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MOLECULAR AND MORPHOLOGICAL CHARACTERIZATION OF SELECTED *MENTHA* SPECIES

ZABTA KHAN SHINWARI[1], SIDRA SULTAN[2] AND TARIQ MAHMOOD[2]

[1]*Department of Biotechnology, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan*
[2]*Department of Plant Sciences, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan*

Abstract

The present study was conducted with the objective of finding out the concordance between morphological data and molecular data of *Mentha spicata* and *Mentha royleana*. Moreover the study was aimed to investigate the relationship between and within the two species. *Mentha* species were collected from QAU, Shadara, Chattar, Donga gali and Qarshi industries, Hattar. For molecular study Random Amplified Polymorphic DNA was performed which was efficient in detecting polymorphism and genetic variation within and between *Mentha* species. Random primer OPC-9 generated a total of 63 bands sharing 93.6% polymorphism in *Mentha spicata* whereas OPC-4 generated 30 bands showing 100% polymorphism in *Mentha royleana.*The morphological and molecular data were analysed using softwares NTSYS pc version 2.02. The results indicated a considerable level of morphological and molecular diversity between the species.

Introduction

Family Labiatae comprises of about 210 genera and some 3,500 species (Davidson, 1999). The family name Labiatae refers to the flowers that have typical petals fused into an upper lip and a lower lip. Although this is still considered an acceptable alternate name, most botanists now use the name "Lamiaceae" in referring to this family. The genus *Mentha* belongs to the family Lamiaceae (Labiatae) consisting of about 25 to 30 species mainly found in temperate regions of Eurasia, Australia , South Africa and North America (Brickell & Zuk, 1997). All mints prefer and thrive in cool, moist spots in partial shade (Bradley, 1992). Mints grow 10 -120 centimeters tall and can spread over an indeterminate sized area. In Botany, mint is the common name for any of the various herbaceous plants and perennial aromatic herbs that are cultivated for their essential oils and culinary purposes. The genus *Mentha* L. (Lamiaceae) produces secondary metabolites such as alkaloids, flavanoids, phenols, gummy polysaccharides. Terpens and quinines are used in food and pharmaceutical, cosmetics and pesticide industries (Khanuja *et al*., 2000). Some members of this genus are also used as herbal teas and condiments both in fresh and dried form due to their distinct aroma (Baser *et al*., 1995).

The most common species of the genus *Mentha* found in Pakistan are *M. pulegium, M. arvensis, M. spicata, M. longifolia, M. piperita* and *M. royleana* (Hedge & Wendelbo, 1978). However Shinwari & Chaudhri (1992) revised the genus and re- examined its taxonomy. They reported 5 species in Pakistan viz., *M. arvensis, M. piperita, M. spicata, M. longifolia* and *M. royleana.* Morphological markers (such as plant height, leaf shape, colour, etc) are among the oldest markers used in the evaluation of genetic variability. However, they are not sufficiently specific and informative because different gene expression in different environments causes wide variability of phenotypic characters in individuals. In some cases congruence between morphology and molecular phyllogenetics were reported. (Shinwari,1995). Genetic diversity refers to the variation at the level of individual gene and provides a mechanism for the plants to adapt in ever changing environment.

Random Amplified Polymorphism DNA (RAPD) markers are a modification of Polymerase Chain Reaction (PCR) used in the late 1980 (Williams *et al*., 1990). Among PCR based molecular markers RAPD is a widely used technique in different plants (Nazar & Mahmood, 2011; Kayani *et al.,* 2011; Mahmood *et al.,* 2011; Mahmood *et al.,* 2010a; Mahmood *et al.,* 2010b). PCR technique is one of the best available DNA-based tools for scoring variations between cultivars within species (Lakshmikumaran & Bhatia, 1998). One probable disadvantage is the degree of reproducibility of these markers, sometimes which can be low (Muralidharan & Wakeland, 1993; Ellsworth *et al*., 1993; Skroch & Nienhis, 1995) particularly between laboratories (Penner *et al*., 1993; Jones *et al*., 1997). This is due to sensitivity of RAPD banding patterns to reaction conditions, and the difficulty in exactly replicating reaction conditions across laboratories, where different brands of thermocyclers may be used. The technique is being successfully used widely for the estimation of genetic variability as well as the cultivar identification / differentiation in various plant species, including rice (Mackill, 1995), Broccoli and cauliflower (Hu & Quiros, 1991), banana (Howel *et al*., 1994), *Brassica* (Jain *et al*., 1994), *Triticum* (Chandrashekhar & Nguyen, 1993), *Medicago* (Yu & Pauls, 1993), *Coffea* (Orozco-Castillo *et al*., 1994) and *Lycopersicon* (Williams & St Clair, 1993) etc. In the present paper, we describe the similarity and diversity in terms of RAPD profiles of two mint species which includes fifteen accessions of each.

Material and Methods

Plant materials: Various species of genus *Mentha* were collected from 5 different sites including Quaid-i-Azam University campus, Shadara, Chattar, Donga gali and Qarshi industries Hattar (Table 1). The voucher was submitted to Herbarium of QAU Islamabad.

Plant DNA extraction: Total genomic DNA was extracted from fresh leaves using CTAB method by Richards (1997).

Amplification and electrophoresis of PCR products: 10 decamer primers of OPC series were used in the present study. RAPD analysis was performed following the protocol of Williams *et al*., 1990 with minor modifications. The amplification mixture for each sample DNA contained 50 ng of each template, 10.5 µl of water nuclease-free, 12.5 µl of Master Mix (MBI, Fermentas), and 1µl (25pM) of primer in a 25µl of total reaction volume. PCR was performed in gradient thermal cycler (Multigene Labnet) with an initial denaturation at 94ºC for 1 minute followed by 44 cycles of denaturation at 94ºC for 30 seconds, annealing at 40ºC for 1minute and extension at 72ºC for 2 minute. Final cycle was same except extension for 7 minute at 72ºC. After that PCR contents were stored at 4ºC till use. Amplification was checked by running the PCR products on 1.5% agarose gel prepared in 0.5X TAE (Tris Acetate EDTA) buffer at constant voltage of 70 volts for 3 hours. The gel was stained for 30 minute with ethidium bromide and visualized using gel documentation system. The molecular weight of amplified fragments was estimated with the help of 100 bp plus (MBI, Fermentas) DNA ladder.

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

Accession No.	Species	Localities	Accession No.	Species	Localities
1	*Mentha royleana*	QAU	16	*Mentha spicata*	QAU
2	*Mentha royleana*	QAU	17	*Mentha spicata*	QAU
3	*Mentha royleana*	QAU	18	*Mentha spicata*	QAU
4	*Mentha royleana*	Shadara	19	*Mentha spicata*	Shadara
5	*Mentha royleana*	Shadara	20	*Mentha spicata*	Shadara
6	*Mentha royleana*	Shadara	21	*Mentha spicata*	Shadara
7	*Mentha royleana*	Chattar	22	*Mentha spicata*	Chattar
8	*Mentha royleana*	Chattar	23	*Mentha spicata*	Chattar
9	*Mentha royleana*	Chattar	24	*Mentha spicata*	Chattar
10	*Mentha royleana*	Donga gali	25	*Mentha spicata*	Donga gali
11	*Mentha royleana*	Donga gali	26	*Mentha spicata*	Donga gali
12	*Mentha royleana*	Donga gali	27	*Mentha spicata*	Donga gali
13	*Mentha royleana*	Qarshi, Hattar	28	*Mentha spicata*	Qarshi, Hattar
14	*Mentha royleana*	Qarshi, Hattar	29	*Mentha spicata*	Qarshi, Hattar
15	*Mentha royleana*	Qarshi, Hattar	30	*Mentha spicata*	Qarshi, Hattar

Data scoring: The amplified products were sored as 1 for presence and 0 for absence respectively. The analysis was plotted in the form of a dendrogram. All computations were carried out using the NTSYS- pc, version 2.2 package (Rohlf, 2005). A dendrogram based on similarity coefficients was generated using the unweighted pair group method of arithmetic means (UPGMA) by NTSYS software.

Results and Discussion

The present study was conducted with the objective of finding out the morphological and molecular diversity to investigate the relationship within and among two species of *Mentha* i.e., *Mentha spicata* and *Mentha royleana*. The amplification profiles produced by five primers gave a total of 215 bands in *M. royleana*, out of which 150 bands were monomorphic while 59 were polymorphic. The percentage polymorphism observed in all *M. royleana* species was 27.44%. Maximum number of bands was produced by primer OPC- 5 (66) and minimum by primer OPC- 6 (16). In *Mentha spicata*, total 277 bands were produced by five primers. Out of which 135 bands were monomorphic while 131 were polymorphic. The percentage polymorphism observed in all *M. spicata* species was 47.2%. In this study OPC-5 generated maximum number of bands (73) while OPC-6 amplified minimum number of bands (41). It was observed that OPC 2 and OPC 9 generated maximum number of bands while OPC 5 amplified minimum number of bands across all the genotypes. The monomorphic bands are constant bands and cannot be used to study diversity while polymorphic bands revealed differences and can be used to examine and establish systematic relationships (Hadrys *et al*., 1992). The variation in the number of bands amplified by different primers are influenced by variable factors such as primer structure, template quantity and less number of annealing sites in the genome (Kernodle *et al*., 1993).

Morphological studies were carried out based on 31 morphological characters including stem, leaf size and leaf colour, inflorescence and other floret characteristics. The data was analyzed by UPGMA cluster analysis based on similarity level and using software NTSYS. The 30 accessions belonging to two different species formed 2 major clusters (Fig. 1). *M. royleana* showed 84% similarity while *M. spicata* showed 88% similarity. On the basis of morphological data *M. royleana* showed 39% similarity while *M. spicata* showed 49% similarity. Fig. 1 shows dendrogram showing morphological relationship among two species i.e., *Mentha spicata and Mentha royleana.* At 35% similarity coefficient, two distinct groups were observed i.e., Group 1 represents *M. spicata* and Group 2 represents *M. royleana.* It was intresting to note that each intact larger group contained samples of single species. This indicated that the status of the species is valid. Group 1 contained all samples from *Mentha spicata* and group 2 contained all the samples of *Mentha royleana.*

In Group 1 there were 2 major Sub groups.Sub group 1 contained accessions 26, 24, 30, 23, 25, 27, 20, 28, 29, 19, 18 showing more genetic similarity while Subgroup 2 included accessions 22, 21, 17, 16, were genetically close to each other. In Group 2, Sub group 3 included only 2 accessions i.e., 6 and 13 while all the rest accessions 10, 4, 3, 8, 11, 5, 12, 7, 15, 2, 9, 14, 1 were clustered in sub group 4.

According to molecular data, UPGMA cluster analysis revealed two major clusters, cluster 1 of *M. spicata* and cluster 2 of *M. royleana* independently (Fig. 2). Cluster 1 included 2 accessions 20 and 28 which showed 88% similarity. There were two distinct sub-clusters in cluster 1: (1) within sub-cluster 1, two subgroups are clearly defined: (1) 19, 24, 29, 22 27, 23. (2) 18, 26, 21, 25 showed 95% similarity. Sub-cluster 2 included 16, 30 and 17. In the sub-cluster 1, maximum similarity was observed between *M. spicata* 19 and *M. spicata* 24 (98%). Cluster 2 included all accessions of *M. royleana* showed greater divergence as compared to *M. spicata*. Cluster 2 divided into two major sub clusters. Cluster 2 included accession 9 which remained unresolved due to greater diversity as compared to other accessions. At 91% similarity coefficient, sub cluster 1 was further divided into two subgroups. Subgroup 1 included accessions 6, 13, 8 and 14. It was observed that 6 and 13 showed 95% similarity with each other while 8 and 14 showed 92% similarity. Subgroup 2 included 2 accessions 10 and 15 which also showed 92%. Sub cluster 2 showed greater diversity as compared to sub cluster 1. Sub cluster 2 showed 86% similarity and included 2 subgroups. Subgroup 1 included two accessions i.e., 2 and 12. They showed 93% similarity with each other. Subgroup 2 included accessions 1, 4, 11, 5 and 3. Accessions 2 and 15 of *Mentha royleana* collected from Quaid-i-Azam University Islamabad and Qarshi industries showed 100% morphological similarity with eachother. Accessions 4 and 11 of *Mentha royleana* collected from Shadara & Donga Gali showed 100% genetic similarity. This suggested that geographical origin is not always a good predictor of genetic structure among populations. The present work is consistent with that of Soltis & Soltis (1991). Study on genetic diversity of *Mentha* species showed that the taxa maintained high levels of genetic

polymorphism among species but not among populations. The polymorphism within populations depicted genotype richness, recombination and gene flow. Clustering of populations based on UPGMA cluster analysis showed some unresolved accessions which were not clustered together. Polymorphisms revealed through RAPD technique may be due to deletion, elimination of primer binding site, an insertion making a fragment too large for polymerization and nucleotide substitutions in the primer annealing site (Fritsch & Rieseberg, 1992). The analysis of genetic variation both within and among plant materials is of fundamental interest to plant breeders. The genetic diversity of *Mentha* species is imprecise and of heterozygote nature. This obscures the determination of genetic diversity patterns based on morphological and phonological observations (Campos-de-Quiroz & Ortega-Klose, 2001).

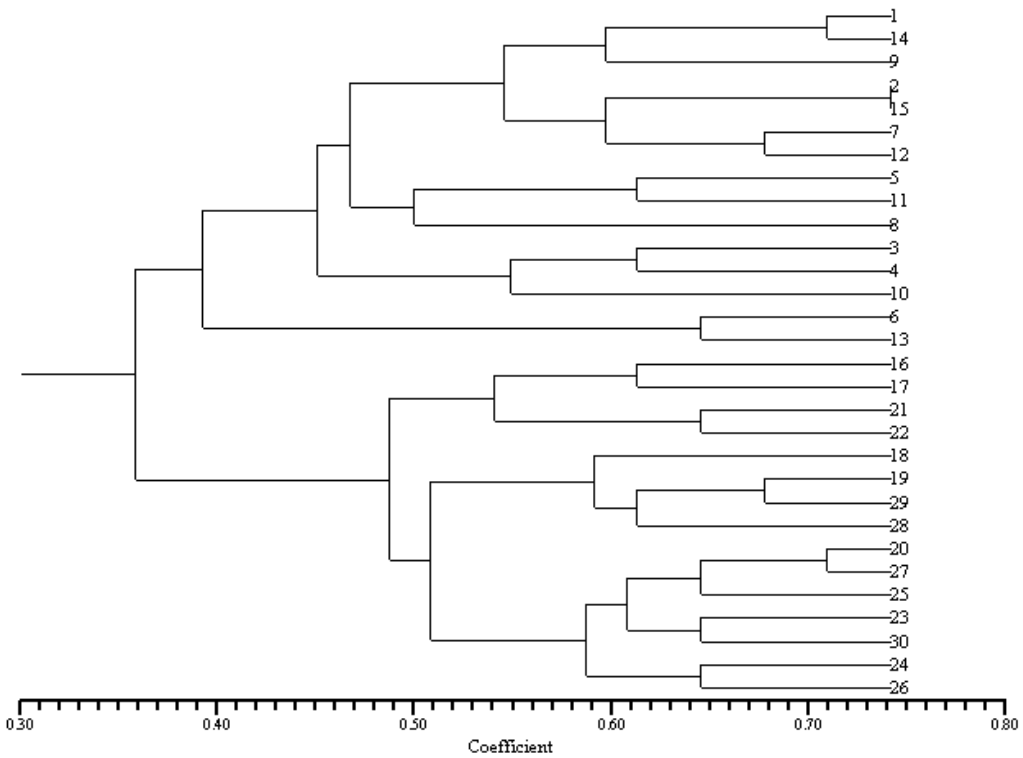

Fig. 1. UPGMA cluster analysis based on morphological data.

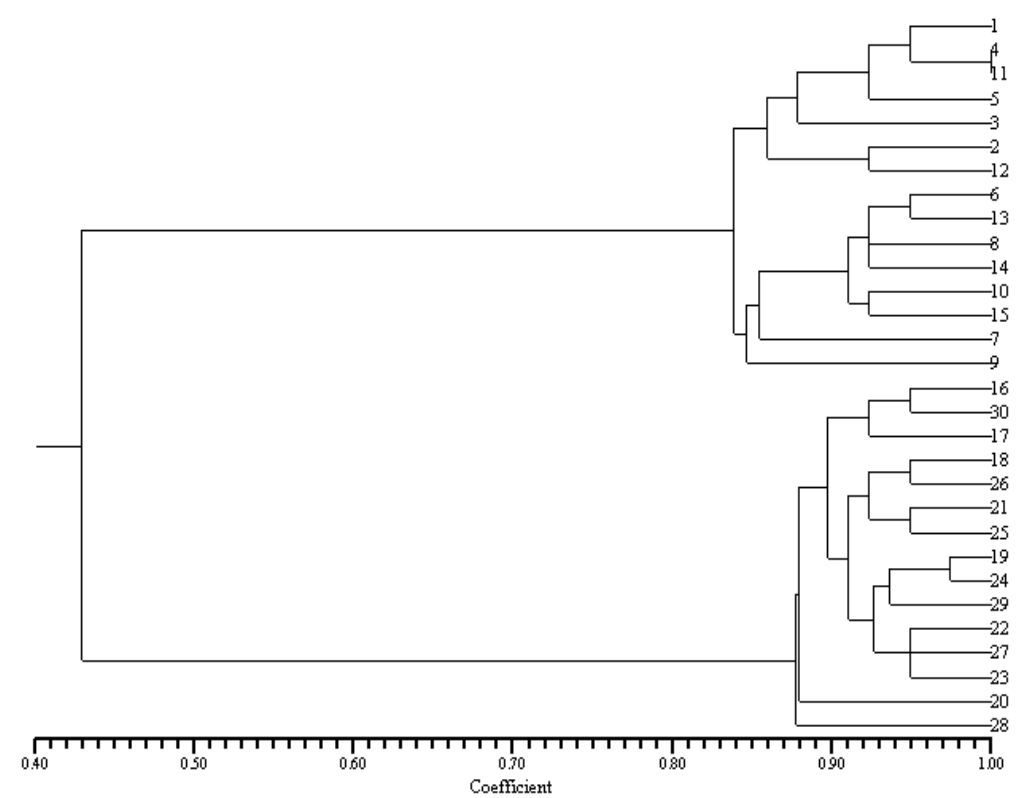

Fig. 2. Dendrogram representing the genetic relationships among all *Mentha* accessions of *M. royleana* and *M. spicata* using NTYSYS clustral analysis generated from five RAPD primers.

Fewer studies have been made on this genus, so a need was felt to explore and study this economically important genus. Considerable morphological and genetic variation was observed among *Mentha* species also showed close affinities with each other which might be due to sharing of almost similar habitat and ecology. The study was a starting point to explore *Mentha* of hilly areas. Further studies need to be done on different aspects including more species ecology, medicinal importance and further molecular studies.

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