12, M16]. Figure 2(A) shows PCR amplification by SSR primer (GAA)7 and (B) shows PCR amplification by ISSR primer (GA9)T, of four *M. piperita* varieties

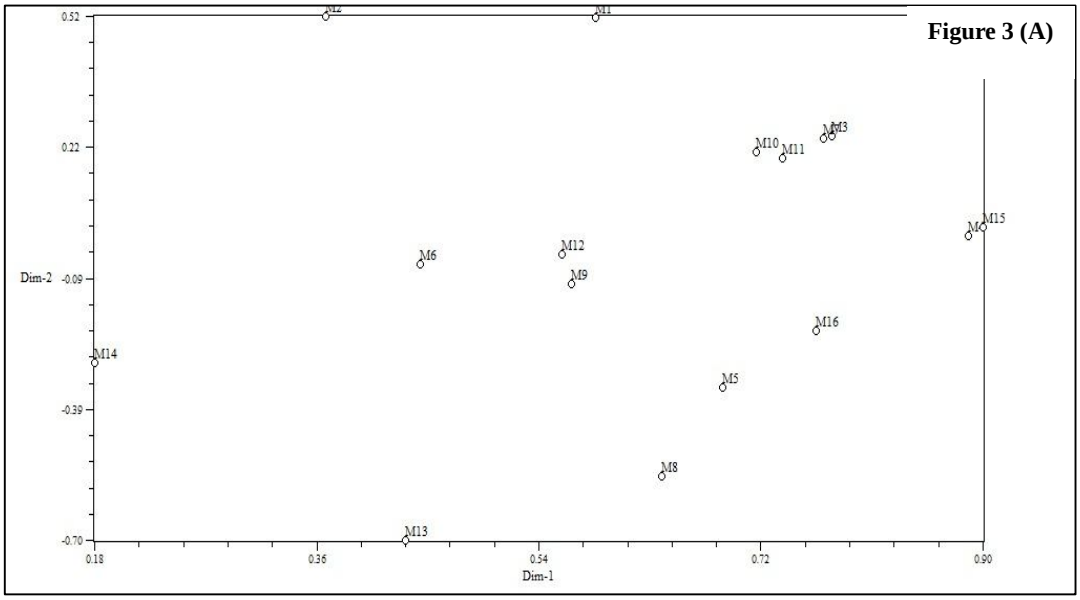
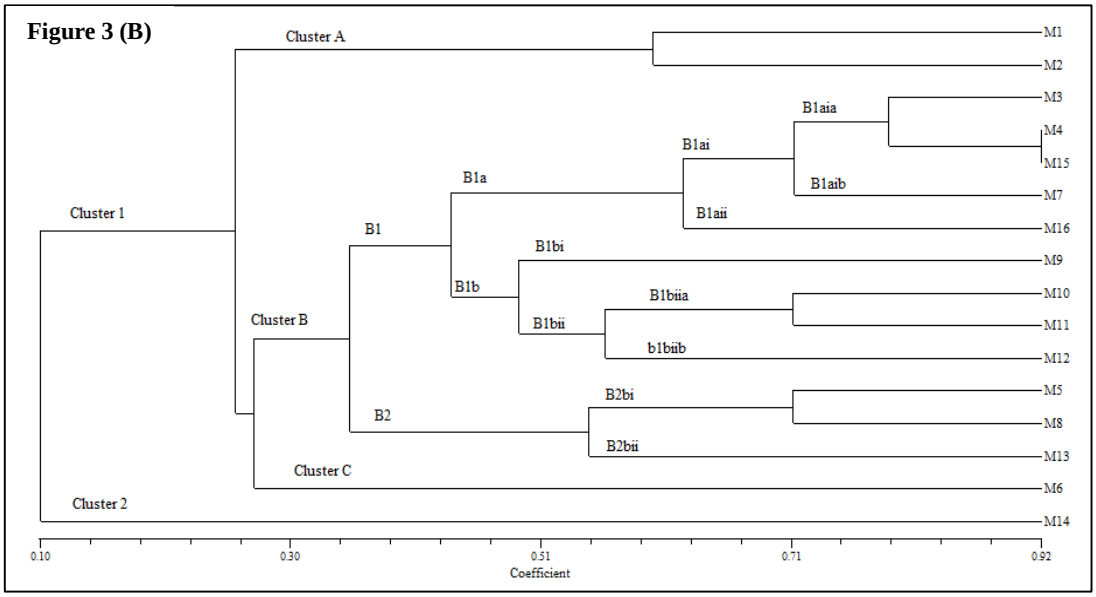


Figure 3 (A)

Figure 3 (B)



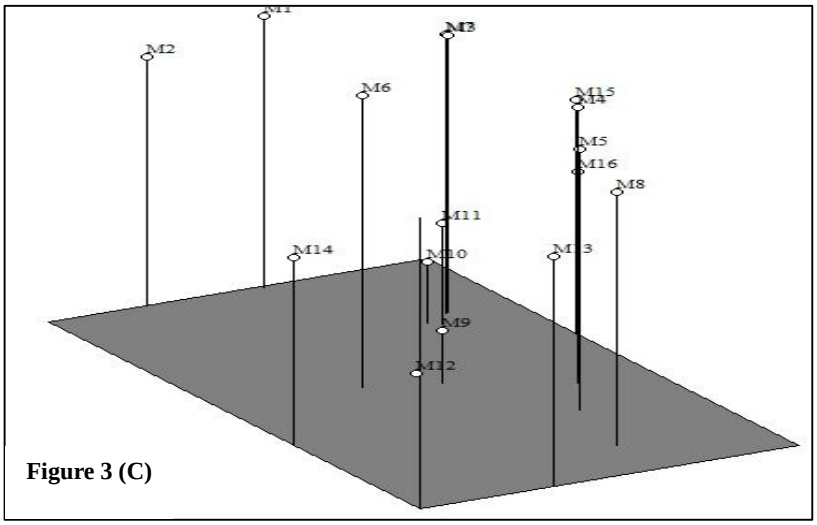


Figure 3 (C)

Rows\Cols	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12	M13	M14	M15	M16
M1	1.0000000															
M2	0.6000000	1.0000000														
M3	0.5000000	0.3000000	1.0000000													
M4	0.4545455	0.2727273	0.7500000	1.0000000												
M5	0.3750000	0.1250000	0.3333333	0.5454545	1.0000000											
M6	0.2857143	0.0000000	0.4000000	0.3636364	0.4285714	1.0000000										
M7	0.5555556	0.3333333	0.7272727	0.6666667	0.5000000	0.3000000	1.0000000									
M8	0.2222222	0.1250000	0.3333333	0.5454545	0.7142857	0.2500000	0.3636364	1.0000000								
M9	0.2352941	0.1176471	0.3000000	0.4210526	0.3750000	0.1764706	0.3157895	0.3750000	1.0000000							
M10	0.3571429	0.2142857	0.5000000	0.5625000	0.3333333	0.2000000	0.5333333	0.2500000	0.5000000	1.0000000						
M11	0.5000000	0.3000000	0.4285714	0.6153846	0.4545455	0.2727273	0.4615385	0.3333333	0.4444444	0.7142857	1.0000000					
M12	0.2000000	0.1428571	0.2777778	0.4117647	0.2666667	0.1333333	0.2222222	0.2666667	0.5263158	0.5882353	0.5333333	1.0000000				
M13	0.0000000	0.0000000	0.1666667	0.3636364	0.4285714	0.1428571	0.1818182	0.6666667	0.2500000	0.1250000	0.1666667	0.2142857	1.0000000			
M14	0.0000000	0.0000000	0.0909091	0.0833333	0.1428571	0.0000000	0.1000000	0.1428571	0.0588235	0.1428571	0.0909091	0.0714286	0.2000000	1.0000000		
M15	0.4166667	0.2500000	0.8333333	0.9166667	0.5000000	0.3333333	0.7500000	0.5000000	0.4000000	0.6250000	0.5714286	0.3888889	0.3333333	0.1666667	1.0000000	
M16	0.2727273	0.2000000	0.5833333	0.6666667	0.5000000	0.3000000	0.5000000	0.5000000	0.3157895	0.4375000	0.4615385	0.4666667	0.4444444	0.2222222	0.7500000	1.0000000

Figure 3 (D)

Figure 3 (A) Shows 2-D plot among four *M. piperita* Cultivars Revealed by RAPD. (B) 3-D PLOT REVEALED by RAPD (C) Phylogenetic Relationship Revealed by RAPD.(D)Similarity Matrix Table by Using Jaccard's Coefficient

Fig-4 (a)

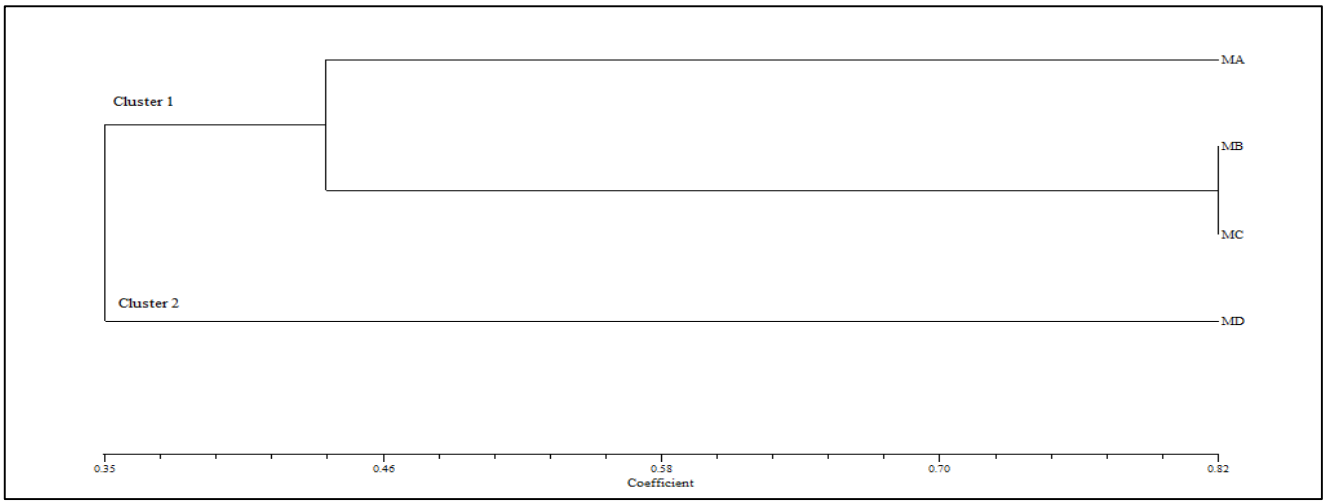
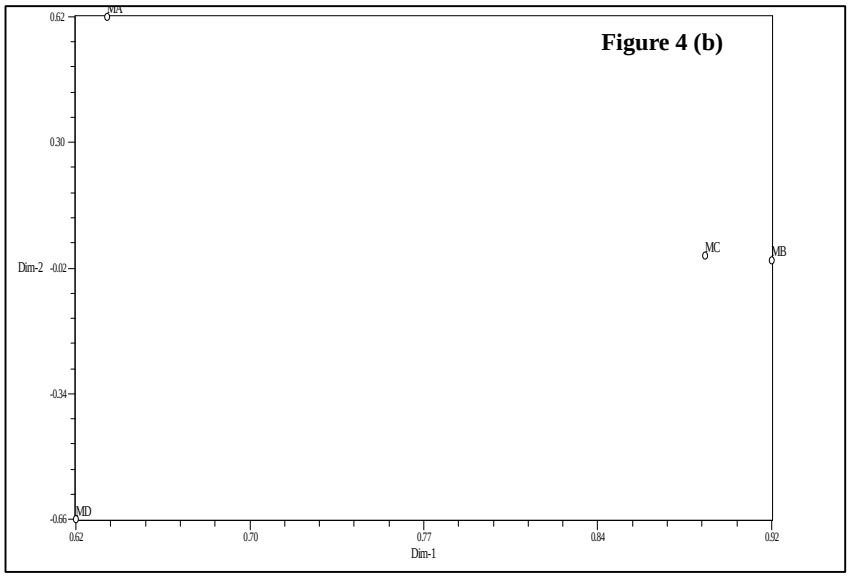


Figure 4 (A)



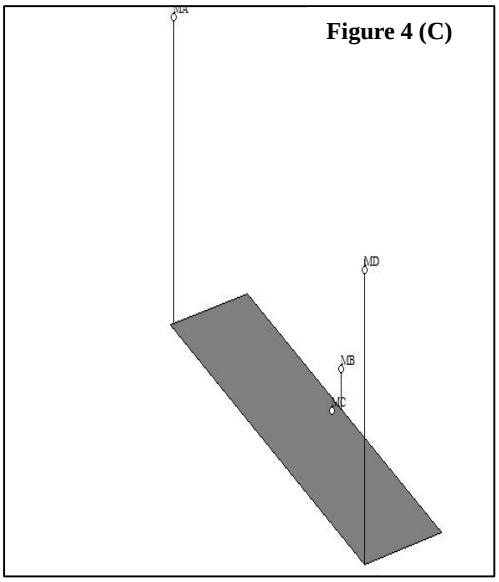


Figure 4 (A) Shows Phylogenetic Relationship Among Four *M. piperita* Cultivars Revealed by SSR. (B) 2-D plot Revealed by SSR (C) 3-D Plot Revealed by SSR

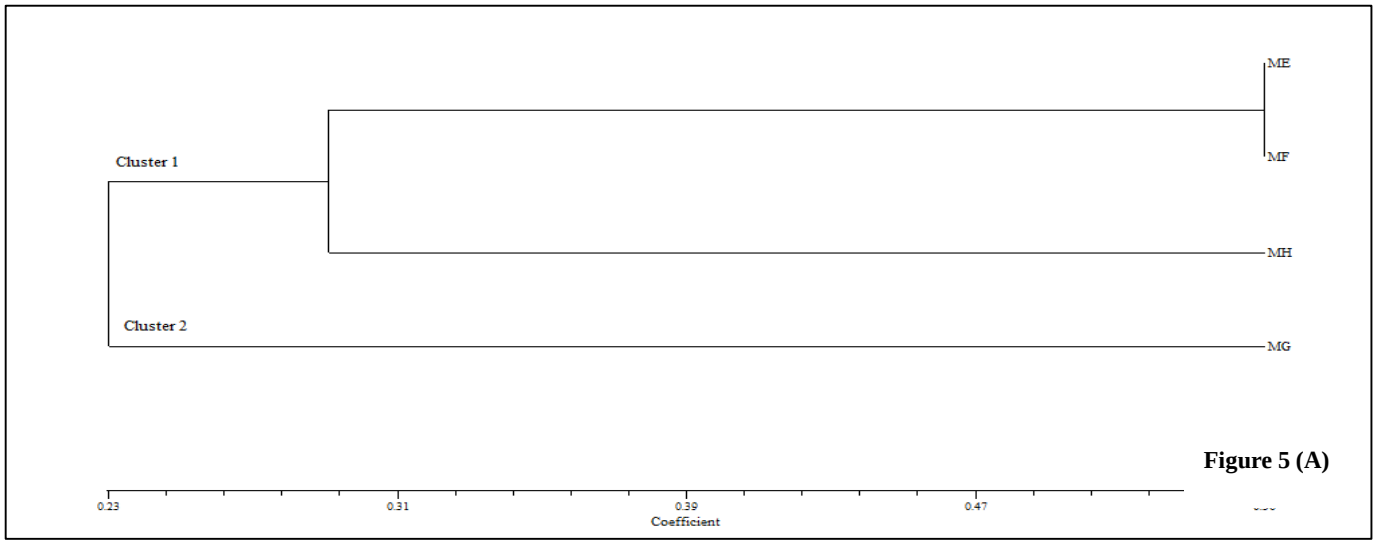


Figure 5 (A)

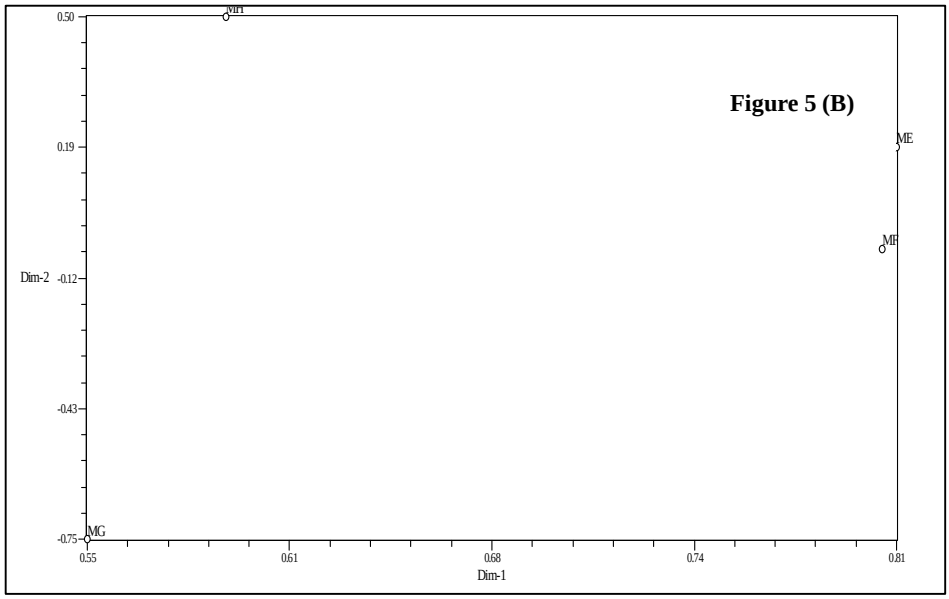


Figure 5 (B)

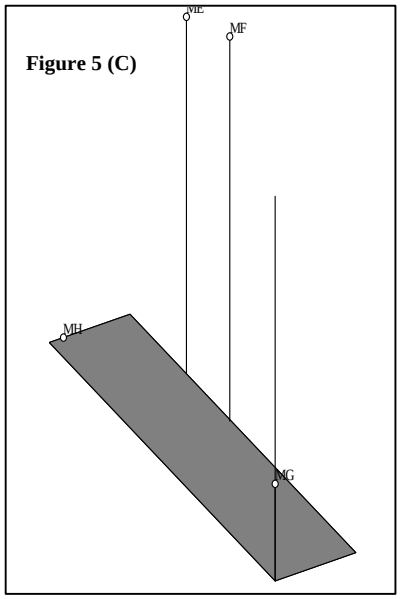


Figure 5 (C)

Figure 5 (A) Shows Phylogenetic Relationship Among Four *M. piperita* Cultivars Revealed by ISSR. (B) 2-D Plot Revealed by ISSR (C) 3-D Plot Revealed by ISSR

Table 2: Detailing of RAPD Primers and Analysis of Amplified Bands in Four Mentha Cultivars

Primer	Nucleotide Sequence	Range of Amplification (in bp)	Total Bands	Total Polymorphic Bands	Total Monomorphic Bands	PIC
OPD8	GTGTGCCCCA	160-1100	29	17	12	0.898929845
RPI6	ACACACGCGCTG	170-990	24	16	8	0.88888889
OPA03	AGTCAGCCAC	190-1210	53	29	24	0.941232323
OPF1	ACGGATCCTG	180-1000	27	23	4	0.902606312
TOTAL		160-1210	133	85	48	0.933459571

Table 3: Detailing of SSR Primer and Analysis of Amplified Bands in Four Mentha Cultivars

Primer	Annealing temperature	Range of Amplification (in bp)	Total Bands	Total Polymorphic Bands	Total Monomorphic Bands	PIC
(GAA)7	54^0C	890-1400	33	25	8	0.912764007

Table 4: Detailing of ISSR Primer and Analysis of Amplified Bands in Four Mentha Cultivars

Primer	Annealing temperature	Range of Amplification (in bp)	Total Bands	Total Polymorphic Bands	Total Monomorphic Bands	PIC
(GA)9T	53 ºC	230-930	21	17	4	0.714285714