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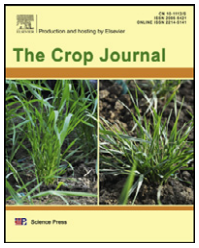

Identification of EST–SSRs and molecular diversity analysis in *Mentha piperita*

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ABSTRACT

EST sequences of *Mentha piperita* available in the public domain (NCBI) were exploited to develop SSR markers. A total of 1316 ESTs were assembled into 155 contigs and 653 singletons and of these, 110 sequences were found to contain 130 SSRs, with a frequency of 1 SSR/3.4 kb. Dinucleotide repeat SSRs were most frequent (72.3%) with the AG/CT (43.8%) repeat motif followed by AT/AT (16.2%). Primers were successfully designed for 68 SSR-containing sequences (62.0%). The 68 primers amplified 13 accessions of *M. piperita* and 54 produced clear amplicons of the expected size. Of these 54, 33 (61%) were found to be polymorphic among *M. piperita* accessions, showing from 2 to 4 alleles with an average of 2.33 alleles/SSR, and the polymorphic information content (PIC) value varied between 0.13 and 0.51 (average 0.25). All the amplified SSRs showed transferability among four different species of *Mentha*, with a highest in *Mentha arvensis* (87.0%) and minimum in *Mentha citrata* (37.0%). The newly developed SSRs markers were found to be useful for diversity analysis, as they successfully differentiated among species and accessions of *Mentha*.

1. Introduction

The genus *Mentha* (mint) is one of the most important taxa of the family Lamiaceae and comprises 25 to 30 species grown in different parts of the world. Only five of them, Japanese/menthol mint (*Mentha arvensis* L. var. *piperascens forma* Holmes), spearmint (*Mentha spicata* L.), peppermint (*Mentha piperita* L.), scotch spearmint (*Mentha cardiaca* Baker.) and Bergamot mint (*Mentha citrata* Ehrh.) are commercially grown in India and other countries [1,2]. These five species are the major natural source of aroma compounds of industrial importance; namely-menthol, menthofuran, carvone, linalool, and linalyl acetate. Because of their cooling, pleasant aroma and flavor, essential oils of mint are used in perfumery, cosmetics, confectionery, and the pharmaceutical industries. The oil of *M. piperita*, known as peppermint oil, is widely used for headache, nerve pain, toothache, oral inflammation, joint conditions, itchiness, allergic rash, repelling mosquitoes, rheumatism, muscular pains, etc. [3,4]. Menthol is the major constituent of the essential oil constituents of peppermint oil [5]. Peppermint oil of globally accepted quality contains high amounts of menthol, moderate amounts of menthone, and very low amounts of pulegone and menthofuran [6,7]. The presence and concentrations of certain chemical constituents of essential oils change

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according to the season, soil, climate, and site of plant growth. Peppermint is cultivated in several parts of India and has great economic value and a strong export potential for its volatile oil extracts.

DNA-based molecular markers have been shown to be an efficient tool to assist conventional plant breeding in various ways, such as by assessing the gene pool for diverse parental lines, hybridity testing, QTL mapping, gene tagging, and marker-assisted selection. Despite the importance of peppermint as an aromatic and medicinal plant, no comprehensive molecular marker systems are available. A few studies have assessed genetic diversity in species of *Mentha* based on RAPD [8–10] and AFLP fingerprinting [11]. There is a complete lack of *Mentha*-specific molecular markers for use in genetic studies and genetic improvement programs.

Simple sequence repeats (SSRs) also called microsatellites, are 1–6-base tandem repeats of DNA sequences, abundant in both prokaryotic and eukaryotic organisms in coding and noncoding regions [12]. SSRs are a preferred marker system owing to their codominant inheritance, multiallelic nature, abundance in the genome, high reproducibility, hyperpolymorphism, and high rate of transferability across genera and species [13–15]. Expressed sequence tags (ESTs) available in the public domain are the easiest and cheapest source for SSR development. EST–SSRs offer various advantages including ease of access, presence in gene-rich regions, and high transferability across species and genera [16], which enable them to serve as anchor markers for comparative mapping and evolutionary studies [15]. Given that no SSR markers are reported in *Mentha*, the present study was undertaken to exploit the EST database of *M. piperita* to (1) analyze the frequency and distribution of SSRs in ESTs, (2) develop and characterize EST–SSRs (3) test their transferability in related species and (4) detect polymorphism/diversity among accessions and species of *Mentha*.

2. Materials and methods

2.1. Plant materials and DNA isolation

The plant material included 13 accessions of *M. piperita*, 5 of *M. arvensis*, 4 of *M. spicata*, and 1 each of *Mentha longifolia* and *M. citrata*. These accessions were previously evaluated for essential oil content and other components. The details of these plant materials are given in Table 1. *M. piperita* is characterized by moderate oil content and high menthofuran. *M. arvensis*, also known as menthol mint, contains comparatively high oil content rich in menthol. The accessions of *M. spicata* and *M. longifolia* contain carvone-rich essential oils and *M. citrata* is rich in linalool and linalyl acetate. These accessions were grown during the 2012–2013 crop season in the experimental field of the Central Institute of Medicinal and Aromatic Plants (CIMAP) Lucknow, Uttar Pradesh, India. Fresh leaves combined from five randomly chosen plants of each accession were used to isolate total genomic DNA with a Qiagen DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. The quality of DNA was assessed on 0.8% agarose gel and quantity was checked with a NanoDrop spectrophotometer ND 1000 (NanoDrop Products, USA). Finally, the DNA was normalized to 10 ng μL^{-1} for PCR amplification.

2.2. Data mining and EST–SSR identification

A total of 1316 raw EST sequences of *M. piperita* were downloaded from the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/dbest/) on January 14, 2013. The 5′ or 3′ end poly A or poly T stretches were removed from the raw EST sequences using EST-Trimmer software (http://pgrc.ipk-

Table 1 – Details of cultivars and species of *Mentha* used in the study.

Species	Cultivars	Type	Oil content (%)	Origin
M. piperita	CIM-Indus	Cultivar	0.25 (menthofuran 15–25%)	CSIR-CIMAP, Lucknow
M. piperita	CIMAP-Patra	Cultivar	0.35 (menthofuran 40–45%)	CSIR-CIMAP, Lucknow
M. piperita	Kukrail	Cultivar	0.50 (menthofuran 5–8%)	CSIR-CIMAP, Lucknow
M. piperita	Madhuras	Cultivar	0.45 (menthofuran 2–5%)	CSIR-CIMAP, Lucknow
M. piperita	MPS-5	Breeding line	0.40 (menthol 65%)	CSIR-CIMAP, Lucknow
M. piperita	MPS-6	Breeding line	0.45	CSIR-CIMAP, Lucknow
M. piperita	MPS-9	Breeding line	0.35	CSIR-CIMAP, Lucknow
M. piperita	MPS-16	Breeding line	0.45	CSIR-CIMAP, Lucknow
M. piperita	MPS-20	Breeding line	0.45	CSIR-CIMAP, Lucknow
M. piperita	MPS-25	Breeding line	0.30	CSIR-CIMAP, Lucknow
M. piperita	MPS-36	Breeding line	0.35 (menthofuran 30–35%)	CSIR-CIMAP, Lucknow
M. piperita	MPP	Landrace	0.40	Purara, Bageshwar
M. piperita	MPS-21	Breeding line	0.55	CSIR-CIMAP, Lucknow
M. arvensis	Kosi	Cultivar	0.85 (menthol rich)	CSIR-CIMAP, Lucknow
M. arvensis	MAS-1	Cultivar	0.85 (menthol rich)	CSIR-CIMAP, Lucknow
M. arvensis	MAS-49	Breeding line	0.90 (menthol rich)	CSIR-CIMAP, Lucknow
M. arvensis	MAS-10-11-45	Breeding line	0.80 (menthol rich)	CSIR-CIMAP, Lucknow
M. arvensis	MAS-13-2-125	Breeding line	0.80 (menthol rich)	CSIR-CIMAP, Lucknow
M. spicata	Neera	Cultivar	0.55 (marvone rich)	CSIR-CIMAP, Lucknow
M. spicata	Neerkalka	Cultivar	0.60 (marvone rich)	CSIR-CIMAP, Lucknow
M. spicata	Arka	Cultivar	0.75 (marvone rich)	CSIR-CIMAP, Lucknow
M. spicata	MSP	Landrace	0.45 (marvone rich)	Nagar, Kullu, H.P.
M. longifolia	MLP	Landrace	0.25 (carvone rich)	Palampur, H.P.
M. citrata	Kiran	Cultivar	0.35 (minalool and minalyl acetate rich)	CSIR-CIMAP, Lucknow