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Reassessment of Mentha Species from Kunhar River Catchment Using Morphological and Molecular Markers

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ABSTRACT: ***Mentha*** *specimens collected from Kunhar River catchment of Hazara region were analyzed through numerical and molecular markers. For numerical analysis twenty two traits were used. Dendrogram analysis of morphological traits assorted the 25* ***Mentha*** *collections into 4 groups viz, Group-A, B, C and D. Group-A showed 98% similarity* ***(M. longifolia****). Group-B showed 98% similarity (****M. spicata****). Group-C showed 92% similarity (****M. arvensis****) and Group-D showed 89% similarity (****M. royleana****). Molecular analyses were carried out through 11 RAPD primers. Primers amplification revealed high level of genetic diversity (0-100%) existed among the* ***Mentha*** *genotypes. Dendrogram analyses based upon the genetic distance estimates conferred the cluster analysis of the morphological traits. Furthermore, the variations among populations of* ***M. royleana*** *need further elaboration through additional marker assisted discrimination for establishing their taxonomic status.*

Key words: Mentha*, CTAB, DNA, PCR, RAPD, dendrogram, trait.*

INTRODUCTION

The genus *Mentha* of family Lamiaceae comprising 25-30 species is widely distributed in the temperate areas (Kokkini *et al*., 1995; Dorman *et al*., 2003; Celenk *et al.,* 2008). The herbaceous, perennial mint plants are cultivated for their essential oils (produced as secondary metabolites) characterized by strong odor and widely used for medicinal and aromatic purposes (Betts, 2001; Bowles, 2003; Broza *et al*., 2009; Pichersky *et al*., 2006). The quality and quantity of the oils may change according to climate, soil composition, plant organ, age and stage in the vegetative cycle (Masotti *et al*., 2003; Angioni *et al.,* 2006).

In Pakistan *Mentha* is represented by six species that is *Mentha pulegium*, *M. arvensis*, *M. longifolia*, *M. piperita*, *M. spicata* and *M. royleana* (Ali and Nasir, 1990; Hedge, 1994). Out of these six species, four species i.e. *M.longifolia*, *M. arvensis*, *M. royleana* and *M. spicata* are commonly found in Kunhar river catchment. The river Kunhar catchment is administratively placed in District Mansehra Khyber Pakhtunkhwa Province located on 34° 14’ to 35° 11’ N latitudes, and 72° 49’ to 74° 08’E longitudes, spreading over an area of 4,579 km^2 (Figure 1). The catchment area is bound by Azad Jammu and Kashmir on the Eastern as well as on Southern side, Chilas and Gilgit on the North and Nandyar Khwarr-Siran River catchment in the West (Jan *et al*., 2008; Haq *et al*., 2010). Climatically the catchment lies in temperate region with distinct seasonal variation (Sultana and Qureshi, 2007).

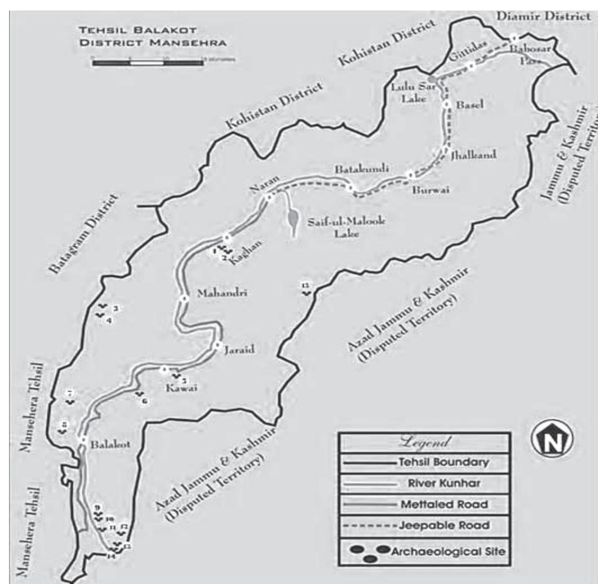

Figure 1. Map of Kunhar River catchment.

Classification of *Mentha* is very complex due to high polymorphism in morphology and great diversity in essential oil composition (Gobert *et al*., 2002). Different methods have been used in the past to examine the diversity of *Mentha* using morphological cytological, chromosomal and chemical markers (Malinvaud, 1880; Singh and Sharma, 1986; Harley and Brighton, 1977; Lawrence, 1978). The polymorphism of the genus needs careful taxonomic reassessment with modern technologies. The present research was conducted to optimize protocol for the DNA isolation from *Mentha* plants and to carry out DNA fingerprinting and phylogenetic elaboration of *Mentha* of Kunhar catchment through morphological and DNA markers.

MATERIALS AND METHODS

Plant specimens were collected from 25 different localities of river Kunhar catchment Mansehra Pakistan. The specimens were rightly pressed, dried, poisoned and mounted on the Herbarium sheets of size 28.75 cm x 32.50 cm. Field data including GPS coordinates, locality, and altitude were documented and submitted into Herbarium Women University of AJK, Bagh (WUAJKB) for future references. The specimens were provisionally identified with the help of Herbarium specimens and available literature (Stewart, 1972; Nasir and Ali, 1975; Xi- Ven and Hedge, 1994).

DNA isolation

The plant specimens were dried and then grinded with pestle and mortar. Genomic DNA was isolated with the help of modified protocols of Doyle and Doyle (1990) and Lodhi, *et al*. (1994). For DNA isolation 0.041 g of the powder material of each specimen were taken in 1.5 ml Eppendorf tube and added 500 µl of Cetyl tri methyl ammonium bromide (CTAB) buffer. The reagent was incubated for 35 minutes at 65°C, cooled at room temperature and added 500 µl of Chloroform: Isoamyl alcohol (24:1 v/v), mixed gently for 1-2 minutes and centrifuged at 6000 rpm for 15 min., supernatant was transferred to another eppendorf and was added to 250 µl of 5 M NaCl. The reagent was then added 500 µl of ice cold PCR grade ethanol and kept at 4-6 °C for 15-20 minutes. It was then centrifuged at 3000 rpm for 3 minutes and at 8000 rpm for 5 minutes. The supernatant was discarded and the pellet was washed with 70% ethanol. The DNA pellet then obtained was dried, dissolved in 20 µl TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0) and preserved at 4 ^{0}C. The DNA was treated with 1 µl RNaseA at 37 ^{0}C to remove the RNA. Quality and quantity of the DNA was checked on 1% agarose / TBE gel. For gel preparation 0.5 g agarose powder was dissolved in 50 ml TBE. The mixture was boiled on hotplate at 100 ^{0}C till the agarose was dissolved completely. The suspension was then cooled at room temperature. 5 µl Ethidium Bromide was added to it. The gel was casted in a Gel Tray with inserted comb. The suspension was allowed to solidify which was then placed in gel tank containing 1X TBE. For loading DNA, 3.00 µl DNA of each sample was mixed with 2 µl loading dye and was then loaded in the wells. Gel was then run at constant voltage of 80 volt and 120 mA for 30 minutes. The DNA was visualized under UV light using "Uvitech" Gel Documentation System.

RAPD analysis

The isolated DNA was checked with Gel electrophoresis and was subjected to dilution according to the quality and quantity of the DNA. The DNA which is visually concentrated for PCR reaction was diluted twice or thrice as needed. For each sample a pre-mix of 12.5 µl was prepared on ice for PCR. The pre-mix for PCR was prepared in 0.5 ml PCR tubes with 0.25 µl of each dNTP (10 mM), 0.5 µl of genomic DNA, 0.5 µl of primer, 1 µl of 10X buffer, 1 µl $MgCl_2$ and 0.5 µl of Taq Polymerase. The volume in the tube was made 12.5 µl by adding 8 µl of H_2O. The PCR reaction was carried out in Applied Biosystems 2720 thermal cycler. All the specimens were run independently with each primer for PCR amplification. Thermocycling condition of PCR was set as 94 ^{0}C for 4 minutes as denaturation temperature, 94 ^{0}C for 1 minute, 30 ^{0}C for 2 minutes, 72 ^{0}C for 1 minute. The cycling was continued for 35 cycles, which amplified a desired DNA sequence. The PCR product was mixed with 3 µl loading dye and electrophoresed in a 1.5 % Agarose gel mixed with Ethidium Bromide for 45 minutes to resolve the bands clearly. Eleven RAPD primers were used for DNA amplification. Details of the primers are given in Table 1.

Quantitative trait analysis

For numerical analysis hierarchical clustering was performed using the Euclidean distance index and Multivariate Cluster Analysis with the computer package MINITAB (Anonymous, 1996). Each taxon was treated as Operational Taxonomic Unit (OTU). Weightage was given to the key characters which are thought to be more informative than others (Abbott *et al*., 1985). For the analysis, total of twenty two characters were selected. These characters were assorted into two groups as quantitative characters and qualitative characters.

Quantitative characters

Seventeen quantitative characters including macromorphological and micromorphological characters were used for characterization. Macromorphological characters comprised of leaf length, leaf width, petiole length, spike length, pedicel length, bract length, peduncle length, leaf length of middle leaf, leaf width of middle leaf, Number of longitudinal veins per leaf and internode length, while micromorphological characterization comprised of length of sepal, length of petal, width of petal, length of style, length of anther and length of filament. For quantitative characters their average values were used (Abid and Qaiser, 2006).

Qualitative characters

Total of five qualitative characters were selected. Qualitative characters were included stem color, flower color, inflorescence, leaf dentation and leaf apex. Qualitative characters were recorded in binary state and in some cases in multiple states. The binary characters were recorded as 1 and 2. Multiple state characters were recorded as 1, 2, 3 and 4.

RESULTS AND DISCUSSION

The PCR amplification results produced from GLA- 11, GLA-15, Gt-2, Gt-4, Gt-5 and Gt-6 gave a total of 492 bands. Only 6 were monomorphic and the remaining 486 were polymorphic bands (98.7%). Gt-4 primer produced 102 (maximum) bands out of which 3 were monomorphic, Gt-5 produced 93 bands, Gt-6 produced 87 bands out of which 3 were monomorphic, Gt-2 gave 85 bands, GLA-11 gave 72 bands with 1 monomorphic while GLA-15 produced 51 (minimum) numbers of bands. The total accessions (1-25) of *Mentha* were clearly differentiated from one another. Further analysis of RAPD could clearly differentiate three varieties of *Mentha royleana* i.e *Mentha royleana var. afghanica, M. royleana var. royleana and M. royleana var. tugidus.*

Gel samples of the PCR amplification of 25 genotypes of *Mentha* collections (1-25) are provided in Figure 2, 3. The similarity coefficient matrix of 25 *Mentha* genotypes based on Multivariate Analysis is shown in Table 2. The

data of 6 RAPD primers using POPGENE 32 software to construct a dendrogram presented in Figure 4. On the base of dendrogram 25 genotypes were assorted into 4 groups (A, B, C, and D). Group A comprised of twelve genotypes; all of them belong to same species (*M. royleana*) but of different geographical regions. Group B comprised of nine genotypes of same species (*M. arvensis*). Group C comprised of two genotypes of same species *(M. longifolia*) and group D also comprised of two genotypes belong to same species (*M. spicata).*

Morphological similarity was estimated among 25 specimens of *Mentha* based on the numerical characterization using MINITAB software to construct dendrogram presented in Figure 5. 25 specimens of *Mentha* were assorted into 4 groups (A, B, C and D). Group A comprised of two specimens which show maximum similarity 98% belong to same species (*M. longifolia*). Group B also comprised of two specimens which also show maximum similarity 98% belong to same species (*M. spicata*). Group C comprised of nine specimens which show similarity up to 92% belong to same species (*M. arvensis*) and group D comprised of 12 specimens, which show similarity up to 89% belong to same species (*M. royleana*).

It is a common observation that isolation of high quality DNA from medicinal plants generally had troublesome due to secondary metabolites which directly or indirectly react with enzymatic reactions to reduce the yield and quality of extracted DNA (Weishing *et al*., 1995). Polysaccharides often react with DNA and thus reduce the action of restriction enzymes, DNA polymerase and ligase during DNA isolation (Sharma *et al*., 2002).

For over all genetic diversity studies, eleven Randomly Amplified Polymorphic DNA (RAPD) primers were used (GLA-11, GLA-15, GLA-18, GLB-12, GLB-14, GLC-20, Gt-2, Gt-4, Gt-5 Gt-6 and Gt-7). Among them, five primers (GLA-18, GLB-12, GLB-14, GLC-20 and Gt-7) did not optimize PCR. The RAPD primers produced different levels of genetic polymorphism. Over all genetic distances ranged from 0 to 66%. The findings strengthened earlier reports that RAPDs can be used for studying genetic polymorphism and tagging of useful genes. It is also evident from the PCR based assays, that RAPD-markers can be used effectively to estimate genetic variability in *Mentha* and could be considered as an easy diagnostic analysis for identifying the over lapping traits. It can also be noted that more molecular analysis is required to reach on a better conclusion regarding genetic variability and more detailed mapping of the *Mentha* genome.

Table 1. Basic information of RAPD primers used for DNA amplification.

S. No	Primer	Sequence (5'-3')	Polymorphic bands	Monomorphic bands	Polymorphism (%)
1	GLA-11	CAATCGCCGT	74	1	
2	GLA-15	TTCCGAACCC	51	-	
3	GLA-18	AGGTGACCGT	-	-	
4	GLB-12	CCTTGACGCA	-	-	
5	GLB-14	TCCGCTCTGG	-	-	
6	GLC-20	ACTTCGCCAC	-	-	
7	Gt- 2	TGCGCGATCG	85	-	
8	Gt- 4	GCGAATTCCG	102	3	
9	Gt- 5	GTGCAATGAG	93	-	
10	Gt- 6	GGATCTGAAC	87	2	
11	Gt- 7	GGACTCCACG	-	-	
Total			492	6	98.7

Source: Alpha DNA Company Canada.

Table 2. Means of Genetic Distances estimated in all the *Mentha* collections analyzed through DNA amplification.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1	-																								
2	0.5	-																							
3	0.5	0.25	-																						
4	0.44	0.22	0.08	-																					
5	0.5	0.22	0.25	0.22	-																				
6	0.5	0.2	0.12	0.05	0.2	-																			
7	0.47	0.51	0.31	0.32	0.51	0.37	-																		
8	0.55	0.63	0.45	0.54	0.65	0.58	0.5	-																	
9	0.5	0.55	0.51	0.49	0.55	0.48	0.61	0.7	-																
10	0.5	0.53	0.45	0.38	0.53	0.33	0.42	0.25	0.6	-															
11	0.61	0.26	0.16	0.19	0.33	0.16	0.41	0.62	0.47	0.5	-														
12	0.55	0.16	0.16	0.19	0.26	0.16	0.41	0.62	0.55	0.5	0.13	-													
13	0.47	0.23	0.04	0.04	0.23	0.09	0.27	0.5	0.50	0.42	0.14	0.14	-												
14	0.44	0.44	0.30	0.22	0.44	0.27	0.15	0.54	0.54	0.38	0.41	0.41	0.26	-											
15	0.41	0.45	0.48	0.45	0.23	0.43	0.40	0.65	0.53	0.53	0.57	0.50	0.47	0.30	-										
16	0.44	0.42	0.28	0.2	0.42	0.25	0.26	0.54	0.52	0.38	0.39	0.39	0.24	0.11	0.38	-									
17	0.4	0.47	0.4	0.33	0.47	0.38	0.09	0.58	0.66	0.44	0.51	0.44	0.37	0.16	0.36	0.27	-								
18	0.47	0.40	0.20	0.21	0.40	0.26	0.16	0.5	0.56	0.42	0.30	0.30	0.16	0.24	0.45	0.31	0.26	-							
19	0.44	0.28	0.15	0.16	0.28	0.20	0.38	0.54	0.53	0.47	0.25	0.17	0.11	0.37	0.50	0.36	0.38	0.26	-						
20	0.55	0.5	0.5	0.52	0.6	0.5	0.47	0.29	0.66	0.16	0.46	0.33	0.47	0.52	0.6	0.52	0.5	0.47	0.44	-					
21	0.55	0.44	0.49	0.46	0.54	0.44	0.20	0.62	0.58	0.5	0.49	0.49	0.48	0.3	0.43	0.41	0.23	0.37	0.58	0.55	-				
22	0.41	0.23	0.48	0.45	0.45	0.43	0.40	0.65	0.61	0.53	0.50	0.40	0.47	0.42	0.22	0.38	0.36	0.45	0.50	0.5	0.33	-			
23	0.46	0.36	0.36	0.31	0.46	0.36	0.36	0.62	0.58	0.5	0.42	0.33	0.34	0.22	0.43	0.11	0.28	0.40	0.37	0.46	0.44	0.33	-		
24	0.5	0.2	0.45	0.42	0.42	0.4	0.23	0.65	0.66	0.53	0.46	0.36	0.43	0.33	0.38	0.44	0.2	0.40	0.55	0.5	0.16	0.16	0.38	-	
25	0.5	0.2	0.45	0.42	0.42	0.4	0.23	0.65	0.66	0.53	0.46	0.36	0.43	0.33	0.38	0.44	0.2	0.40	0.55	0.5	0.16	0.16	0.38	0	-

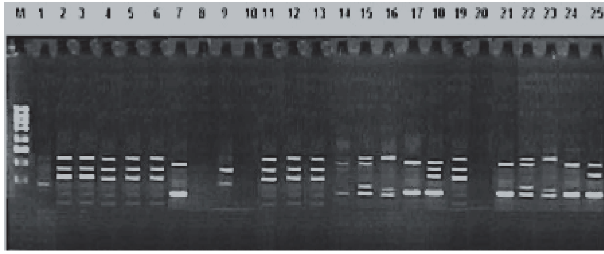

Figure 2. Gel picture of *Mentha* samples amplified with Gt-2 primer viewed under gel documentation system.

It is further evident from morphological study that numerical taxonomy can also be used as a best tool for identification in those species whose taxonomy is complicated by continuous polyploidy and stabilization of novel forms by ease of vegetative propagation. It is further noticed that there was no conclusion at varietal level in the present dendrogram. Therefore, it is strongly recommended that more morphological characters and molecular markers have to be used to refine the species up to varietal level.

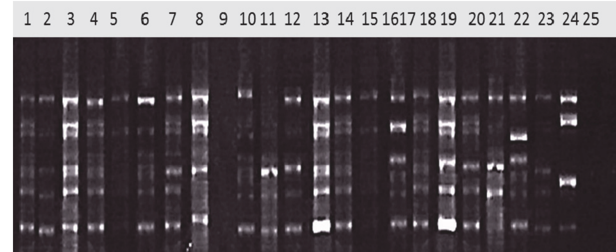

Figure 3. Gel picture of *Mentha* samples amplified with Gt-4 primer viewed under gel documentation system.

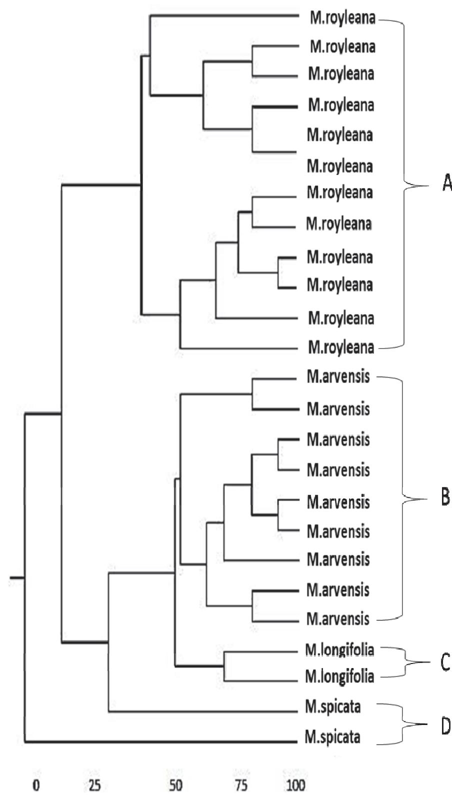

Figure 4. Dendrogram of *Mentha* collections obtained from RAPD-DNA bands analyzed by using Pop Gene 32.

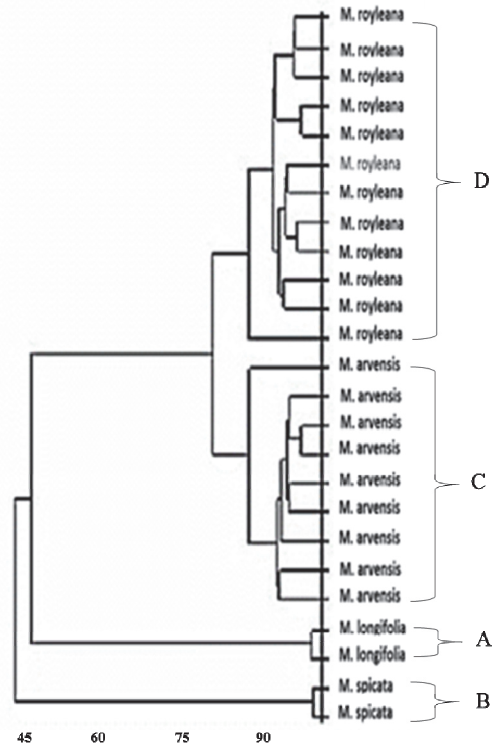

Figure 5. Dendrogram based on numerical data obtained *Mentha* collections computed by using MINITAB program.

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