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Review Article

Role of Molecular Markers in Assessing Genetic Diversity in *Mentha*: A Review

Abstract

Morphological, phytochemical and genetic differences were studied to evaluate the level and distribution of diversity among thirteen genotypes of *Mentha* using both agro-morphological traits and ISSR markers. Our findings indicated that there were significant differences for agro-morphological traits and essential oil variations in mint species. Cluster analysis based on these traits grouped the genotypes into 8 separate clusters. Highest and lowest polymorphism was shown by *M. piperita* and *M. cardica* respectively. Plants have evolved various strategies for survival and reproduction during the long evolutionary course, one of which is the production of an array of small molecular weight compounds, also known as secondary metabolites or natural products. The potent biological activity of secondary metabolites has also led to their exploitation as pharmaceuticals and many other functions. Although much progress has been made, a lot more still need to be learned about the mechanisms responsible for the formation of most of the important secondary metabolites. SCoT analysis also revealed high polymorphism with the primers generating polymorphic profiles.

Introduction

Mints are herbaceous plants and perennial aromatic herbs that are cultivated for their essential oils used both for medicinal and culinary purposes. The essential oil from *M. arvensis* contains menthol, *M. spicata*- carvone, d-limonene, dihydrocarvone, *M. piperita* - menthol, menthone, methyl esters, menthylacetate. Mint species are not clearly distinct as hybridization between some of them occurs naturally. Plants belonging to the genus *Mentha* L. (Lamiaceae) have evolved in nature through natural hybridization and selection showing substantial variation in terms of their natural habitats, growth characteristics, and aromas. Morphological, molecular and biochemical markers are complementary in determining the genetic similarity of inter- and intra-species and the relationship between the populations. Genetic variation is fundamentally involved in the ability of a species to adapt to biotic and abiotic changes and in its evolution. Recognition of the levels and distribution of genetic variation within and among populations of a species is the base for development and selection of plant genotypes in breeding programs and increases the understanding of the historical processes underlying the genetic diversity providing information for the management and preservation of endangered and geographically restricted species.

The major volatile components were Linalyl acetate and

linalool in *Mentha citrata* and Piperitone oxide in *Mentha longifolia*. Both nutraceuticals of *Mentha citrata* and *Mentha longifolia* possess antioxidant and anticancer effect that could be attributed to the presence of phytosterols, phenolic compounds, unsaturated fatty acids and specific volatile constituents [1]. *Mentha* is one of the most common herb which has been known for its medicinal and aromatherapeutic properties since ancient times and in the last few decades, the insecticidal potential of leaf extracts has also been investigated. The insecticidal activity of *Mentha* against various stored grain pests and vectors. Insecticidal properties of different *Mentha* species are commonly inherent in its essential oils or plant extracts which is correlated with their chemical composition. *Mentha* species produce valuable secondary metabolites that scavenge toxic free radicals. The free radical scavenging potential (1,1-diphenyl-2-picrylhydrazyl scavenging activity) in *Mentha* species were investigated to evaluate and explore new potential sources for natural antioxidants [2].

Applications of molecular markers in plant genome analysis and breeding

Molecular markers have been looked upon as tools for a large number of applications ranging from localization of a gene to improvement of plant varieties by marker-assisted selection. They have also become extremely popular

markers for phylogenetic analysis adding new dimensions to the evolutionary theories. If we look at the history of the development of these markers, it is evident that they have been improved over the last two decades to provide easy, fast and automated assistance to scientists and breeders. Genome analysis based on molecular markers has generated a vast amount of information and a number of databases are being generated to preserve and popularize it.

Multi locus probes

A major step forward in genetic identification is the discovery that about 30–90% of the genome of virtually all the species is constituted by regions of repetitive DNA, which are highly polymorphic in nature. These regions contain genetic loci comprising several hundred alleles, differing from each other with respect to length, sequence or both and they are interspersed in tandem arrays ubiquitously. The repetitive DNA regions play an important role in absorbing mutations in the genome. Of the mutations that occur in the genome, only inherited mutations play a vital role in evolution or polymorphism. Thus repetitive DNA and mutational forces functional in nature together form the basis of a number of marker systems that are useful for various applications in plant genome analysis. The markers belonging to this class are both hybridization-based and PCR-based.

Microsatellites and minisatellites

Both are multilocus probes creating complex banding patterns and are usually non-species specific occurring ubiquitously. They essentially belong to the repetitive DNA family. Fingerprints generated by these probes are also known as oligonucleotide fingerprints. The methodology has been derived from RFLP and specific fragments are visualized by hybridization with a labelled micro- or minisatellite probe. These loci contain tandem repeats that vary in the number of repeat units between genotypes and are referred to as variable number of tandem repeats (VNTRs) or hypervariable regions (HVRs). Microsatellites and minisatellites thus form an ideal marker system creating complex banding patterns by simultaneously detecting multiple DNA loci. Some of the prominent features of these markers are that they are dominant fingerprinting markers and codominant STMS (sequence tagged microsatellites) markers. Many alleles exist in a population, the level of heterozygosity is high and they follow Mendelian inheritance.

Genetic diversity analysis

Genetic diversity refers to the total number of genetic characteristics in the genetic makeup of a species. It is distinguished from genetic variability, which describes the tendency of genetic characteristics to vary. Genetic diversity serves as a way for populations to adapt to changing environments. With more variation, it is more likely that some individuals in a population will possess variations of alleles that are suited for the environment. Those individuals are more likely to survive to produce offspring bearing that allele. The population will continue for more generations because of the

success of these individuals. Morphological characterization does not require expensive technology but large tracts of land are often required for these experiments making it possibly more expensive than molecular assessment. These traits are often susceptible to phenotypic plasticity; conversely this allows assessment of diversity in the presence of environmental variation.

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 include 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 [3].

Molecular analysis comprise a large variety of DNA molecular markers which can be employed for analysis of variation. Different markers have different genetic qualities. Molecular markers work by highlighting differences (polymorphisms) within a nucleic sequence between different individuals. These differences include insertions deletions translocations duplications and point mutations. They do not however encompass the activity of specific genes. Molecular marker are widely used to detect and characterize somaclonal variations at the genetic level. Molecular marker are suitable for generating DNA profile have proved to be an effective tool in assessing the genetic stability of regenerated plants. These markers are not influenced by environmental factors and generate reliable and reproducible results.

Genotyping of cultivars

The repetitive and arbitrary DNA markers are markers of choice in genotyping of cultivars. Microsatellites and mini satellites have been employed in DNA fingerprinting for the detection of genetic variation, cultivar identification and genotyping. This information is useful for quantification of genetic diversity, characterization of accessions in plant germplasm collections and taxonomic studies. Microsatellites have been useful for generation of STMS markers, revealing polymorphisms within closely related cultivars. The first application of microsatellites in plants has been in cultivar identification, wherein microsatellites have been used to genotype unequivocally diverse materials like rice, wheat, grapevine (*Vitis unifera*) [4], soybean [5] etc. This is important especially for protection of proprietary germplasm. Microsatellite markers have also been advantageous in pedigree analysis as they represent single locus. The multi allelism of these markers facilitates comparative allelic variability detection reliably across a wide range of germplasm and allows individuals to be ubiquitously genotyped, so that gene flow and paternity can be established.

Mapping and tagging of genes

Plant improvement, either by natural selection or through the efforts of breeders, has always relied upon creating, evaluating and selecting the right combination of alleles. The manipulation of a large number of genes is often required for improvement of even the simplest of characteristics [6]. With the use of molecular markers it is now important to trace valuable alleles in a segregating population and mapping them.

Inter simple sequence repeat (ISSR) technique is a PCR based method, which involves amplification of DNA segment present at an amplifiable distance in between two identical microsatellite repeat regions oriented in opposite direction. The technique uses microsatellites, usually 16–25 bp long, as primers in a single primer PCR reaction targeting multiple genomic loci to amplify mainly the inter-SSR sequences of different sizes.

A novel marker system called start codon targeted (SCoT) polymorphism that is based on the short conserved region in plant genes surrounding the ATG translation start (or initiation) codon that has been well characterized in previous studies. 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 [7]. SCoT markers are highly reproducible as longer primers are used. ATG translation codon is more advantageous than the intersimple (ISSR), mini satellite (VNTR) or SSR regions due to conservation of ATG translation start site and flanking sequences in plant genes.

This method uses single 18-mer primers in single primer polymerase chain reaction (PCR) and an annealing temperature of 50°C. PCR amplicons are resolved using standard agarose gel electrophoresis. This method was validated in rice using a genetically diverse set of genotypes and a backcross population. Start codon targeted (SCoT) markers were generally reproducible but exceptions indicated that primer length and annealing temperature are not the sole factors determining reproducibility. SCoT marker PCR amplification profiles indicates dominant marker like RAPD markers. Gene-targeted markers are preferable for numerous applications in plant molecular genetics especially QTL mapping since recombination levels between marker and gene/QTL are generally lower compared with 'indirect random markers' such as RAPDs, ISSRs, or SSRs.

These markers once mapped enable dissection of the complex traits into component genetic units more precisely, thus providing breeders with new tools to manage these complex units more efficiently in a breeding programme [8]. Genetic maps have been constructed for several other crops like potato, barley, banana and members of Brassicaceae family [9]. Once the framework maps are generated, a large number of markers derived from various techniques are used to saturate the maps as much as possible. Microsatellite markers, especially STMS markers, have been found to be extremely useful in this regard. About 30 microsatellites have already been assigned to five

linkage groups in Arabidopsis, while their integration into the genetic linkage maps is still in progress in rice, soybean, maize, etc. The most recent microsatellite map has been generated for potato [10]. Similar to microsatellites, looking at the pattern of variation, generated by retrotransposons, it is now proposed that apart from genetic variability, these markers are ideal for integrating genetic maps [11].

Once mapped, these markers are efficiently employed in tagging several individual traits that are extremely important for a breeding programme like yield, disease resistance, stress tolerance, seed quality, etc. A large number of monogenic and polygenic loci for various traits have been identified in a number of plants, which are currently being exploited by breeders and molecular biologists together, so as to make the dream of marker-assisted selection come true. Tagging of useful genes like the ones responsible for conferring resistance to plant pathogen, synthesis of plant hormones, drought tolerance and a variety of other important developmental pathway genes, is a major target. Such tagged genes can also be used for detecting the presence of useful genes in the new genotypes generated in a hybrid programme or by other methods like transformation, etc.

SCoT markers have proved their importance as markers for gene tagging and are very useful in locating and manipulating quantitative trait loci (QTL) in a number of crops. 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. Allele-specific associated primers have also exhibited their utility in genotyping of allelic variants of loci that result from both size differences and point mutations. Some of the genuine examples of this are the waxy gene locus in maize [12] the Glu D1 complex locus associated with bread making quality in wheat [13], the *Lr1* leaf rust resistance locus in wheat [14], the *Gro1* and *H1* alleles conferring resistance to the root cyst nematode *Globodera rostochiensis* in potato [15], and allele-specific amplification of polymorphic sites for detection of powdery mildew resistance loci in cereals [16]. A number of other traits have been tagged using ASAPs in tomato, lettuce, etc.. Besides ASAPs, AFLP and SSR markers have been identified to be associated with quantitative resistance to *Globodera pallida* (stone) in tetraploid potato, which can be very well employed in marker-assisted selection [17].

STMS markers have displayed a potential use as diagnostic markers for important traits in plant breeding programmes, e.g. (AT) 15 repeat has been located within a soybean heat shock protein gene which is about 0.5 cM from (Rsv) a gene conferring resistance to soybean mosaic virus (Yu et al 1994). Several resistance genes including peanut mottle virus (Rpv), phytophthora (Rps3) and Javanese rootknot nematode are clustered in this region of the soybean genome. Recently, ISSRs, which too belong to the arbitrary marker category, but are found to be devoid of many of the drawbacks shared by RAPD class of markers, have been employed as a reliable tool for gene tagging. An ISSR marker (AG) 8YC has been found to be linked closely (3.7 ± 1.1 cM) to the rice nuclear restorer gene, RF1 for fertility. RF1 is essential for hybrid rice production and

this marker would be useful not only for breeding both restorer and maintainer lines, but also for the purity management of hybrid rice seeds [18]. Similarly ISSR marker (AC)₈ YT has been found to be linked to the gene for resistance to fusarium wilt race 4 in repulsion at a distance of 5.2 cM in chickpea.

Molecular profiling in *Mentha*

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 [3]. Assessment of molecular variation among different romanian and foreign barley cultivars was done to determine the level of genetic similarity among them [19]. ISSR markers are highly polymorphic and are useful in studies on genetic diversity, phylogeny, gene tagging, genome mapping and evolutionary biology. The technique uses microsatellites, usually 16–25 bp long, as primers in a single primer PCR reaction targeting multiple genomic loci to amplify mainly the inter-SSR sequences of different sizes. ISSRs segregate mostly as dominant markers following simple mendelian inheritance. However, they have also been shown to segregate as co-dominant markers in some cases thus enabling distinction between homozygotes and heterozygotes.

Due to the basis of SCoT primer design, SCoT markers is distributed within gene regions that contain genes on both plus and minus DNA strands. It is also possible that pseudogenes and transposable elements may be used as primer binding sites by SCoT polymorphism technique. An important factor is the distance in base pairs between primer binding sites of the template. Therefore, a relatively long extension time of the thermal cycle is important. For QTL mapping, SCoT markers could be integrated into existing framework maps to increase the marker density or to target specific chromosomal regions. In another analogy with RAPD, we propose that important SCoT markers, such as those identified to be tightly linked to a gene or QTL of interest, could be converted into sequence characterized amplified regions markers or sequence tagged site markers in order to make the marker single locus and improve robustness. The use of ISSR fingerprints could be a powerful tool to assess the genetic diversity in *Mentha* [20]. Morphological, phytochemical and genetic differences were studied to evaluate the level and distribution of diversity among thirteen genotypes of *Mentha* using both agro-morphological traits and ISSR markers [20].

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 [21]. Genetic diversity in *Mentha cervina* was analysed based on morphological traits, essential oils profile and ISSRs markers [20]. Characterization of twenty wheat varieties have been analysed by ISSR Markers [22]. Assessment of genetic diversity in *Mentha* using RAPD markers has been done previously [23] but assessment using ISSR markers in *Mentha* have not been reported yet.

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.

Concluding Remarks

There was a considerable genetic variation among the studied genotypes based on the agro-morphological traits and generated ISSR and 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 ISSR markers set used. Primers were synthesized taking into consideration monoterpene biosynthetic pathway genes.

Larger inter than intra cluster distances implies the presence of higher genetic variability between the genotypes of different groups. The generated dendrogram based on ISSR profiles divided the genotypes into 7 groups. Species specific bands could also be recognized from some ISSR primers in this study indicating the high potential use of this marker for discrimination of genotypes. SCoT analysis also revealed high polymorphism with the primers generating polymorphic profiles. Species-specific and genotype specific markers can be useful for introgression studies where plant breeders want to transfer some desirable traits from one species into another. Genetically distinct cultivars can be identified that could be potentially important sources of germplasm. ISSR and SCoT markers can then be used for linkage mapping of Quantitative Trait Loci and for indirect selection (Marker assisted selection) for genetic improvement in *Mentha*. Localization of these markers on the chromosomes would be useful for keeping track of important traits that need to be transferred.

Key message

The use of ISSR and SCoT fingerprints could be a powerful tool to assess the genetic diversity in *Mentha*. Genotypes of two clusters with a good amount of genetic divergence and desirable agronomic traits were detected as promising genotypes for hybridization.

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Author contribution segment

NK carried out data analysis and prepared manuscript. SSD conceptualize the overall design, draft and revised the manuscript.

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