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DNA barcoding and phylogenetic analyses of mentha species using *rbcL* sequences

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Abstract

The genus, Mentha (Mint) is the most important aromatic plant, belonging to the family, Lamiaceae. It is cultivated worldwide for its commercially and medicinally important essential oils. Due to vast morphological variations, molecular characterization of mentha species is essential for its proper identification. For this purpose, DNA barcode *rbcL* primer was amplified to assess genetic divergence within and between species, using various matrices. In this study, we observed 100% PCR amplification efficiency to recover a quality sequence. The phylogenetic tree from the *rbcL* data demonstrated that all the mentha species considered for the study, formed monophyletic cluster except hybrid species, *M. piperita*. The study proves the efficiency of *rbcL* primer to classify mentha species phylogenetically.

Key words: DNA barcoding, phylogenetic, mentha, *rbcL*

1. Introduction

The medicinal plants have been used for treatment of diseases since the prehistoric human civilization. Even today, in rural and tribal communities, plants are widely used as ethnomedicine for human healthcare. Secondary metabolites of plants such as alkaloids, glycosides, steroids or other active compounds are used for cure of various diseases like cancer, malaria, diabetes and dysentery (Biradar, 2015). For development of novel healthcare products from plants, traditional knowledge about medicinal plants is essential. Approximately 1200 plants are used for medicinal purpose in Indian traditional medicinal systems (Subramoniam, 2014). The herbal industry experiences substitution and debasement of therapeutic herbs with related species. The adequacy of the medicine diminishes in the event that it is contaminated, and becomes toxic when it is substituted with harmful adulterants. Henceforth, the right definition is vital for the therapeutic herb to be successful.

The customary techniques for restorative plant recognizable proof incorporate organoleptic strategies (identification by the senses: taste, sight, smell, touch), naturally visible and microscopic systems and chemical profiling such as TLC, HPLC-UV, HPLC-MS (Soni *et al*., 2013). The chemical constituents of bioactive components of plants vary from one plant species to another species. Understanding of genetic relationships and genetic variation among individuals is crucial for accurate management and conservation of medicinal plants (Thiyagarajan and Venkatachalam, 2015). To reveal the genetic variation in plants at the level of inter-species and intra-species, molecular techniques are more reliable (Sheeba *et al*., 2014). Verification at the DNA level assure greater reliability in comparison to RNA and protein because DNA is a steady macromolecule that is not influenced by environmental effect and is found in all tissues. ISSR, RAPD and AFLP are some commonly used DNA-based markers, used for identification of plants as well as confirmation of herbal ingredients in products. However, some limitations associated with these markers such as dominant nature, requirement of high quality and large amount of DNA during analysis of sample, which might be some time not possible for dried or processed materials. The possibility of fungal contamination in market samples may not avoid that could alter the results of these markers. Limitations of these markers can be overcome by developing of species specific marker (Ganie *et al*., 2015). DNA barcoding primers based on short DNA sequences may be applied for sheared and low quality DNA. These primers are highly species specific and useful for the amplification of contaminated DNA.

The genus, Mentha (Mint) is an important aromatic plants, belonging to the family, Lamiaceae. It consists of 25 to 30 species, cultivated mostly in temperate and sub-temperate regions and known for its commercially and medicinally important essential oils. In India, *Mentha arvensis* L., *Mentha spicata* L., *Mentha piperita* L., *Mentha cardiac* L. and *Mentha citrata* L. are the most commonly cultivated species (Patra *et al*., 2000). These five species are widely cultivated to obtain commercially valuable aroma compound such as menthol, menthofuran, carvone, linalool, and linalyl acetate. These aromatic compounds are frequently used in the cosmetics, pharmaceuticals, food, confectionery and liquor industries (Khanuja *et al*., 2000). The chemical constituents of essential oils of mentha are not similar in all the species and also depend upon seasonal variations, soil types, *etc*. Due to morphological plasticity and variation in active components of essential oil of mentha species, accurate identification is essential for explanation of phylogenetic relatedness and distinctive marker profiles at DNA level. These studies will be important for selection of superior parental combination to develop desired recombinant hybrid genotypes.

DNA barcoding proposed by Hebert *et al*. (2003) is molecular and computational-based approach where short and standardized section

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of genome works as an exclusive identifier for species. The portion applied for recognition is termed as a DNA barcode. This approach is frequently used in integrated taxonomic system and in biodiversity research for accurate identification of existing species, discovery of unknown species and development of phylogenetic relationship among them. DNA barcoding has also been used for the authentication and quality assurance of raw plant material (Li *et al*., 2011).

Among different DNA barcode segments, the plastid ribulose-1, 5-biphosphate carboxylase (*rbcL*) gene sequences are frequently used to analyze the origin, phylogenetics, evolution, biogeography, population genetics and systematics of plants (Sheng-Guo *et al*., 2008; Doyle*et al*., 1997). In polymerase chain reaction (PCR), *rbcL* gene had shown higher amplification efficiency and sequencing success rate compared to other selected plant characterization genes (CBOL Plant Working Group, 2009; Parveen *et al*., 2012). Due to this unique property of *rbcL* gene, it is ideal for studying the genetic variations of the plants species (Hamdam *et al*., 2013). Our study aimed to determine the genetic variation and phylogenetic relationship of mentha species based on *rbcL* sequencing.

2. Materials and Methods

2.1 Plant materials

Seventeen mint accessions belonging to four morphologically identified plant species of genus mentha (*Mentha spicata* L., *Mentha arvensis* L., *Mentha citrate* L. and *Mentha piperita* L.) were collected from different states of India (Table 1).

2.2 DNA extraction and quantification

Genomic DNA was isolated from plant samples, using the DNeasy Isolation and Purification kit (Qiagen, Italy) to obtain metabolites free good quality DNA to avoid any interference with DNA amplification. The quantity, quality and integrity of isolated DNA were checked by gel electrophoresis and on a UV-spectrophotometer.

2.3 PCR amplification and sequencing

Analysis of polymerase chain reaction (PCR) was carried out, using *rbcL*1-F (CTTAATTCATGAGTTGTAGGGAGGG) and *rbcL*1-R (CATCGGTCCACACAGTTGTC) primers (Integrated DNA Technology, USA). Each amplification reaction for testing the PCR amplification efficiency contained 1µl DNA template (25 ng), 1 µl 10X reaction buffer, 0.7 µl $MgCl_2$ (50 µM), 0.2 µl dNTPs mix (10 mM), 0.5 µl forward (10 µM), 0.5 µl reverse (10 µM) primers, 0.2 µl Taq polymerase (5 U/µl) and the final volume 10 µl was adjusted with molecular grade water. The PCR conditions were started with pre-initiation at 95°C for 5 min, followed by a total of 35 cycles of initiation at 94°C for 40 s, annealing at 55°C for 1 min, extension at 72°C for 30 s. The reaction was completed with a final extension at 72°C for 5 min. PCR reactions were performed in a thermal cycler (Agilent Sure Cycler 8800, USA). The PCR amplification efficiency was verified by 1.5% agarose (Sigma, USA) gel electrophoresis using 1X TAE buffer. After verification of the success of PCR amplification, the reaction volumes were increased up to 50 µl for sequencing. The final PCR products were sequenced on an ABI 3730XL sequencer (Applied Biosystems Inc., USA), using the amplification primers. All sequences generated in the present study were deposited in GenBank and accession numbers assigned by GenBank (Table 1).

2.4 Data analysis

2.4.1 *Sequence analysis and estimation of nucleotide variations*

DNA sequences obtained from *rbcL* were aligned with ClustalW (Thompson *et al*., 1994) and alignments were consequently accustomed manually, using BioEdit (Hall, 1999). The length of sequence and GC% of every sample was intended in MEGA 6 (Tamura *et al*., 2013). Pairwise distances were calculated with the simplest K2P model, using MEGA 6. Intra and interspecific variations were calculated in an attempt to define the levels of molecular variability within each group species. Nucleotide polymorphism, as measured by θw (Watterson, 1975) and diversity, as measured by π (Nei, 1978) were calculated using DnaSP v4.5 (Rozas and Rozas, 1999).

2.4.2 *Phylogenetic analysis*

To evaluate whether species were recovered as monophyletic with *rbcL* barcode primer, we used Neighbor-joining (NJ) method for the construction of phylogenetic trees. The output data were processed using MEGA 6 to draw the phylogenetic trees. A total of 1000 bootstrap replicates were considered for the NJ tree construction.

3. Results and Discussion

3.1 Amplification and sequencing success

Species identification based on single-gene sequence similarity or phylogenies are most widely used techniques (Ross *et al*., 2008). During the present investigation, we could get 100% PCR amplification efficiency and recovered a quality sequence and our results are not the contradict of previous results which reported *rbcL* is the most effective loci in terms of sequence quality and recovery (Roy *et al*., 2010; CBOL, 2009). *rbcL* primer amplified single and intact amplicon within desired size range. The amplified products were used for sequencing and further analysis.

3.2 Sequence analysis and estimation of sequence variations

In this study, all 17 *rbcL* sequences of mentha species were included in final analysis. The characteristics of these sequences are summarized in Table 1. The sequence lengths of the *M. arvensis*, *M. piperita*, *M. spicata* and *M. citrata* species were 217bp, 213bp, 202bp and 212bp whereas the average GC content were 46.60, 46.90, 47.00, and 47.10 %, respectively. Overall pairwise distance was 0.003 (Table 2). The intraspecific genetic distance was 0 whereas interspecific genetic distance was ranged from 0 to 0.005. Data matrix has a total number of 217 characters, of which invariable (monomorphic) sites were found 200, variable (polymorphic) sites 1 (total number of mutations 1), singleton variable sites 0 and parsimony informative sites 1. Polymorphism was observed among the populations (π= 0.00263, θw= 0.00147 and total variance 0.199).Whereas, π represents nucleotide diversity (Nei and Li, 1979) and θw Watterson's parameter (Watterson, 1975), respectively.

PCR amplification with *rbcL* primer was used to assess genetic divergences within and between species, using various matrices. A suitable barcode must exhibit high interspecific but low intraspecific divergence. However, no significant difference between the intra-species and inter-species variation was observed in present study, due to sequence homology in *rbcL* sequence of mentha species. This limitation of *rbcL* gene was also reported in closely related species of *Ardisia* and *Sweretia chirayita* (Kshirsagar *et al*., 2015; Liu *et al*., 2013).

Table 1: List of mentha species used in this study with collection site, GenBank accession number and characteristics feature of *rbcL* sequence.

Species name	Code	Collection site	Latitude	Longitude	Accession No.	Sequence length (bp)	G+C contents (%)
Mentha arvensis	MA1	CIMAP, Lucknow	26° 55' N	80° 59' E	KX356044	217.0	46.60
Mentha arvensis	MA2	Pantnagar	28° 582N	79° 242E	KX356045	217.0	46.60
Mentha arvensis	MA3	Amarkantak	22° 40' N	81° 45' E	KX356046	217.0	46.60
Mentha arvensis	MA4	Jabalpur	23° 10' N	79° 59' E	KX356047	217.0	46.60
Mentha arvensis	MA5	Raipur	21° 15' N	81° 41' E	KX356048	217.0	46.60
Mentha piperita	MP1	CIMAP, Lucknow	26° 55' N	80° 59' E	KX356049	213.0	46.90
Mentha piperita	MP2	Amarkantak	22° 40' N	81° 45' E	KX356050	213.0	46.90
Mentha piperita	MP3	Jabalpur	23° 10' N	79° 59' E	KX356051	213.0	46.90
Mentha piperita	MP4	Pantnagar	28° 582N	79° 242E	KX356052	213.0	46.90
Mentha spicata	MS1	CIMAP, Lucknow	26° 55' N	80° 59' E	KX356053	202.0	47.00
Mentha spicata	MS2	Raipur	21° 15' N	81° 41' E	KX356054	202.0	47.00
Mentha spicata	MS3	DMAPR, Anand	22° 32' N	73° 00' E	KX356055	202.0	47.00
Mentha spicata	MS4	Amarkantak	22° 40' N	81° 45' E	KX356056	202.0	47.00
Mentha citrata	MC1	CIMAP, Lucknow	26° 55' N	80° 59' E	KX356057	212.0	47.10
Mentha citrata	MC2	Pantnagar	28° 582N	79° 242E	KX356058	212.0	47.10
Mentha citrata	MC3	Amarkantak	22° 40' N	81° 45' E	KX356059	212.0	47.10
Mentha citrate	MC4	Raipur	21° 15' N	81° 41' E	KX356060	212.0	47.10

Table 2: Pairwise distance matrics among different mentha species based on *rbcL* barcodes sequences

MA1																
MA2	0.000															
MA3	0.000	0.000														
MA4	0.000	0.000	0.000													
MA5	0.000	0.000	0.000	0.000												
MP1	0.005	0.005	0.005	0.005	0.005											
MP2	0.005	0.005	0.005	0.005	0.005	0.000										
MP3	0.005	0.005	0.005	0.005	0.005	0.000	0.000									
MP4	0.005	0.005	0.005	0.005	0.005	0.000	0.000	0.000								
MS1	0.000	0.000	0.000	0.000	0.000	0.005	0.005	0.005	0.005							
MS2	0.000	0.000	0.000	0.000	0.000	0.005	0.005	0.005	0.005	0.000						
MS3	0.000	0.000	0.000	0.000	0.000	0.005	0.005	0.005	0.005	0.000	0.000					
MS4	0.000	0.000	0.000	0.000	0.000	0.005	0.005	0.005	0.005	0.000	0.000	0.000				
MC1	0.005	0.005	0.005	0.005	0.005	0.000	0.000	0.000	0.000	0.005	0.005	0.005	0.005			
MC2	0.005	0.005	0.005	0.005	0.005	0.000	0.000	0.000	0.000	0.005	0.005	0.005	0.005	0.000		
MC3	0.005	0.005	0.005	0.005	0.005	0.000	0.000	0.000	0.000	0.005	0.005	0.005	0.005	0.000	0.000	
MC4	0.005	0.005	0.005	0.005	0.005	0.000	0.000	0.000	0.000	0.005	0.005	0.005	0.005	0.000	0.000	0.000

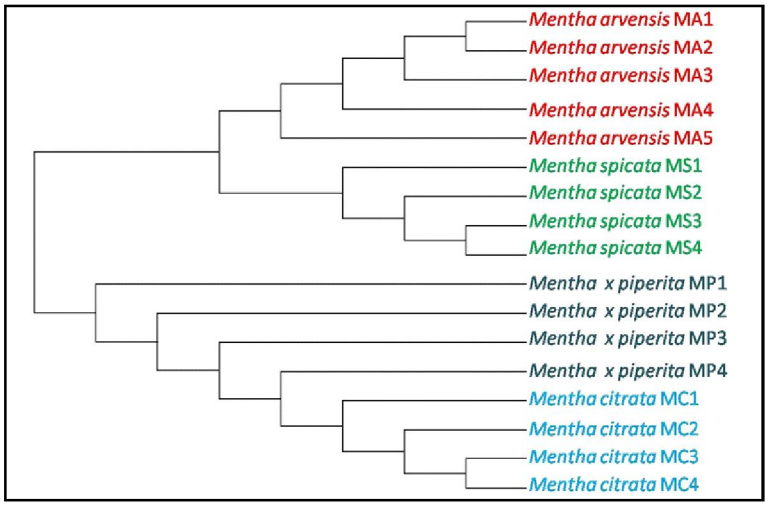

Figure 1: Phylogenetic tree from partial *rbcL* sequence of 17 samples of mentha species

3.3 Phylogenetic analysis

Phylogenetic tree was analyzed for the *rbcL* sequences, using Neighbor-Joining (NJ) method. The phylogram could be separated into two distinct clusters. Cluster one contained *M. arvensis* and *M. spicata* whereas *M. piperita* and *M. citrate* were grouped in second cluster.The phylogenetic tree from the *rbcL* sequences showed that species, *M. arvensis* and *M. spicata* in cluster one formed clearly distinctive clades whereas in second cluster, only *M. citrate* form clearly distinctive clades while *M. piperita* species not positioned within the clade (Figure 1).

In NJ tree method, a species was considered to be resolved if the accessions under the species form a monophyletic group. Our study showed that species identification in genus mentha is possible, using phylogenetic analysis based on *rbcL* sequences. The phylogenetic tree from the *rbcL* data showed that all the species formed monophyletic group except hybrid species *M. piperita*. Evolution of *M. piperita* as triple hybrid from hybridization of spearmint (*M. spicata*) and water mint (*M. aquatica*) and resultant further hybridized with *M. longifolia* and *M. rotundifolia* is reported in previous results (Anonymous, 1962; Fleming, 1998). This evolution may be a cause of differences at genetic level among *M. piperita* accessions, used under study, therefore, *M. piperita* failed to group with monophyletic clade in phylogenetic tree. In our study, we were able to get 100% PCR amplification efficiency and established phylogenetic relationship among mentha species, using *rbcL* DNA barcode primer.

4. Conclusion

During the present investigation, the relationship between four mentha species was established based on the nucleotide polymorphism and phylogenetic tree analyses, using *rbcL* marker. Identification of the species through phylogenetic study is an easy and efficient method, using *rbcL* sequences. However, using higher quantity of samples collected from diverse locations and try to get big sized amplicons of *rbcL* gene can improve the importance of such methodology. Combining data from phytochemical analysis could help to standardize the species identification process of mentha species.

Conflict of interest

We declare that we have no conflict of interest.

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