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Development of species specific SCoT markers and analysis of genetic diversity among *Mentha* genotypes

Nidaf khan[1], Saudan Singh[1], Sunita Singh Dhawan[1]

CSIR- Central Institute of Medicinal and Aromatic Plants, P.O CIMAP, Lucknow , Uttar Pradesh, India

Abstract

Mentha is a genus of aromatic plants in the family Lamiaceae. The essential oils from *M.spicata* contains d-limonene, carvone and dihydrocarvone. *M.piperita* with high menthol content also contains and menthone, methyl esters particularly menthyl acetate. Mint species are not clearly distinct as hybridization between some of them occurs naturally. The objective of the study was to develop SCoT markers used for genetic mapping for germplasm resources. We have developed a set of SCoT in mint species viz., *M.piperita, M.spicata, M.cardiaca, M.viridis.* Genetic differences were studied to evaluate the level and distribution of diversity among genotypes of *Mentha* using SCoT markers. The results showed significant differences among 13 genotypes. The 10 SCoT primers chosen for molecular analysis revealed 171 bands, of which 117 were polymorphic. Percentage of polymorphic bands ranged from 75 to 100% according to primers tested. The generated dendrogram based on SCoT profiles divided the genotypes into 5 groups. Cluster analysis based on these traits grouped the genotypes into 8 separate clusters. MPST 13 and MPST 14 SCoT primers tested in this study yielded highly informative patterns. The generated SCoT fragments sizes ranged from 200-600 bp. Genotypes of two clusters with a good amount of genetic divergence and desirable agronomic traits were detected as promising genotypes for hybridization. Probes developed were then in future utilized for molecular breeding.

Keywords: SCoT markers, Probe, Polymorphism, Genetic Diversity

1. Introduction

The genus *Mentha* commonly known as mints belongs to the family Lamiaceae, which is comprised of a group of species. Mint species possess high chemical diversity,as the aerial parts of the mints yield essential oils constituted of various aroma chemicals. For instance the essential

oils from *M. arvensis* contains high menthol, *M.spicata* contains carvone, D-limonene, dihydrocarvone, *M. piperita* contains menthol, menthone, methyl esters, methylacetate. These chemicals have high commercial importance and are used in pharmaceutical, food, flavor, cosmetics and beverage industry considering the importance of this crop. Therefore assessment of genetic diversity is a prerequisite to develop genetic markers thus aiding genetic improvement of this species, however the exact distinction between the species is not clear.

Table 1: Details of development and origin of selected genotypes of *Mentha* species and their accessions used in the study

Species/Accession	Common name	Origin/Development
Mentha piperita Var.Kukrail	Pippermint	Induced mutagenesis in local strains
Mentha piperita Var. Tushar		Induced mutagenesis
Mentha piperita Var.Madhuras		Selection from Kukrail
Mentha piperita Var.Pranjal		Induced mutagenesis
Mentha piperita Var.Indus		Induced mutagenesis
Mentha spicata Var. Neera	Spearmint	Induced mutagenesis
Mentha spicata Var. Arka		Induced mutagenesis
Mentha spicata Var. Neerkalka		Interspecific hybridization
Mentha viridis Var. MSS-5		Variety developed by clonal selection
Mentha cardiaca Var. Mukta	Gingermint/Redmint	Inter-specific hybridization
Mentha cardiaca Var. Prateek		Inter-specific hybridization
Mentha viridis Var. Ganga	Spearmint	Variety developed by clonal selection
Mentha viridis Var. Supriya		Variety developed by clonal selection

At CSIR-CIMAP, many elite accessions have been developed during the last decade which constitutes the major gene pool to serve as the usable source of genetic variability in the national genebank for these commercially important taxa of *Mentha*. These accessions of the germplasm have been produced through introductions, clonal selections, mutant selections and selected half-sibs from the progenies of superior genotypes. The success of a breeding program depended on the genetic variability available into the germplasm of the crop. The assessment of genetic diversity at DNA level for these accessions has been considered as the desirable step in the

process of developing taggable markers to aid genetic improvement in the variety development programme in addition to estimating strength of the gene pool (Maria Lucília et al. 2011).

SCoT technique is a type of targeted molecular marker technique with the ATG context as one part of a functional gene, markers generated from SCoT marker technique may be mostly correlated to functional genes and their corresponding traits. SCoT markers are highly reproducible as longer primers are used. ATG translation codon is more advantageous than the intersimple (ISSR), minisatellte (VNTR) or SSR regions due to conservation of ATG translation start site and flanking sequences in plant genes. The diversity information generated by the RAPD, SSR or ISSR is based on the non-coding regions of DNA, so it is not that useful in the context directly. But it is useful only when it is linked strongly to some trait. Whereas in SCoT markers as they reveal the genetic diversity at the level of genes thus have the possibility of finding new alleles among a given germplasm collection.So the information generated by this marker is far more crucial, as it is derived from either the gene itself or its immediate flanking sequences.

The aim of the study was to determine morphological and genetic diversity among 12 mint accessions belonging to 4 species of mints. Assessment of the genetic diversity in cultivated species is of interest to support the selection of genetic materials for breeding of the crop, broadening of the genetic base and conservation of genetic resources. The estimate of genetic variation will be more useful if agro-morphological traits and molecular markers which cover both ends of the path of gene expression from genome to phenotype are applied together. The relatively lower cost and simplicity of the agro-morphological characterization make it an interesting tool to study genetic variation of the germplasm, but the effect of environmental factors may limit its application. In this context, molecular markers can augment the agronomical characterization and provides more accurate assessments of genetic variation since they are independent of environmental conditions and the developmental stage of the organism.

Genetic improvement(s) in *Mentha* species leading to wider adaptation, higher herbage and/or essential oil yield(s) and better quality of oil will permit economical production of mint related commodities. Currently, much emphasis is being laid on conservation of plant germplasm (varied genetic resources) as valuable bio-resource. These collections are expected to serve as repositories of biodiversity for numerous species available as utilisable sources of desired genes

in plant improvement programmes. The importance of molecular markers exists for not only authentication of the genotypes of released cultivars of medicinal and aromatic plants, but also in assessing and exploiting the genetic variability through molecular approaches. Developing DNA markers/fingerprints of all the genetic resources of the medicinally and industrially important plants, is a necessity for generating a molecular database to catalogue as well as to utilize the information in a systematic way. *Mentha* species, which represent a substantial variability in origin as well as chemotype properties for the oil and its components, do need to be documented well for establishing phylogenetic relationships and unique marker profiles at DNA level. These molecular relationship and markers can be useful for designing strategies for gene introgression and breeding programmes to produce desired recombinant hybrid genotypes with both oil quality and yield.

2. Materials and Methods

2.1. Plant material

Thirteen genotypes/accessions of *Mentha* obtained from the collection centre of CSIR-CIMAP, Lucknow. These accessions developed through clonal selection, mutant selection, interspecific hybridization from progenies of superior genotypes (see Table 1).Field experiments were carried out with these genotypes as experimental farms at CSIR-CIMAP.

2.2. Quantitative and qualitative analyses of essential oil components by gas chromatography

The chemical composition of the essential oils obtained from *Mentha* fresh plant leaves were studied by GC.A microprocessor based GC systerm (Chromatography and Instrument Co. Pune India) fitted with a 3.2 -5.1 mm stainless steel column packed with 10% carbowax treated with 20000 molecular weight tetraphthalic acid (20 M_ TPA) on Chromosorb W(WA) 80/100 mesh was used. Argon was used as the carrier gas at an inlet pressure of 110.4 KPa. The temperatures were programmed as follows: injector/ detector 200- 250, 8^{o}C and column were maintained isothermally at 115, 8 ^{o}C. Data were processed in the electronic integrator Varian 4400 and the identification was based on retention time of spiked authentic samples of different compounds and retention indices calculations. The essential oil extracted from regenerated plants were compared with control as

concerns the qualitative changes in oil constituents such as D-limonene, carvone and dihydrocarvone.

2.3 Trichome analysis

Fresh leaves (four to five) were collected from all *Mentha* genotypes, and their trichome numbers were counted with the help of a microscope (LEICA KL 200 LED, Schott, Germany) with adjusted 1-mm2 grids at its eyepiece lens at ×40 magnification. Glandular and non-glandular trichomes were counted on both abaxial and adaxial surfaces of leaves. Leaves were analyzed at different parts, i.e., tip, middle and base and the total leaf area was also measured with a leaf scanner. Grids covering a 1-mm2 area were fixed at the eyepiece lens; therefore, only those trichomes were counted which were present inside the grid.

2.4. Molecular analysis

2.4.1. DNA extraction, amplification and gel electrophoresis

DNA extraction was done with the help of protocol developed by Khanuja *et al.* (1999).Fresh leaf tissue of *Mentha* were grinded into liquid nitrogen and then transferred to a tube containing extraction buffer (0.5 M CTAB 20%,100 mM Tris HCl,0.5 M EDTA, 5 M NaCl, PVP 1%, β-mercaptoethanol)). During lysis the suspension was gently mixed and incubated at 65° C for 2 hours in water bath. The suspension was then cooled to room temperature and an equal volume of chloroform: isoamyl alcohol (24:1) was added and the mixture was centrifuged at 10,000 rpm for 15 minutes at 25°C. The clear upper aqueous phase was transferred to a new tube and 1.5 ml 0.5M NaCl was added and mixed properly with 0.6 volume of ice-cooled isopropanol and mix properly incubated at room temperature for 1 hour and centrifuged for 15 min at 10,000 rpm . The pellet was air dried and 400µl of TE was added and the whole mixture was placed at 42ºC for 30 min. An equal amount chlorophorm: isoamyl alcohol (24:1) added and centrifuged for 10 min for 13000 rpm and upper aqueous phase was transferred to new tube and double volume of absolute ethanol were mix for ppt (incubate for 4-5 hours in -20^0c) .The resulting pellet was washed twice with 80% ethanol. The pellet was air-dried and dissolved in 30µl of nuclease free water. DNA concentration and purity was determined by measuring the absorbance of diluted DNA solution at 260 nm and 280 nm on nanodrop spectrophotometer ND-1000 (Thermo fisher scientific, Wilmingtons, DE, U.S.A.).The

quality of the DNA was determined using agarose gel electrophoresis stained with ethidium bromide. This method provided good quality and as well as good quantity of DNA, which was then subjected to PCR amplification for developing SCoT markers.

2.4.2 SCoT Analysis

Primers were designed according to the short conserved region surrounding the ATG translation start (or initiation) codon (or translational start site, TSS). Primers were designed from consensus sequences derived from the studies by Joshi et al. (1997) and Sawant et al (1999). For primer design, the ATG codon (+1, +2, and +3), G' at position +4, and A, C, and C at positions +7, +8, and +9, respectively, were fixed. All primers were 18-mer and ranged in GC content between 50% and 72% There were no degeneracies. Primers were checked for dimers and hairpin loops using the program 'FAST PCR'. PCR was optimized for testing the SCoT method (Bertrand et al). The PCR reaction was performed using a Techne cycler (TC512, UK) which programmed to 1 cycle of 4 min at 94°C (initial denaturation) followed by 43 cycles of 1 min at 94°C, 1 min at particular temperature for each primer (annealing) and 2 min at 72°C (extension) ending with 1 cycle of 5 min at 72°C (final extension). Reactions were performed in a 20 µl volume containing 2 µl of DNA template (30 ng), 2 µl of 10× buffer, 1 µl of 200 µM dNTP, 2 µl of of MgCl2, 2 µl of each primer (10 pM concentration), 0.2 U of Taq polymerase.

2.5.4. Statistical Data analysis

For each SCoT marker, total amplified bands, number of polymorphic bands, and percentage of polymorphic bands were recorded. SCoT bands were scored as present (1) or absent (0) for the estimation of the similarity among all the analysed samples. The matrices of similarity (Nei and Li, 1979) were calculated and a phylogenetic tree was obtained by clustering according to the Unweighted Pair-Group Method with Arithmetic averages (UPGMA). Polymorphism percentages were estimated by dividing the number of polymorphic bands over the total number of bands. The average similarity matrix was used to generate a tree for cluster analysis with UPGMA NTSyS-pc Version 2.02 softwares.

3. Results

3.1.1 Herbage and essential oils yield of all the *Mentha* species

Like in any other aromatic crop, the yield and the essential oil composition of mint species are influenced by interaction between the genotype and environment, method of distillation, kind of storage, crop age, time of harvest, and season (Kofidis et al., 2004). The herbage yield and essential oil yield were estimated and it was found to be highest in *M.piperita* var.Kukrail and *M.spicata* var. Neerkalka followed by *M.cadiaca* var. Prateek and lowest yield was recorded for *M.viridis var.MSS-5* as described in Table 2.

Table 2: Showing herbage and essential oil yield of all the genotypes of *Mentha*

S.No	Mentha species	Fresh weight biomass per 100 m^2	Essential oil yield/ 100 m^2
1	***M. piperita var.* Kukrail**	51.7 kg	723.8ml
2	**var. Tushar**	64.7 kg	388.2 ml
3	**var. Madhuras**	36.7 kg	440.4ml
4	**var. Indus**	66.1 kg	528.8ml
5	**var. Pranjal**	57.3 kg	515.7ml
6	***M. spicata* var. Neera**	52.9 kg	423.2ml
7	**var. Arka**	66.1 kg	661ml
8	**var. Neerkalka**	66.1 kg	793.2ml
9	***M. viridis* var. Ganga**	58.8 kg	529.2ml
10	**var. Supriya**	75 kg	675ml
11	**var. *MSS-5***	60.2 kg	361.2 ml
12	***M.cardiaca* var. Prateek**	63.2 kg	505.6 ml
13	**var. Mukta**	51.4 kg	411.2ml

3.1.2 Trichome analysis of all the selected genotypes of *Mentha* species

Glandular trichomes are specialized hairs found on the surface of about 30% of all vascular plants and are an important source of essential oils. Mint oil obtained from mint plants, is synthesized and gathered in glandular hairs and its quality and quantity depend on cultivated

genotype. Trichomes and their exudates can be harvested relatively easily, and this has permitted a detailed study of their metabolites, as well as the genes and proteins responsible for them. This knowledge now assists classical breeding programs, as well as targeted genetic engineering, aimed to optimize trichome density and physiology to facilitate customization of essential oil production or to tune biocide activity to enhance crop protection. Ratio of GST/NST was found to be highest in *M. piperita* (var. Kukrail) followed by *M. cardiaca* (var. Prateek) as shown in Table 4 and there was considerable variation in essential oil constituents as shown in Table 3.

Table 3: Comparative analysis of Trichome patterning of glandular trichomes (GT) and non-glandular trichomes (NGT) in all the selected genotypes of *Mentha* species at abaxial and adaxial surfaces of leaves

***Mentha* species**		**Number of GST on dorsal surface**	**Number of GST on ventral surface**	**Number of NST on Dorsal surface**	**Number of NST on Ventral surface**	**Ratio of GST/NST on dorsal surface**	**Ratio of GST/NST on ventral surface**
1	***M. cardiaca*** *var*. Mukta	2002 ± 28.8	711 ± 2.1	609 ± 2.4	252 ± 2.0	2002/609	711/252
2	*var*. Prateek	2360± 11.5	310 ± 1.8	752 ± 3.6	288 ± 2.0	2360/752	310/288
3	***M. piperita*** *var*. Kukrail	2380 ± 37.0	820 ± 4.3	800 ± 1.6	340 ± 2.9	2380/800	820/340
4	*var*. Tushar	2335 ± 19.9	940 ± 2.8	150 ± 1.6	260 ± 1.7	2335/150	940/260
5	*var*.Madhuras	2255± 17.5	832 ± 2.5	755 ± 3.4	334 ± 1.8	2255/755	832/334
6	*var*. Indus	2230 ± 18.6	931 ± 4.6	531 ± 2.9	226 ± 1.6	2230/531	931/226
7	*var*. Pranjal	2440± 27.4	763 ± 3.5	390 ± 2.6	135 ± 2.3	2440/390	763/135

8	***M.viridis*** *var*. Ganga	2124± 44.5	775 ± 9.9	549± 22.9	222 ± 1.6	2124/549	775/222
9	*var*. *MSS-5*	2007 ± 30.6	102 ± 2.9	149 ± 3.7	350 ± 3.3	2007/149	102/350
10	*var*. Supriya	2880± 14.9	800 ± 4.9	742 ± 2.0	358 ± 2.8	2880/742	800/358
11	***M. spicata*** *var*. Neera	2030± 42.0	705 ± 22.8	681± 22.6	327 ± 2.0	2030/681	705/327
12	*var*. Arka	2125± 28.5	711± 4.5	721 ± 8.5	349 ± 3.6	2125/721	711/349
13	*var*.Neerkalka	2310± 12.5	728 ± 1.6	783 ± 2.4	302 ± 2.9	2310/783	728/302

Table 4 Essential oil chemical constituents analysis

S.No	Oil components	Kukrail	Tushar	Madhuras	Indus	Pranjal	Mukta	Pratek	Ganga	Mss-5	Supriya	Neera	Arka	Neerkalka
1.	α-pinene	0.5	0.5	0.5	0.4	0.5	0	0.8	0	0	0	0	0	0
2.	β-pinene	0.9	0.8	0.9	0.9	0.9	0	1.04	0	0	0	0	0	0
3.	sabinene	0.6	0.6	0.6	0.5	0.6	0	0.4	0	0	0	0	0	0
4.	β-myrcene	0	0	0	0	0	0.8	0.8	0	0	0	0	0	0
5.	limonene	2.3	1	3.7	3.7	4	15	18	16	6	18	17	17	6.9
6.	Linalyl acetate	0	0	0	0	0	0	0	0	0	0	0	0	0
7.	1,8 cineole	4.3	1	4.5	4.3	33	1	0.2	2	0	9.4	32	2	1
8.	Menthofuren	11.6	12	13	12	4	0	0	0	0	0	0	0	0
9.	Isomenthene	3.9	0	3.7	3.5	4	0.1	0.1	0	0	0	0	0	0
10.	Menthyl acetate	2.4	4.8	5	6	2.2	0	0	0	0	0	0	0	0
11.	Pulegone	6.5	9	8	22	5	0	0	0	0	0	0	0	0
12.	Menthol	30	46	26	23	29	0	0	0	0	0	0	0	0

13.	Piperitenone	4	1	4	3	2	0.4	0.4	0	2	2	0.4	0	0
14.	Piperitenone oxide	0	0	0	0	0	0	0	0	77	1.5	0	0	0
15.	Linalool	0	0	0	0	0	0	0	0	0	0	0	0	0
16.	Carvone	0	0	0	62	0	74	70	62	60	36	61	62	57
17.	Menthone	25	20	18	21	15	0	0	0	0	0	0	0	0
18.	Dihydrocarvone	0	0	0	0	0	0.8	1	4	0	2	3.8	0	0
19.	Germacrene-d	0	0	0	0	0	0.3	0.2	0	1.9	0	0	1	0
20.	β-caryophyllene	0	0	0	0	0	0.5	0.3	0	0	0	0	0	0
21.	α-terpenol	0	3	0	0	3	0	0	0	0	0	0	0	4.8

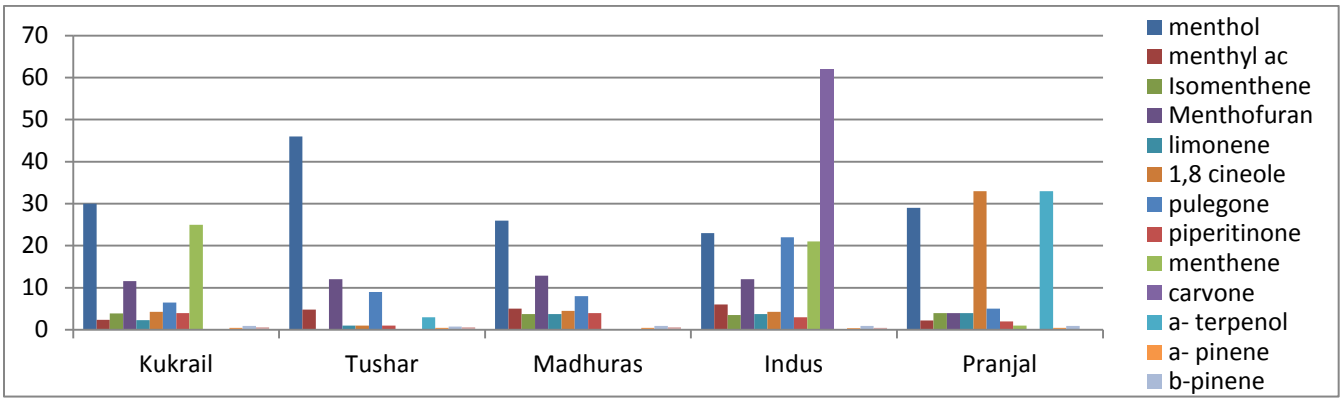

Fig1: Differential graphical representation of essential oil constituents in different accessions of *Mentha piperita* genotypes

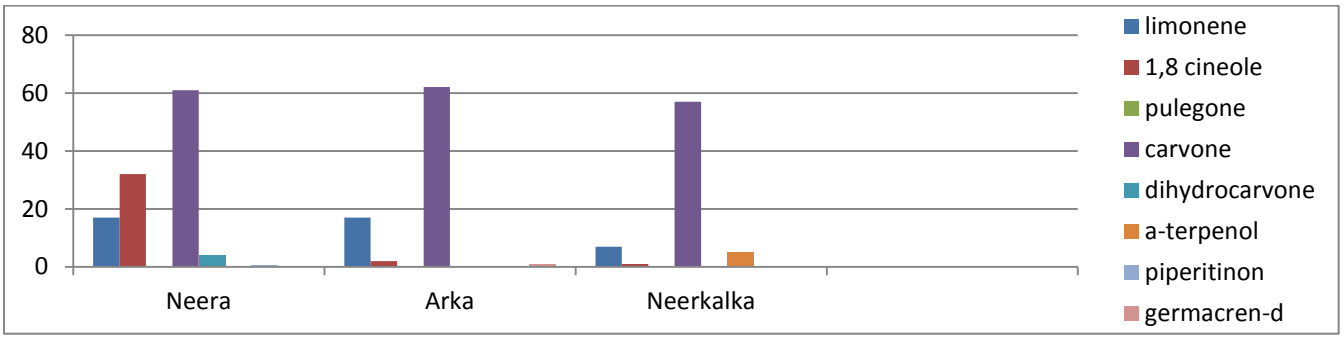

Fig 2: Differential graphical representation of essential oil constituents in different accessions of *Mentha spicata* genotypes

3.1.3. Molecular profiling of *Mentha* genotypes through SCoT

Molecular techniques have also had critical roles in studies of phylogeny and species evolution, and have been applied to increase our understanding of the distribution and extent of genetic variation within and between species. Molecular markers may or may not correlate with phenotypic expression of a genomic trait. They offer numerous advantages over conventional, phenotype-based alternatives as they are stable and detectable in all tissues regardless of growth, differentiation, development, or defense status of the cell. Fig. 3 shows comparative DNA profile in which *Mentha* genotypes showed common as well as differentiating band patterns with MPST 2.

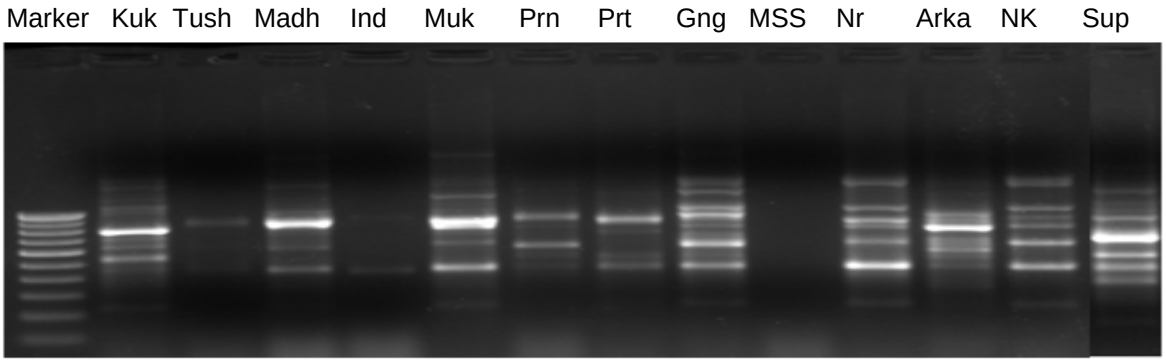

Fig 3: Differentiating profiles of *Mentha* genotypes by using MPST 2

4. Supplementary data (Tables and figures)

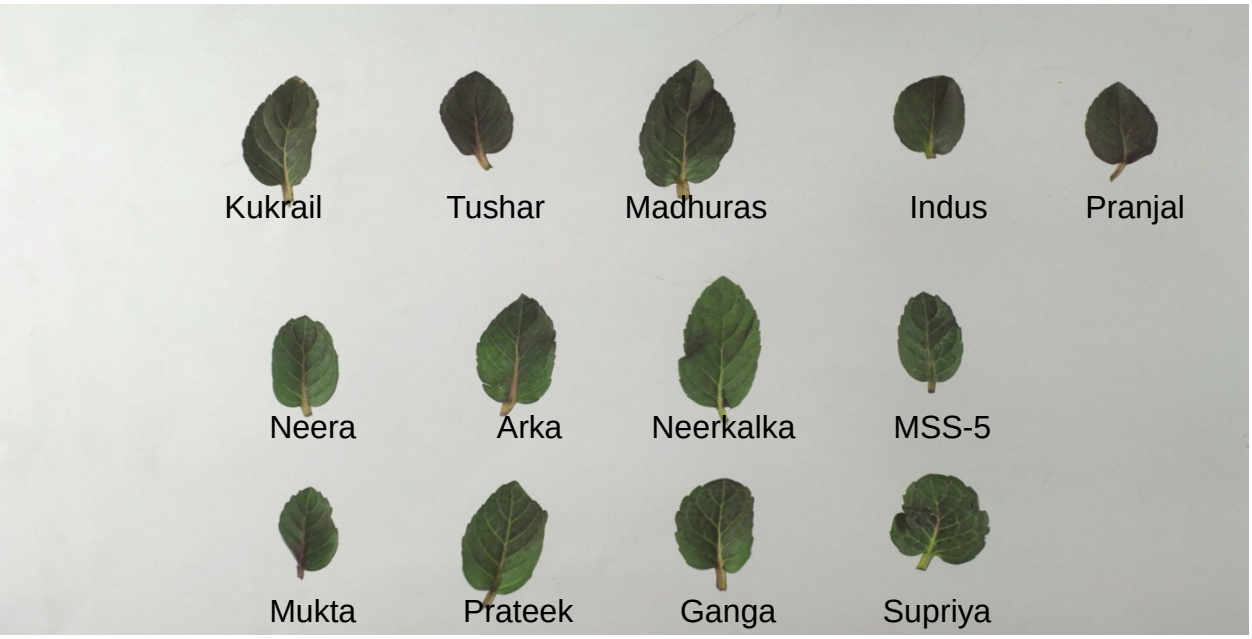

Fig.4 Showing clear difference in leaf structure

Table 5 Showing percentage of polymorphism obtained by SCOT primers

Primer number and name	Primer sequence 5'-3'	Total number of bands with single primer	Length of amplification products (bp)	Monomorphic bands	Unique bands	Polymorphic bands	% Polymorphism
MPST 11	**GGTGTTGATGGCGACCT**	6	400-600	0	0	6	100%
MPST 16	**GATTTGAAATGGCTACCA**	3	350	0	0	3	100%
MPST 30	**CCATGGCTACCACCGCAC**	8	100-300	0	2	6	75%
MPST 2	**ACCACAAAATGG**	19	400-600	1	1	17	89.4%

	CGACCTA						
MPST 17	**ATGGCTACCCTTAGCATG**	12	400-600	0	0	12	100%
MPST 12	**TTTGTTGATGGCGACCG**	10	200-300	0	0	10	100%
MPST 13	**GACAGCATGGCTACCAT**	18	300-400	0	0	18	100%
MPST14	**ATGAGCATGGCTACCGA**	20	400-500	0	0	20	100%
MPST27	**CAACAATGGCTACCACCC**	12	200-300	0	0	12	100%
MPST 18	**TTAGCATGCATGGCTACC**	13	300-400	0	0	13	100%

Kukrail												
Tushar	0.53731											
Madhuras	0.49383	0.45890										
Indus	0.55357	0.48649	0.6083									
Mukta	0.55914	0.54563	0.5283	0.59854								
Pranjal	0.45783	0.41463	0.47917	0.64567	0.53704							
Prateek	0.48544	0.49021	0.48276	0.66667	0.71875	0.57627						
Ganga	0.33333	0.18868	0.32836	0.22449	0.25316	0.31884	0.24719					
MSS-5	0.43038	0.46154	0.36957	0.53659	0.44231	0.48936	0.57895	0.36923				
Neera	0.42553	0.43011	0.50467	0.52174	0.53782	0.45872	0.55814	0.17523	0.45714			

Arka	0.32609	0.30769	0.43816	0.52941	0.52991	0.50467	0.66142	0.30769	0.60194	0.61017		
Neerkalka	0.23188	0.32353	0.34146	0.30088	0.38298	0.28571	0.30769	0.18182	0.17556	0.37895	0.3871	
Supriya	0.14085	0.14286	0.2619	0.29565	0.33333	0.23256	0.33962	0.14035	0.26829	0.41237	0.42105	0.3215

Fig 5: Similarity matrix as deduced by SCoT markers

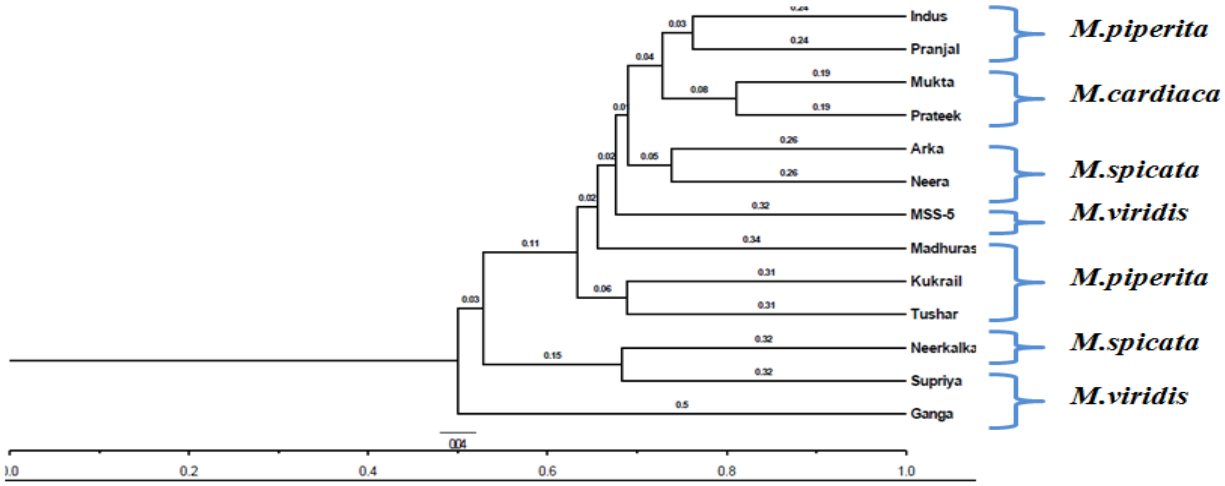

Fig 6: Phylogenetic tree showing relatedness of *Mentha species* and genotypes as deduced by SCoT markers

Table.6. SCOT markers specific to *Mint*

S.No	Genus and species	Species specific or genotype specific band
1	***M.piperita* var.Madhuras**	350 bp with MPST 16
2	***M.piperita* var.Indus**	350 bp with MPST 16
3	***M.piperita* var.Pranjal**	350 bp with MPST 16
4	***M.spicata* var. Neera**	300 bp species specific band with MPST 30
5	***M. spicata* var. Arka**	300 bp species specific band with MPST 30

6	***M.arvensis and M.spicata* (Hybrid) var. Neerkalka**	100bp and 150 bp genotype specific band and 300 bp species specific band with MPST 30

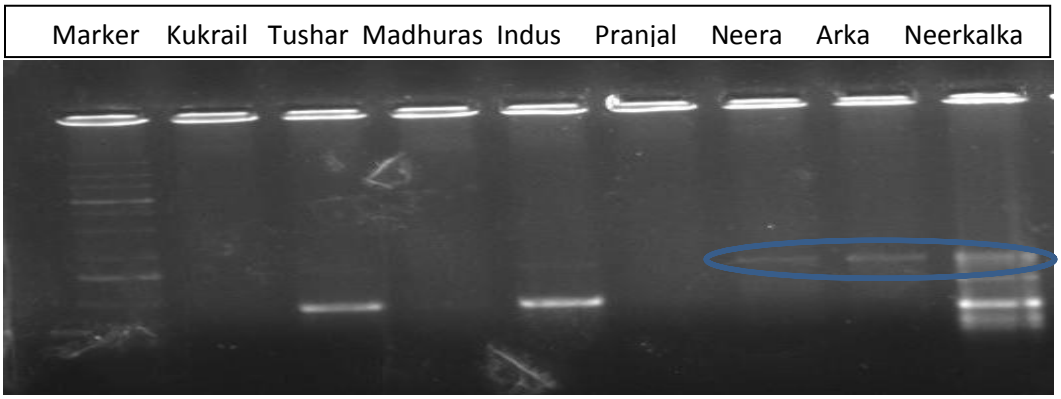

Fig 7. Species specific (*M.spicata*) SCoT bands with MPST 30

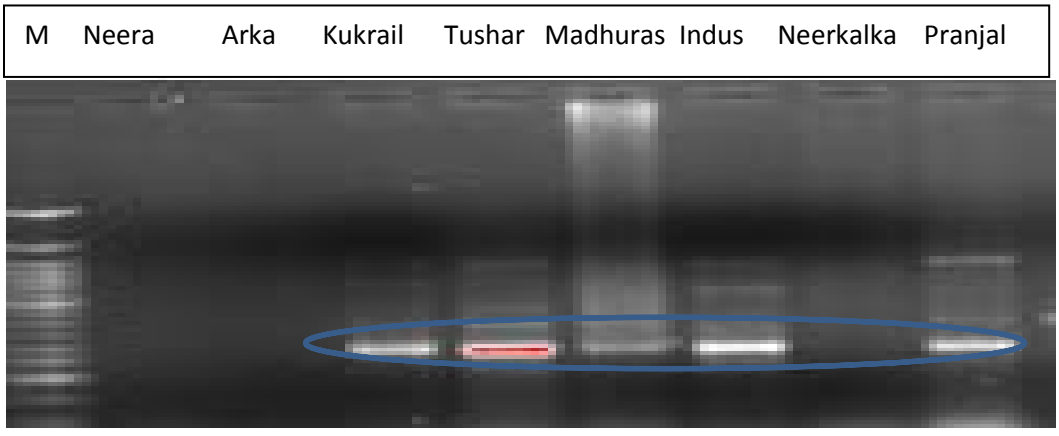

Fig 8. Species specific (*M.piperita*) SCoT bands with MPST 16

13 accessions of *Mentha* were amplified with 20 SCoT primers out of which 10 primers which produced clear and reproducible bands were selected that produced reproductive result. A total of 171 amplified bands were obtained and 98.5 % were found to be polymorphic. In SCoT analysis 97.68% of scored loci were polymorphic. Most of the PCR products were in the size range of 200-600 bp (Table 5). Highest percentage of polymorphism was 100% with 8 SCoT primers and lowest was 75% with SCoT 30. The marker index for SCoT markers was calculated to characterize the capacity of each primer to detect polymorphic loci among the genotypes.

These polymorphic amplified bands together were able to distinguish the taxa and also different accessions from each other. Cluster analysis based on these traits formed the similarity matrix (Fig. 7) and grouped the genotypes into 8 separate clusters as shown in Fig.8. Recognition of specific bands from some SCoT primers in this study indicates the high potential use of this marker for discrimination of *Mentha* species and genotypes. Reasonable number of polymorphic bands even with the use of a limited set of SCoT primers indicates a diverse genetic base of the studied genotypes and the high capacity of SCoT technique to reveal genetic diversity in *Mentha*. The taxa-specific bands can be utilised for defining the uniqueness, which will be helpful in species and taxa identification.

Table 7. Qualitative data of *Mentha cardiaca* and *Mentha viridis*

Genus and species	***M.cardiaca*** **var. Mukta**	***M.cardiaca*** **var.Prateek**	***M. viridis*** **var. Ganga**	***M.viridis*** **Var.Supriya**
Growth habit	Erect to Creepy	Creepy	Creepy	Erect
Stem	Round green	Round green	Round green	Round green
Leaf colour	Green	Green	Light Green	Green
Texture	Thin	Thick	Thin	Thick
Surface	Rough	Rough	Rough	Rough
Shape	Ovate	Ovate	Ovate	Ovate
Margin	Serrated	Serrated	Serrated	Serrated
Tip base	Acute	Acute	Acute	Pointed
Size	Moderately broad	Moderately broad	Broad	Broad

Inflorescence	Verticillaster	Verticillaster	Verticillaster	Slender spikes
Pedicel	Green	Green	Green	Smooth
Calyx	Broad	Broad	Broad	Broad

Table 8. Qualitative data of *Mentha piperita*

Genus and species	***M.piperita* Var.Kukrail**	***M.piperita* var.Tushar**	***M. piperita* var.Madhuras**	***M.piperita* var.Indus**	***M.piperita* var. Pranjal**
Growth habit	Herbaceous,Erect and branched	Herbaceous,Erect and branched	Herbaceous,Erect and branched	Herbaceous,Erect and branched	Herbaceous,Erect and branched
Stem	Quadangular,purplish green	Quadangular,purplish green	Quadangular,purplish green	Quadangular,purplish green	Quadangular,purplish green
Leaf colour	Green,simple,opposite,decussate	Green,simple,opposite,decussate	Green,simple,opposite,decussate	Green,simple,opposite,decussate	Green,simple,opposite,decussate
Texture	Chartaceous	Chartaceous	Chartaceous	Chartaceous	Chartaceous
Surface	Smooth	Rough	Glabrous dorsal surface,minute hairs on ventral surface	Rough	Rough
Shape	Ovate-oblong or spathulate	Ovate- oblong or spathulate	Ovate-oblong or spathulate	Ovate- oblong or spathulate	Ovate-oblong or spathulate
Margin	Serrated	Serrated	Serrated	Serrated	Serrated
Tip base	Acute	Acute	Acute	Acute	Pointed
Size	Medium sized	Medium sized	Medium sized	Medium sized	Medium sized
Inflorescence	Verticillaster	Verticillaster	Verticillaster	Verticillaster	Verticillaster
Pedicel	Smooth	Smooth	Smooth	Smooth	Smooth
Calyx	Broad	Broad	Broad	Broad	Broad

Table 9. Qualitative data of *Mentha spicata*

Genus and species	***M.spicata* var.Neera**	***M.spicata* var. Arka**	***M. spicata* var. Neerkalka**	***M.spicata* var.MSS-5**

Growth habit	Erect	Creepy	Erect	Erect sturdy main stem
Stem	Round green	Round green	Round green	Round green
Leaf colour	Green (faint)	Green	Green	Green
Texture	Thin	Thick	Thin	Thin
Surface	Smooth	Smooth	Rough	Rough
Shape	Lanceolate	Ovate	Lanceolate	Ovate
Margin	Serrated	Serrated	Serrated	Serrated
Tip base	Pointed	Acute	Pointed	Acute
Size	Small	Small	Broad	Broad
Inflorescence	Slender spikes	Slender spikes	Slender spikes	Verticillaster
Pedicel	Smooth	Smooth	Smooth	Green
Calyx	Broad	Broad	Broad	Broad

4. Discussions

In recent years, there has been a significant increase in the application of molecular genetics methods for assessing the conservation and use of plant genetic resources. Molecular techniques have been applied in the analysis of specific genes, as well as to increase understanding of gene action, generate genetic maps and assist in the development of gene transfer technologies. Additionally, they are not confounded by environmental, pleiotropic and epistatic effects.

Genetic diversity across the natural populations of three montane plant species in the Western Ghats (India), *Symplocos laurina*, *Gaultheria fragrantissima* and *Euryanitida* using inter simple sequence repeat (ISSR) markers have been analysed (Deshpande et al.). Genetic diversity in *Mentha cervina* was analysed based on morphological traits, essential oils profile and ISSRs markers (Leandra Rodrigues et al.2013). Characterization of twenty wheat varieties have been analysed by ISSR Markers (Abou-Deif et al 2013). Assessment of genetic diversity in *Mentha* using RAPD markers has been done previously (Khanuja et al.2000) but assessment using ISSR markers in *Mentha* have not been reported yet. In the present paper, we describe the similarity and diversity in terms of ISSR profiles of the released cultivars of four different mint species which includes thirteen accessions of *Mentha.* ISSRs are highly polymorphic and are useful in studies on genetic diversity, phylogeny, gene tagging, genome mapping and evolutionary biology. *M. piperita* showed best results as compared to other species of *Mentha*. The present investigation of DNA profiling in mint species clearly demonstrates that it is possible to analyse

the SCoT patterns for correlating their similarity and distances between species and accessions by which one can predict the origin of the species and cultivars to a great extent.

Highest and lowest polymorphism was shown by *M. piperita* and *M.cardiaca* respectively. Genetic improvement of *Mentha* should be based on morphological as well as molecular differences. Accessions appearing to be in the same group morphologically, many times show different molecular groupings. The molecular diversity database can prove to be directly useful as attempted in the present study, for *Mentha* breeders to develop and analyse novel intra as well as inter-specific hybrids as the morphological data alone may be limiting and misleading.

In future, analysis with more markers is required to find out more specific and unique sequences in *Mentha* species. This comparative analysis and correlation among different genotypes of *Mentha* species would be helpful as the probes developed would be utilized for genetic improvement of *Mentha* to increase the yield of secondary metabolites by development of new and improved genotypes.

Conclusion

There was a considerable genetic variation among the studied genotypes based on SCoT profiling. To quantify genetic diversity among the genotypes based on agro-morphological traits, cluster analysis was performed using Euclidean distance matrix by UPGMA method and the genotypes were clearly grouped into 8 main clusters. As it was expected, the inter cluster distances in all cases were more than the intra cluster distance indicating higher genetic variability between the genotypes of different clusters. Genetic variation at the DNA level was also considerable and a high level of polymorphism (100%) was detected with SCoT markers set used. Reasonable number of polymorphic bands even with the use of a limited set of SCoT primers indicates a diverse genetic base of the studied genotypes and the high capacity of SCoT technique to reveal genetic diversity in *mentha*. Recognition of unique bands from some SCoT primers in this study indicates the high potential use of this marker for discrimination of genotypes. Present investigation suggests the effectiveness of SCoT marker system to estimate the genetic diversity among *Mentha* species and it can be seen as a preliminary point for future research on the population and evolutionary genetics of *Mentha* species.

Species-specific and genotype specific markers can be identified that would be useful for introgression studies where plant breeders want to transfer some desirable traits from one species into another. Localization of these markers on the chromosomes would be useful for keeping track of important traits that need to be transferred. SCoTs target potential coding genomic regions producing a dominant marker-system, but several co-dominant markers are also generated. Despite the high potential of SCoTs for targeted fingerprinting or QTL mapping purposes, those characteristics also consent their use for genetic diversity assessment.

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