PHYTOCHEMICAL PROFILING AND GENETIC DIVERSITY ANALYSIS OF *Mangifera indica* Linn. VARIETIES IN KERALA

By

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(2015-09-002)

THESIS

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requirement for the degree of

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KERALA, INDIA

2020

DECLARATION

I hereby declare that the thesis entitled **"Phytochemical profiling and genetic diversity analysis of *Mangifera indica* Linn. varieties in Kerala"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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CERTIFICATE

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LIST OF ABBREVIATIONS

%	Percentage
°C	Degree Celcius
µL	Microlitre
µm	Micrometre
A_{260}	Absorbance at 260 nm wavelength
A_{280}	Absorbance at 280 nm wavelength
AFLP	Amplified Fragment Length Polymorphism
bp	Base pair
cm	Centimetre
CTAB	Cetyl Trimethyl Ammonium Bromide
Da	Dalton
DNA	Deoxyribonucleic acid
EDTA	Ethylene Diamine Tetra Acetic acid
et al.	et alia
EtBr	Ethidium Bromide
FDA	Food and Drug Administration
g	Gram
GC-MS	Gas Chromatograph-Mass Spectrometer
H_2O_2	Hydrogen peroxide
HCA	Hierarchical Cluster Analysis
ISSR	Inter Simple Sequence Repeats
KAU	Kerala Agricultural University
kb	Kilo bases
kg	Kilogram
L	Litre
Linn.	Linnaeus
m	Metre
M	Molar
mg	Milligram
min	Minute

mL	Millilitre
mm	Millimetre
m*M*	Millimolar
NaCl	Sodium chloride
ng	Nanogram
nm	Nanometre
OD	Optical Density
PCR	Polymerase Chain Reaction
pH	Potential of Hydrogen
PVP	Poly Vinyl Pyrrolidone
RAPD	Random Amplification of Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RNase	Ribonuclease
rpm	revolutions per minute
s	second
SCAR	Sequence Characterized Amplified Region
SCoT	Start Codon Targeted marker
SNP	Single Nucleotide Polymorphisms
SSR	Simple Sequence Repeats
STR	Short Tandem Repeats
TAE	Tris Acetate EDTA buffer
TE	Tris-EDTA buffer
Tm	Melting temperature
Tris HCl	Tris (Hydroxy methyl) aminomethane hydrochloride
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
UV	Ultra violet
V	Volt
ver.	Version
α	Alpha
β	Beta
γ	Gamma
δ	Delta

INTRODUCTION

The limitless abilities of plants from providing fresh air to synthesizing aromatic secondary metabolites, mostly phenols or their oxygen-substituted derivatives make them different among other lives on the planet. Demand for the products of natural origin is rising everyday as the deleterious effects of synthetic additives began a major concern. This growing demand widens the utility of aromatic plants and their derived products like essential oils in food, feed, pharmaceutical and cosmetic industries. There are huge number of plant species producing diverse bioactive molecules with different chemical scaffolds. Such molecules provide plants, the defense against predators and microbial pathogens, repellence to herbivores and protection against abiotic stress. Meanwhile these phytochemicals are also necessary for the communication of plants with other organisms (Schafer *et al*., 2009), but are not much essential in the plant growth and development (Rosenthal *et al*., 1991).

Mango (*Mangifera indica*) is a versatile crop having huge commercial importance in terms of production, marketing and consumption. The appealing appearance, pleasant aroma, delicious taste and high nutritious value of mangoes made them, the choicest fruit of tropics. The mango fruit aroma whose key constituents being its volatiles, is a major factor that influences the quality and consumer acceptance of mango and its products. These hydrophobic, odorous volatiles seen in highly concentrated forms are called essential oils that can be isolated from all parts of the plant like flowers, buds, leaves, bark, wood, fruits and roots. Essential oils are complex mixtures of secondary metabolites mainly consisting of low-boiling-point phenylpropenes and terpenes. These metabolites often contain certain characteristic compounds that give specific aroma for different mango varieties which makes identification easier.

Diversity analysis of cultivars using morphological characters as in the past is inaccurate and unreliable since these characters are often limited in number and are influenced by habitat variation as well as environmental and development changes (Karihaloo *et al*., 2003; Rahman *et al*., 2007). This arouses the need for more systematic and reliable approaches like chemical profiling which has been proved as an essential

tool for chemosystematics of *M. indica* varieties in many studies. The volatile chemical profiles of the plant studied through GC-MS analysis of leaf essential oils can be used for authenticating the varieties which are in the verge of extinction.

Meanwhile, molecular markers based on PCR techniques are advanced tools presently available that could improve the accuracy of diversity analysis. These markers are limitless and are not influenced by environmental and developmental changes. SSRs have proved to be the most potential marker system for genetic diversity analysis and parentage evaluation in mango (Schnell *et al*., 2006). However, SSRs require the aid of polyacrylamide gels or capillary electrophoresis genetic analyzers and prerequisite sequence knowledge. Collard and Mackill (2009) introduced a novel and simple molecular marker technique called Start Codon Targeted (SCoT) Polymorphism which uses 18-mer single primer in PCR and an annealing temperature of 50°C. The easiness in designing the SCoT primers based on the conserved region flanking the translation initiation codon, ATG (Joshi *et al*., 1997; Sawant *et al*., 1999) without the need of genomic sequence information made them widely acceptable. Moreover, resolving the PCR products using standard agarose gel electrophoresis, increases its simplicity. Unlike RAPD, AFLP and ISSR, SCoT is a gene targeted marker that can generate more information linked with the biological traits.

The present study aims at the volatile chemical profiling of selected local varieties of *M. indica* in Kerala using GC-MS analysis and their genetic diversity analysis using SCoT marker polymorphisms.

2. REVIEW OF LITERATURE

2.1. DISTRIBUTION OF *Mangifera* SPECIES

Anacardiaceae, also called Mango family or Cashew family, is a family of flowering plants, including 80 genera and over 600 species (Jones and Liechsinger, 1987). The members are chiefly tropical but occurs in S. Europe, temperate Asia and America also. Though several reports have shown that the genus *Mangifera* spreads throughout the world, its natural area is confined to India, Ceylon, Philippines, New Guinea, the Himalayas and Yunnan (China).

Apart from the Indo-Burma region, the centre of origin of mango, the crop is being cultivated commercially as a major crop or as a mixed plantation in about 89 countries. The major mango growing countries of the world include India, Pakistan, Bangladesh, Myanmar, Sri Lanka, Florida and Hawaii of USA, Australia, Brazil, Thailand, the Philippines, Malaysia, Vietnam, Indonesia, Fiji Islands, Egypt, Israel, South Africa, Sudan, Somalia, Kenya, Uganda, Tanzania, Niger, Nigeria, Zaire, Madagascar, Mauritius, Venezuela, Mexico, West Indies Islands and Cambodia (Yadav and Singh, 2017).

2.2. HISTORY

According to the observed level of genetic diversity, 'Indo-Burma' region is known as the centre of origin of mango (Vavilov, 1926). Mukherjee (1972) supported this finding by showing the presence of wild varieties of mango and its related species along with the cultivated varieties in large number in the region.

India's history of mango cultivation is about 4000 years old as stated in the historical writings by successive botanists to 6000 years (de Candolle, 1883). There are also archaeology reports showing frequent discoveries of mangoes. One of the most fine among them is Bharhut sculpture of ~110 BC. Babar, the Mughal emperor began giving mango, the present importance in India after recognising it as the premier fruit of India. The cultivation was followed in his heir with the aid of advanced grafting techniques and growing technologies which yielded enormous collection of mango varieties in India. Akbar (1556 – 1605 AD) developed an orchard of 1,00,000 mango trees, the 'Lakh Bagh' near Darbhanga. A written encyclopedia, Ain-e-Akbari during the reign of Akbar

accounts the detailed information about varying features of mango fruit. This shows the fine knowledge of Indian people about this fruit even during the 16th century. However specified researches in this valuable crop started during the 19th century only (Yadav and Singh, 2017). There are more than 1000 cultivars of mangoes in India (Kumar *et. al*., 2001).

2.3. TRADITIONAL USES

The plant is known as Kalpavraksha since its leaves are used as offerings to god in marriage ceremonies according to Hindu religious beliefs. Furthermore the whole plant is used for various traditional uses. Raw fruit is being used for making pickles, chutney and curry. Fresh ripen fruits are used for making juice drinks and sharbats. In some parts of India the seed is eaten in the form of grounded starchy flour or as boiled vegetable. The young boiled leaves are also edible and they have a gained taste due to their resinous savour.

Mangos are also used for making alcoholic beverages like wines and liquors in India as well as Australia. Their fragrant flowers are used for flavouring teas occasionally. Seeds and by-products from the fruit processing are being used as feed for cattle, poultry and pigs. Moreover a yellowish-brown dye employed in silk production is extracted from the mango bark (Bally, 2006).

Apart from these common traditional uses, *M. indica* is being widely in traditional medicine possess several medicinal properties also. Dried flowers of the plant is being used as a cure for wasp sting. Resinous gum from trunk is used for applying on skin cracks of feet. Young leaves are used in several diseases such as burning sensation, diarrhoea, dysentery haemorrhoids, hiccough hyperdipsia, ulcer, kidney stone and wound. Leaves are also used for air blackening, piles, jaundice, vomiting, urinary diseases, liver disorder, constipation, bloody dysentery and as anti-microbial. The plant roots can be used against diarrhoea, leucorrhoea, pneumonia and rheumatism. Inner bark and young leaves are used by tribals against diabetes (Khandare, 2016). Seeds are used in heart problems, amebiosis, nasal bleeding, liver disorder, teeth diseases, acidity, uterus problems, fistula and also against poisonous biting such as scorpion, makadi, honeybee etc (Ainslie, 1813 Govindachari, 1983).

2.4. PHARMACOLOGICAL PROPERTIES

Numerous studies describing the pharmacological properties of *M. indica* have been reported globally. Different parts of *M. indica* possess various pharmacological properties including anti-cancer, anti-inflammatory, anti-diabetic, anti-oxidant, anti-bacterial, antifungal, anthelmintic, gastro-protective, hepato-protective, immune-modulatory, anti-plasmodial and anti-hyperlipemic effects.

Mangiferin is a xanthonoid characteristic to *M. indica*, with numerous pharmacological properties. The bark and leaves of the plant are rich in this polyphenol. Crude extracts of the plant are more potent than refined mangiferin which indicates its synergism with other mango polyphenols. This property enhances the activity of mangiferin.

2.5. ANTIOXIDANT ACTIVITIES

According to Ajila and Rao (2008) mango peel extract possess protective effect against hydrogen peroxide induced oxidative damage since the peel is rich in polyphenols, carotenoids and anthocyanins. Acetone extracts of ripe and unripe mangoes showed protection against lipid peroxidation, membrane protein degradation and morphological changes caused by H_2O_2 in rat erythrocytes. The acetone-water, methanol-water and ethanol-water extracts of mango peel were also found to possess good antioxidant potential (Dorta *et al.*, 2012). In a similar finding the acetone extract of mango peel powder exhibited strong radical scavenging activity (Thambi *et al*., 2016). Moreover mango peel extracts are more potent than mango flesh extracts in terms of anti-oxidant activity (Kim *et al.*, 2010).

Pitchaon *et al*. (2011) demonstrated that the mango kernel extracts prepared by acid hydrolysis possess high anti-oxidant potential than the ethanol extracts of mango kernel. A study conducted on the antioxidant potential of mango fruit pulp of four commercial cultivars (Haden, Tommy Atkins and Uba) grown in Brazil, at the ripening stage showed that all the varieties represent a potential source of natural antioxidants (phenolic compounds, ascorbate and β-carotene). Among them the variety Uba showed a better performance with enhanced antioxidant activity for juice and pulp, the quality that help preservation naturally without the addition of any synthetic antioxidants (Ribeiro *et al.*, 2007).

Several polyphenols such as mangiferin, propyl and methyl gallate, catechins, anthocyanins, kaempferol, rhamnetin, gallic and ellagic acids, quercetin, protocatechuic acid and benzoic acid are reported in *M. indica* with significant antioxidant activities (Masibo and He, 2008).

2.6. ANTIDIABETIC ACTIVITY

The leaves, fruit flesh, fruit peel, seed kernel and bark of *M. indica* have been abundantly studied for their anti-diabetic properties. The remarkable lowering of blood glucose was observed in hyperglycemic rats, with the administration of root and bark extracts of mango (Oliver, 1986). Wadood *et al*. (2000) reported the anti-diabetic activity of alcoholic extract of mango leaves at doses of 50, 100,150 and 200 mg/kg body weight in rabbits. . In a clinical investigation of the alcoholic extract and aqueous extract of *M. indica* leaves at high (1g/kg/d) dose showed a remarkable lowering of the average glucose concentration in plasma after two weeks of administration (Waheed *et. al.*, 2006). There are reports suggesting that the young leaves of *M. indica* were found to be more effectual than the matured leaves in anti-diabetic activity where they used methanolic extracts of the leaves for evaluation (Mohammed and Rizvi, 2017).

Perpetuo and Salgado (2003) studied the effect of consumption of mango fruit pulp flour on blood glucose level in normal and diabetic rats where they found a 66% decrease of glucose level and a 64% increase of hepatic glycogen level in rats consuming mango compared to the control rats.

The anti-diabetic potential of ethanolic extract of mango fruit peel were also reported, in streptozotocin induced diabetic rats in which the treatment showed a significant decrease in the level of fructosamine and glycated haemoglobin, the status indicators of diabetes (Gondi and Rao, 2015).

The streptozotocin induced diabetic rats were employed for the anti-diabetic potential assessment of *M. indica* seed kernel as well, at an extract dose of 200 mg/kg body weight. Long term (21 days) administration of the aqueous as well as methanolic extracts were found to be effective in lowering the blood glucose level and regularizing the other biochemical parameters in diabetic rats. A similar study conducted with a flour

supplement prepared from mango kernel also showed effective reduction in the blood glucose level of diabetic rats (Irondi *et al*., 2016).

2.7. ANTI INFLAMMATORY ACTIVITY

Inflammation refers to any natural reaction by body tissues against infections, toxins or injuries as an aid in the healing process. Anti-inflammatory agents are drugs or supplements that reduce the pain and swelling associated with inflammation by blocking the production of prostaglandin, a key compound involved in the inflammatory response. *M. indica* have been reported as a natural source of several bioactive compounds that act as anti–inflammatory agents. Dhananjaya and Shivalingaiah (2016) reported that the standard aqueous steam bark extract of *M. indica* owns anti-inflammatory potential by the inhibition of inflammatory phospholipase A_2 (PLA_2). The study also suggests that the inhibition is irreversible and is independent of the substrate and calcium concentration.

Ulcerative colitis is a chronic inflammatory bowel disease in which several studies regarding the anti–inflammatory property of mango extracts have been reported. Kim *et al*. (2017) reported the potential of mango polyphenols in reducing inflammation associated with intestinal colitis both *in vitro* and *in vivo*. The reduction in the inflammatory response is through modulation of the mTOR pathway and associated post-translational mechanisms.

An evaluative study conducted on the seed kernel, peel and pulp of three *M. indica* varieties revealed that the seed kernel and peel possess good anti-inflammatory activity, where aspirin was used as the standard. . Human Red Blood Cell membrane, stabilized through heat induced haemolysis was used in the study since it is analogous to lysosomal membrane (Kuganesan *et al.,* 2017).

2.8. PHYTOCHEMISTRY OF *Mangifera indica*

Mangifera indica is one among the most popular, high prized and choicest fruit of tropics, mainly due to its delicious flavour and nutritional value. Being an aromatic plant, the phytochemistry of mango can be divided into volatile and non-volatile chemicals.

2.8.1. Volatile phytochemistry of *Mangifera indica*

Aroma is one of the chief parameters in determining the quality of a product. Variation in aroma of different varieties of *M. indica* indicates the variation in volatile chemical composition among the varieties. The volatile aroma of the plant is basically characterized by low molecular weight compounds (<400 Da) having functional groups. These compounds possess high vapour pressure by which they diffuse faster in air, water and soil (Sharifi and Ryu, 2018). These include monoterpenes, sesquiterpenes and their oxygenates along with esters, lactones, alcohols, aldehydes, ketones and volatile fatty acids which are generally present in minute amounts in the plant (Bender *et al.*, 2000; Pino *et al*., 2005; Lebrun *et al*., 2008; Pandit *et al.*, 2010; Li *et al*., 2017). The volatile aroma composition of a plant is generally studied through GC–MS analysis of the essential oil obtained from the plant tissue. The quantity and quality of these compounds vary according to varieties, plant tissue used and their maturity stage (MacLeod and Pieris, 1984; Adedeji *et al*., 1992; Andrade *et al*., 2000; Lalel *et al.*, 2003a; Lalel *et al*., 2003b; Lalel *et al.*, 2003c). The method of isolation or extraction, analysis and identification can also affect the composition of volatiles.

Flavour compositions of mango have been extensively studied in numerous varieties in different parts of the world. These studies revealed the presence of more than 300 volatiles from different cultivars across the world. Conventional hydro-distillation, solvent-extraction, vacuum steam distillation followed by solvent-extraction, simultaneous distillation-extraction, static headspace, solid-phase extraction and headspace solid-phase micro-extraction are the different types of techniques used in the extraction of volatile compounds from mango. Among them, conventional hydro-distillation is the most common technique used in the essential oil isolation from medicinal herbs and other plants.

Singh *et al.* (2004) identified more than 285 volatiles in mango fruit which included 7 acids, 55 alcohols, 31 aldehydes, 26 ketones, 14 lactones, 74 esters, 69 terpene hydrocarbons and 9 other compounds. Monoterpenes were reported as the most abundant class of volatiles in the mango germplasms from China, America, Thailand, India, Cuba, Indonesia and Philippines (Li *et al*., 2017).

MacLeod and Pieris (1984) analysed the volatile aroma composition of three mango cultivars of Sri Lanka (Willard, Jtina, and Parrot) through GC–EIMS and CIMS. About 76 volatiles were detected from these Sri Lankan varieties, where terpenes were the major volatiles with monoterpene hydrocarbons contributing to the highest percentage followed by sesquiterpene hydrocarbons.

Mangifera indica leaf essential oil, obtained for the evaluation of its repellent effect on the host-seeking female *Anopheles gambiae*, was analyzed through GC and GC–MS. Among the 26 volatiles identified from the oil, α-pinene showed the greater composition followed by α-phellandrene, limonene, p-cymene, heptane, β-pinene, ledene, (-)-α-gurjunene, β-myrcene, γ-terpinene, (+)-2-carene and trans (β)-caryophyllene in the order (Alwala *et al.* 2010).

MacLeod and Snyder (1985) analysed the volatile components of two cultivars of mango from Florida (Tommy Atkins and Keitt) through GC-MS. Monoterpene hydrocarbons were the major group of volatiles in both cultivars and car-3-ene was their most abundant component. The study also identified seven other volatile components, ethyl-cyclohexane, ethanol, α-fenchene, camphene, sabinene, 1,l-diethoxyethane and sabinyl acetate, that were not been reported as mango constituents previously.

A similar analysis of volatile chemical constituents was conducted on the mango fruit pulp of some Kenyan cultivars using GC and GC-MS. About 58 compounds were identified in the analysis, where monoterpenes (66.1%) contributes to the major composition followed by esters (19.7%), sesquiterpenes (4.8%), non-terpenoid hydrocarbons (3.1%) as well as oxygenated volatiles like ethers, alcohols, acids, and carbonyls (6.3%). α-Pinene, myrcene, δ-3-carene, E-caryophyllene, ethyl butanoate were found as the dominant aroma volatiles in these studied varieties (Wetungu *et al.*, 2018).

Several drawbacks in the conventional methods such as long duration of extraction, loss of the volatile components and high energy consumption can be overcomed by new extraction techniques like microwave-assisted extraction (MAE), supercritical fluid extraction (SFE) and ultrasonic extraction which are rapid, sensitive, safe, and energy efficient and thereby shorten the duration of extraction, decrease the use

of organic solvent, improve the efficiency of extraction, enhance the quality of extract, prevent pollution, and reduce the costs of sample preparation.

Wang *et al.* (2010) compared the microwave-assisted and conventional hydro-distillation in the extraction of essential oils from mango flowers. The oil yield in microwave-assisted hydro-distillation was higher (0.16%) in comparison to conventional hydro-distillation (0.11%). The main content in the essential oil was identified by GC-MS analysis as monoterpene hydrocarbons like terpinolene, δ-3-carene, limonene and α-terpinene. Monoterpene hydrocarbons were followed by sesquiterpenes, their oxygenated forms and other hydrocarbons like alkanes and esters.

2.8.2. Non-volatile phytochemistry of *Mangifera indica*

Apart from the aroma volatiles, *M. indica* is a rich source of many non-volatile bio-active compounds, of which the plant have been studied over the decades. Such a study analysed the flavonol O- and xanthone C-glycosides, anthocyanins, and pectin contents of the fruit peels and flesh of several mango varieties through high performance liquid chromatography (HPLC)-diode array detection-electrospray ionization mass spectrometry (ESI-MS) (Berardini *et al.*, 2005). The separation of phenolic compounds by HPLC and its LC MS analysis indicates that mangiferin is the predominant compound in most cultivars. The concentration of the flavanols and xanthones spotted in the varieties ranged from 360 mg/kg to 4860mg/kg. Two flavonol glycosides were isolated through preparative HPLC whose structures were elucidated on the basis of Elecrospray Ionization–Mass Spectrometry and Nuclear Magnetic Resonance spectroscopy. These flavonol glycosides were thus confirmed as rhamnetin 3-O-β-galactopyranoside and rhamnetin 3-O-β-glucopyranoside. Flavonol glycoside profile was used for authenticity control since they are highly characteristic to mango. The mango peels were found to be a good source of pectin, but a very poor source of anthocyanins. Thus mango peel can be treated as a good alternative to apple pomace, the common source of pectin which is facing scarcity.

Hydroxybenzoic and hydroxycinnamic acid derivatives, the two major classes of phenolic acids seen in plants have been reported in mango pulp also. These compounds were found to be free as well as combined, with either quinic acid or glucose (Mattila and Kumpulainen, 2002; Burton-Freeman *et al.*, 2017). While the hydroxyl-benzoic

acids found in mango flesh include gallic acid, vanillic acid, syringic acid, protocatechuic acid, and p-hydroxybenzoic acid, the hydroxycinnamic acid derivatives include p-coumaric acid, chlorogenic acid, ferulic acid, and caffeic acid (Masibo and Qian, 2008; Ediriweera *et al.*, 2017).

2.8. GENETIC DIVERSITY STUDIES

Genomic DNA is the basic experimental material in genetic relationship studies. Hence, isolation of genomic DNA is the primary step in such studies. Quantity and quality of DNA are the two key factors to be taken care of while dealing with the techniques in molecular biology. Plant genomic DNA isolation is more laborious to conduct in comparison with DNA isolation from animal tissues. Lysis of cell wall and purification of DNA from polysaccharides, polyphenols and other organic compounds are the two basic steps that makes plant genomic DNA isolation more tedious. Conventional methods involving phenol/chloroform mixtures are still in use because of its consistency in the production of high-quality DNA (Hillis *et al*., 1996).

The development of molecular markers paved the way for genetic mapping and marker–assisted selection in plant breeding (Peng *et al*., 2007). A molecular marker can be described as a segment of DNA characteristic to the variations at the genome level which may or may not correspond with the phenotypic expression of a character. Molecular markers are widely accepted as an important tool over the conventional phenotype based genetic markers, due to their stability and invariable detection in all tissues regardless of growth, differentiation, development, or defence status of the cell. These markers remain unaffected by the environment, pleiotropic and epistatic effects also (Agarwal *et al*., 2008). Molecular marker techniques can be broadly classified into non-PCR based techniques and PCR based techniques.

The only marker system representing non-PCR based technique or hybridization based technique is Restriction fragment length polymorphism (RFLP). In RFLP, polymorphisms are generated by digesting the genomic DNA into fragments of varying size using restriction enzymes. The fragments differ in size because of point mutation, insertion/deletion, translocation, inversion or duplication. The polymorphism is detected by hybridizing a complementary radio-labelled or chemically labelled probe to a Southern blot of the separated DNA fragments (Amom *et al*., 2017). The limitations of

RFLP technique like time consuming, requirement of high quality and quantity of DNA, expensive radioactive probes, involvement of complex Southern blotting method and prerequisite sequence knowledge for developing labelled probes were cleared by later evolved PCR based markers.

Polymerase Chain Reaction (PCR), the exponential amplification of target DNA using *Taq* DNA polymerase was invented by Kary Mullis in 1983. PCR based markers are more advantageous, since they require very little and not necessarily good quality DNA, along with their high reproducibility, more reliability and higher polymorphism in short time. These markers include RAPD, AFLP, SSR, ISSR, SCoT, SNPs, SCAR and so on.

While the polymorphisms are detected using arbitrary chosen primers (usually 8-10 bp long) in RAPD, AFLP combines the potential of RFLP and flexibility of PCR-based technique by ligating adapters to the digested DNA and thereby selectively amplifying the restricted fragments using a limited set of primers (Agarwal *et al*., 2008). Microsatellites or short tandem repeats (STRs) or simple sequence repeats (SSRs) are monotonous repetitions of very short sequence motifs. These genetic markers are highly popular due to their co-dominant inheritance, high availability, wide extent of allelic diversity, and the ease of determining the repeat size variation through PCR using pairs of flanking primers.

ISSRs are segments of DNA seen at an amplifiable distance between two oppositely oriented identical SSRs. These ISSRs make use of SSRs with di, tri, tetra or penta-nucleotide core sequences as primers for amplification in different sizes.

SNPs are the most abundant molecular markers in the genome since they represents single nucleotide variations of varying frequencies and distributions in the genome sequence of individuals of a population. Advanced genotyping methods like DNA chips, allele-specific PCR and primer extension approaches, which are highly efficient make single nucleotide polymorphisms (SNPs) more appealing.

Sequence Characterized Amplified Regions (SCARs) are PCR-based markers that represent single, genetically defined loci that are amplified by PCR amplification of genomic DNA comprising pairs of specific oligonucleotide primers. They were derived

from eight RAPD markers linked to disease resistance genes in lettuce (Paran and Michelmore, 1993).

Start codon targeted (SCoT) polymorphism involves a novel marker system developed based on the short conserved regions surrounding the translation initiation codon ATG in plant genome. SCoT uses single primer of 18-mer sequence which act as both forward and reverse primers with annealing temperature at 50°C (Collard and Mackill, 2009). SCoT markers are simple, less time consuming and do not require prior sequence knowledge. They generate polymorphism linked to functional genes and their corresponding traits (Xiong *et al*., 2011).

2.8.1. Genetic Diversity of *Mangifera indica*

According to Rao and Hodgkin (2002) genetic diversity represents the heritable variation within and between populations of organisms in a species. Thus, this genetic variation, especially plant genetic diversity is crucial for present and future human well-being. *Mangifera indica* being a commercially important fruit crop in the world, several genetic diversity studies have been reported in different regions of the world with healthy mango germ pools (Sherman *et al*., 2015; Ahmedand *et al*., 2015). Since India represents the biggest mango germ pool in the world, such studies on Indian cultivars were necessary to gather precise information on the genetic relationships within such germplasm diversity (Pandit *et al*., 2007).

Karihaloo *et al*. (2003) studied the genetic diversity of Indian mango cultivars using RAPD markers. A total of 29 mango cultivars representing different regions of the country and some advanced cultivars were included in the study. The PCR amplification using 24 primers generated 314 bands with 91.4% polymorphisms. UPGMA dendrogram of the cultivars clustered northern and eastern regions together and distinct from southern and western regions.

Pandit *et al*. (2007) reported the genetic diversity among 70 mango cultivars analysed using 33 ISSR markers. Among the total 420 bands generated by the primers, 408 were polymorphic. Clustering of the cultivars done using Dice and Jaccard coefficients with bootstrapping showed that non-Indian mango cultivars are genetically diverged from Indian mango germ pool.

The efficiency of SCoT and ISSR markers in analyzing the genetic relationships among mango cultivars were estimated by Luo *et al.* (2011). The selected SCoT and ISSR primers developed a total of 158 bands and 104 bands with 65.82% and 55.77% polymorphisms respectively. The twenty three varieties were assembled into two major groups based on the SCoT analysis and three major groups based on the ISSR analysis. The results of the study indicate that SCoT analysis better represented the actual genetic relationships, thus showing its edge over ISSR markers.

Molecular characterization and genetic diversity studies associated with fruit quality of indigenous mango varieties from western part of India were carried out using SCoT markers. The primers showed 79.57% polymorphism with an average of 5.05 polymorphic fragments per primer. Among the 19 SCoT primers which developed polymorphisms, 17 primers produced 25 and 9 cultivar-specific unique fragments for the identification of 15 indigenous cultivars and 5 popular cultivars respectively. The results also demonstrated that the clustering pattern correlated well with the physical and/or biochemical traits of the fruits suggesting that SCoT marker system is an effective tool in cultivar identification and genetic diversity analysis of mango cultivars based on their biological traits (Gajera *et al*., 2014).

In a recent study, the genetic diversity of 168 mango seedling germplasms were analysed using fourty five SCoT markers. A total of 337 bands with an average of 7.49 bands per primer were generated through amplification, where 244 amplicons (72.4%) were polymorphic, which shows an average of 5.42 amplicons per primer. The dendrogram developed using the UPGMA clustered the 168 germplasms into two major groups. Most of the germplasms were grouped with their potential parents and the genetic diversity (89%) within each population was found to be higher than between the populations (11%). The effectiveness of SCoT marker system in analysing the genetic diversity of mango cultivars were revealed in this study too (Zhou *et al.*, 2019).

In addition to the efficiency in amplification of functional gene and their corresponding traits, high polymorphism and reproducibility of SCoT markers make them a good choice for fingerprinting studies in mango.

3. MATERIALS AND METHODS

The present study entitled "Phytochemical profiling and genetic diversity analysis of *Mangifera indica* Linn. varieties in Kerala" was carried out at the Phytochemistry and Phytopharmacology Division of Jawaharlal Nehru Tropical Botanic Garden and Research Institute, Palode, during the academic year 2019 – 2020. Details regarding the experimental materials used and procedures followed in the study are elaborated in this chapter.

3.1. SAMPLE COLLECTION

Fresh leaves (mature leaves for phytochemical profiling and tender leaves for genetic diversity analysis) of 10 local varieties of *Mangifera indica* were collected from JNTBGRI, Palode and nearby areas (Table 3).

3.2. CHEMICALS AND REAGENTS

Diethyl ether, anhydrous sodium sulphate, liquid nitrogen, CTAB, PVP, mercaptoethanol, proteinase K (Origin, Kerala), EDTA, Tris Base, sodium chloride, phenol, chloroform, isoamyl alcohol, isopropanol, absolute ethanol, sodium acetate, 70% ethanol, RNase A (Origin, Kerala), PCR reaction mix (Takara Emerald), agarose, EtBr

3.3. INSTRUMENTS AND EQUIPMNTS

Weighing balance, Clevenger-type apparatus, heating mantle, Gas Chromatograph-Mass Spectrometer, thermal cycler, electrophoresis unit, gel documentation system, autoclave, centrifuge

3.4. PHYTOCHEMICAL STUDY

3.4.1. Volatile Chemical Profiling

3.4.1.1. Isolation of Essential Oil

Fresh leaves (250 g each) were subjected to hydro–distillation using a Clevenger-type apparatus for 3 to 4 hours. The essential oils obtained were dried over anhydrous sodium sulphate and stored at 4°C in sealed glass bottles till further analysis.

3.4.1.2. GC- MS Analysis of Essential Oil

GC-MS analysis of essential oil was carried out in a Shimadzu Gas Chromatograph Mass Spectrometer (QP2020C NX) fitted with a cross bond 1,4-bis(dimethylsiloxy) phenylene dimethyl polysiloxane Rxi-5 Sil MS capillary column (30 m x 0.32 mm, film thickness 0.25 µm) coupled with Shimadzu single quadrupole 8030 series mass selective detector. 1µL of the essential oil diluted in diethyl ether (1:10 dilution) was injected in split mode (1:50). The injector temperature was 240°C, and the oven temperature was 60-250°C at the rate 3°C/minute. The ion source temp of the mass detector was 240°C, and the interphase temp was 260°C.

3.4.1.3. Identification of the Components

The relative retention indices (RRI) of the essential oil components were calculated using standard series of C_8-C_{30} hydrocarbons (Aldrich Chemical Company, USA) (Dool and Kratz, 1963).

$$RRI=100[(E_x-H_n)/(H_{n+1}-H_n)]+100n$$

where E_x is the retention time of the volatile compound, H_n and H_{n+1} are retention times of reference standard hydrocarbons with n and n+1 carbon items respectively.

The calculated relative retention indices (RRI), MS library (NIIST 17, Wiley 275) and literature reference (Adams, 2007) were used for the identification of constituent compounds.

3.4.1.4. Statistical Analysis

Intra-species relationship among the mango varieties were established through multivariate statistical analysis, *i.e.*, hierarchical cluster analysis (HCA) using the essential oil composition obtained from the present study. SPSS ver. 16.0 software was used for multivariate statistical analysis.

3.5. GENETIC DIVERSITY STUDY

3.5.1. Isolation of Genomic DNA Using CTAB Method

Genomic DNA from leaf samples of 10 local varieties of *M. indica* were isolated by CTAB method.

3.5.1.1. Procedure

- 150 mg of fresh leaf sample was grinded to fine powder using mortar and pestle in the presence of liquid nitrogen.
- The tissue powder was quickly transferred into an autoclaved 1.5 mL microfuge tube containing prewarmed (65°C) CTAB buffer and was mixed well. (2 mL beta mercaptoethanol and a pinch of PVP was priorly added to the CTAB buffer)
- A 4 µL protease K was then added to the tube and mixed again by inversion for 1 minute.
- The mixture was incubated at 65°C for 30 minutes with regular mixing by tube inversion.
- The tube contents were then centrifuged at 13000 rpm for 5 minutes.
- Supernatant was transferred to a clean microfuge tube.
- An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the tube and mixed by inversion.
- The tube contents were then centrifuged at 10000 rpm for 5 minutes and the supernatant was transferred to a clean microfuge tube.
- An equal volume of chloroform:isoamyl alcohol (24:1) was added to the tube, mixed by inverting the tubes repeatedly to form an emulsion and centrifuged at 13000 rpm for 10 minutes.
- Supernatant was carefully transferred to a clean tube and precipitated by adding equal volume of chilled isopropanol.
- Mixed the tube contents by gentle inversion and incubated at ¯80°C for 1 hour.
- Sample was then centrifuged at 13000rpm for 15 minutes and the supernatant was discarded.
- The obtained pellet was washed with 5µL sodium acetate and 100µL absolute ethanol (10000 rpm, 5 minutes)
- The pellet was again washed with 70% ethanol (100 µL), for 2 or 3 times (10000 rpm, 5 minutes).
- Supernatant was decanted and the pellet was air dried at room temperature.
- The air dried pellet was then resuspended in 100 µL nuclease free water and stored at ¯20°C.

- The DNA sample was later treated with RNAase A (OrionX) and incubated at room temperature for 30 minutes before gel electrophoresis.

3.5.2. Agarose Gel Electrophoresis

Agarose gel electrophoresis is a technique used to separate DNA fragments based on their molecular weight. The negatively charged DNA fragments in the gel matrix move towards the positive electrode in the applied electric field. Since all DNA fragments have the same charge per mass ratio, the smaller fragments move faster through the gel compared to the larger ones. It can be used to check the quantity and size of the DNA fragments produced and is an essential part of all experiments carried out in molecular biology.

0.8% agarose gel was used to check the quality and integrity of isolated genomic DNA. 0.8 g of agarose was weighed and dissolved in 100 mL 1X TAE buffer and casted in a gel tray after adding 4 µL EtBr. 3 µL of each DNA sample was mixed with 1 µL of tracking dye and loaded into the wells of the prepared gel. The gel was run at 70 V in a horizontal gel electrophoresis unit until the dye front reached $3/4^{th}$ of the gel. The genomic DNA bands in the gel were visualized under EC3 Chemi HR 410 imaging system (UVP, U.K.).

3.5.3. Quantification of DNA

The quantity and quality of the isolated DNA was checked using a Nanophotometer (Implen, Germany). Nuclease free water (1 µL) in which the DNA dissolved was used to calibrate the machine to blank i.e. zero absorbance and 1 µL of the DNA samples were used to measure the optical density in specified parameters.. The quantity of DNA was determined at OD_{260} and the purity was determined by OD_{260}/OD_{280} ratio.

While A_{260}/A_{280} ratio around 1.8 indicates a good quality DNA, the ratio less than 1.8 indicates protein contamination and the ratio greater than 2.0 indicates RNA contamination.

3.5.4. PCR Amplification

Dilution of stock DNA to be used as template and PCR parameters like annealing temperature were optimized in the first step. Most of the SCoT marker anneals at 50˚C. Ten randomly selected SCoT primers were used in the preliminary screening to check the reliability and reproducibility of the bands.

Table 1. List of SCoT primers used

SCoT	Primer Sequence	% G-C
21	ACGACATGGCGACCCACA	61
22	AACCATGGCTACCACCAC	56
24	CACCATGGCTACCACCAT	56
25	ACCATGGCTACCACCGGG	67
29	CCATGGCTACCACCGGCC	72
32	CCATGGCTACCACCGCAC	67
35	CATGGCTACCACCGGCCC	72
36	GCAACAATGGCTACCACC	56
40	CAATGGCTACCACTACAG	50
45	ACAATGGCTACCACTGAC	50

The PCR amplification of isolated genomic DNA was performed in a Veriti thermal cycler (Applied Biosystems, California) using the SCoT primers and other reagents of following concentrations.

Table 2. PCR reaction mix

Reagents	Volume
Master mix (2X)	7.5 µL
Primer (0.8mM)	1.2 µL
Template DNA (40ng)	3 µL
Water	3.3 µL
Total Volume	15.0 µL

The amplifications were performed with a preliminary cycle of 5 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 50°C, 2 min at 72°C and a final extension of 10 min 72°C. The amplified products were resolved in 1.5 % agarose gels with ethidium bromide, in 1X Tris-borate EDTA buffer and were analyzed using a gel documentation system (UVP, UK). A 100bp DNA ladder (OrionX) was loaded along with the samples to compare the size of resultant bands.

3.5.5. Data Analysis

The band patterns obtained were scored as absent (0) or present (1). Jaccard's similarity coefficient (Guha *et al*., 1999) was calculated to evaluate the genetic similarity among the varieties and the cluster analysis was performed using the UPGMA algorithm. Principal Co–ordinate Analysis (PCA) analysis was carried out as per the software package NTSYS-pc version 2.1 (Rohlf, 2000).

4. RESULTS

The present study entitled "Phytochemical profiling and genetic diversity analysis of *Mangifera indica* Linn. varieties in Kerala" was focused on the analysis of morphological, phytochemical and genetic variations among the selected 10 local varieties of *M. indica* (Kilichundan, Chambavarikka, Kottoorkonam, Pulinji, Chakka manga, Thaali, Moovandan, Neelam, Karpooram, Vellari). The results recorded in the study are as follows:

4.1. MORPHOLOGICL STUDY

General morphological features of mango tree, especially leaves were recorded in the initial phase of the study. The evergreen tree grow up to a height of 30-45 m with dark green mature leaves and pale green young leaves with reddish tinge. Detailed data regarding the morphological features of mango leaves are given below (Table 3, Plate 1)

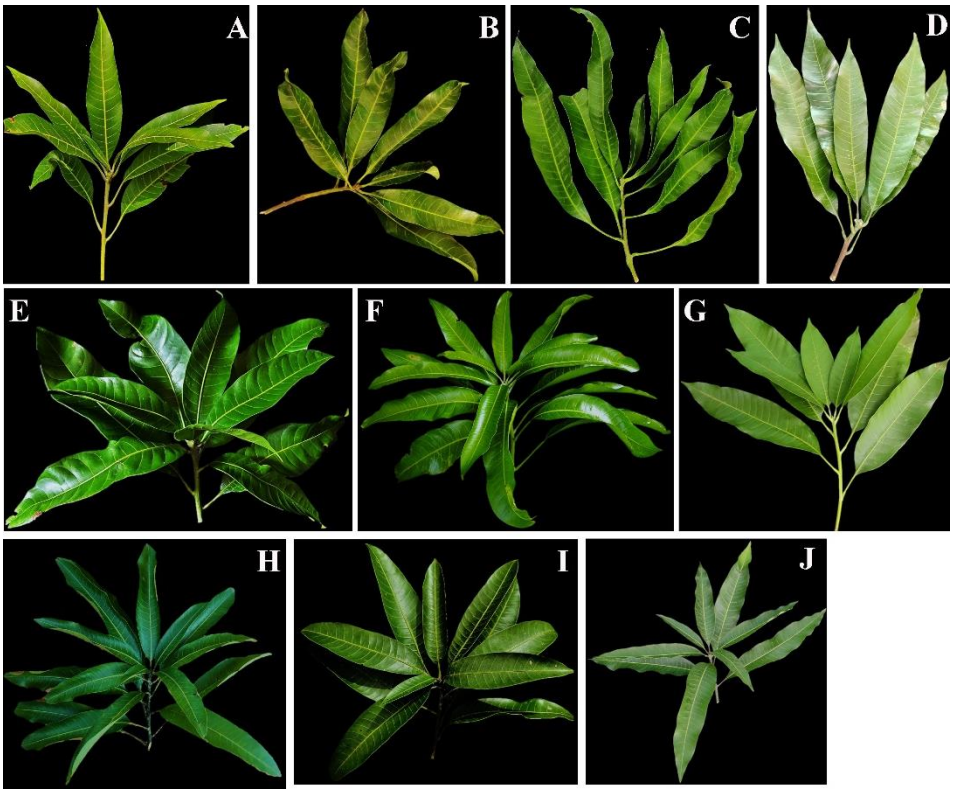

Plate 1. Leaf samples of *Mangifera indica* varieties selected for the study

(A) Kilichundan (B) Chambavarikka (C) Kottoorkonam (D) Pulinji (E) Chakka manga (F) Thaali (G) Moovandan (H) Neelam (I) Karpooram (J) Vellari

Table 3. Morphological features of the leaves of different varieties of *Mangifera indica*

Sl. No	Variety	Length (cm)	Breadth (cm)	L/B ratio
1	Kilichundan	26.0	6.0	4.3
2	Chambavarikka	22.0	5.5	4.0
3	Kottoorkonam	25.0	5.8	4.3
4	Pulinji	32.5	8.0	4.1
5	Chakka manga	40.0	11.0	3.6
6	Thaali	20.5	5.5	3.7
7	Moovandan	23.0	6.5	3.5
8	Neelam	19.0	5.4	3.5
9	Karpooram	20.0	5.0	4.0
10	Vellari	23.5	5.3	4.4

4.2. PHYTOCHEMICAL ANALYSIS

Mangifera indica leaf oil was pale yellow in colour and was lighter than water.

Table 4. Leaf oil yields of selected varieties of *Mangifera indica*

Sl. No.	Sample name	Sample code	Oil yield (v/w) (%)
1	Kilichundan	MI KLC	0.07
2	Chambavarikka	MI CVA	0.03
3	Kottoorkonam	MI KTK	0.03
4	Pulinji	MI PLJ	0.04
5	Chakka manga	MI CKM	0.30
6	Thaali	MI THL	0.04
7	Moovandan	MI MVN	0.03
8	Neelam	MI NLM	0.03
9	Karpooram	MI KRP	0.03
10	Vellari	MI VLR	0.03

4.2.1. Analysis of Essential Oil

55 volatile constituents were identified through GC-MS analysis of the leaf essential oil of different varieties of *M. indica* (Figure 1, Table 5). Twelve major compounds were identified from all the varieties (Figure 2, Figure 3). The percentage contributions of each volatile compounds in all the varieties were used to develop the similarity indices (Table 6), which set the base for dendrogram construction (Figure 4).

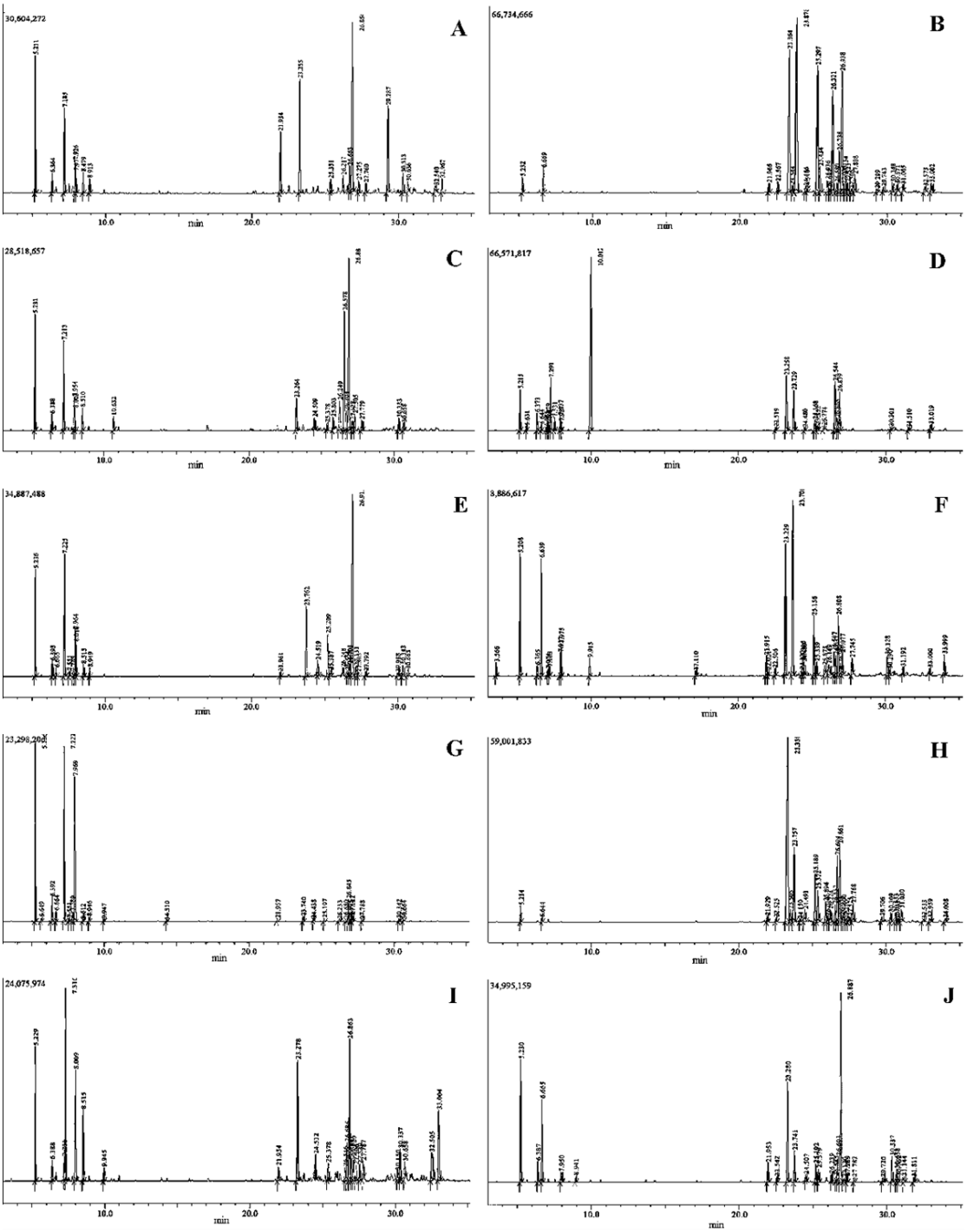

Figure 1. Gas chromatographs of the leaf essential oils of the *Mangifera. indica* varieties: **(**A) Kilichundan (B) Chambavarikka (C) Kottoorkonam (D) Pulinji (E) Chakka manga (F) Thaali (G) Moovandan (H) Neelam (I) Karpooram (J) Vellari

Table 5. Chemical composition of the leaf essential oils of *Mangifera indica* varieties

Sl. No.	RRI $_{lit}$	RRI $_{cal}$	Compound	MI KLC (%)	MI CVA (%)	MI KTK (%)	MI PLJ (%)	MI CKM (%)	MI THL (%)	MI MVN (%)	MI NLM (%)	MI KRP (%)	MI VLR (%)
1.	932	932	α-Pinene	11.0	0.8	9.7	4.3	10.3	8.3	23.4	1.3	8.1	10.8
2.	946	948	Camphene				0.4			0.5			
3.	974	977	β-Pinene	1.7		1.6	1.9	1.5	0.7	3.4		1.3	2.1
4.	988	986	Myrcene		1.3		0.6	0.8	8.5	1.4	0.3		8.4
5.	1001	1000	δ-2-Carene				0.4						
6.	1001	1003	3E-Hexenyl acetate				1.3		0.5				
7.	1002	1006	α-Phellandrene	7.6		8.9	0.6	12.5	0.6	25.9		1.5	
8.	1008	1006	δ-3-Carene				6.1					12.3	
9.	1014	1016	α-Terpinene				1.5	0.5		0.5			
10.	1022	1023	o-Cymene					0.5		1.4			
11.	1024	1027	Limonene	3.2		3.8	2.1	5.5	1.7	28.3			1.3
12.	1025	1028	β-Phellandrene	1.5		1.6	0.5	2.8	2.4			8.9	
13.	1044	1043	E-β-Ocimene	1.9		2.3		1.3		0.2		4.9	
14.	1054	1055	γ-Terpinene	1.3				0.9		0.7			0.7
15.	1086	1083	Terpinolene				39.8		1.5	0.3		1.0	
16.	1100	1103	n-Nonanal			1.6							
17.	1186	1193	α-Terpineol							0.2			
18.	1260	1259	2E-Decenal						0.5				
19.	1374	1372	α-Copaene	7.3	0.7			0.5	2.0	0.5	0.7	1.2	2.6
20.	1383	1374	E-β-Damascenone						0.5				
21.	1389	1386	β-Elemene		0.8		0.5		0.7		0.4		0.6
22.	1409	1405	α-Gurjunene	13.7	16.4	4.3	8.9		13.0		36.2	10.0	13.6
23.	1417	1415	E- Caryophyllene		22.0		6.8	10.2	17.9	0.9	9.4		4.3
24.	1430	1424	β-Copaene								0.3		
25.	1432	1429	α-trans-Bergamotene						0.4				
26.	1437	1431	α- Guaiene		0.5				1.5	0.3			
27.	1439	1433	Aromadendrene		0.2	1.2	0.4	2.2	1.2		1.7	1.4	0.8
28.	1452	1450	α-Humulene		14.6		1.7	6.2	6.1	0.6	6.1		2.5
29.	1458	1454	allo-Aromadendrene	1.5	2.0	0.9	0.8	0.8	1.5		3.9	1.5	1.9

30.	1471	1464	4,5 di-epi-Aristolochene			1.6	0.6						
31.	1475	1467	γ-Gurjunene		1.1				0.9		2.6		0.9
32.	1478	1470	γ-Muurolene		0.4				1.8		0.7		
33.	1484	1475	Germacrene D	1.8	10.1	4.2		0.8		0.6	1.8		
34.	1489	1482	β-Selinene		0.8	17.3	8.5	0.7	2.5		2.2	1.2	0.7
35.	1496	1486	Viridiflorene	3.1	4.5	3.5	2.2	1.9	2.0	0.4	9.4	3.7	4.1
36.	1498	1489	α-Selinene						6.5				
37.	1500	1491	Bicyclogermacrene	21.9	12.6	26.1	6.4	32.1		4.5	10.2	12.4	30.9
38.	1500	1493	α-Muurolene		0.5				1.2		0.7	1.9	
39.	1505	1502	E,E-α-Farnesene	1.5		2.3		0.5				1.3	
40.	1508	1501	Germacrene A		0.8						0.3		0.1
41.	1509	1496	α-Bulnesene		1.5	1.7		1.6	2.2	1.2	1.1	2.2	0.8
42.	1513	1507	γ-Cadinene		0.3						0.6	1.5	
43.	1522	1513	δ-Cadinene	1.2	1.4	1.6		0.7	1.6	0.3	2.2	1.5	0.7
44.	1559	1563	Germacrene B	11.0	0.3								0.8
45.	1567	1564	Palustrol		0.5						0.7		
46.	1577	1570	Spathulenol					0.5					
47.	1582	1575	Caryophyllene oxide						2.3			1.1	
48.	1590	1579	Globulol	2.4	0.8	1.7	0.6	1.6	0.6	0.5	1.1	2.5	3.7
49.	1592	1587	Viridiflorol	1.1	0.6	1.1		1.0		0.3	0.3	1.3	1.9
50.	1595	1590	Cubeban-11-ol										0.8
51.	1600	1600	Rosifoliol										0.8
52.	1602	1596	Ledol		0.5				0.7		1.6		
53.	1638	1636	epi-α-Cadinol	1.4	0.6						0.5	5.0	
54.	1651	1648	Pogostol				0.9		0.4				
55.	1652	1649	α-Cadinol	1.9	1.0						0.6	6.5	
Total percentage				98.0	97.6	97.0	97.8	97.9	92.2	96.3	96.9	94.2	95.8
Monoterpene hydrocarbon				28.2	2.1	27.9	58.2	36.6	23.7	86	1.6	38.0	23.3
Monoterpene oxygenated										0.2			
Total monoterpene				28.2	2.1	27.9	58.2	36.6	23.7	86.2	1.6	38.0	23.3
Sesquiterpene hydrocarbon				63.0	91.5	64.7	36.8	58.2	63.5	9.3	90.5	39.8	65.3
Sesquiterpene oxygenated				6.8	4.0	2.8	1.5	3.1	4.0	0.8	4.8	16.4	7.2
Total sesquiterpene				69.8	95.5	67.5	38.3	61.3	67.5	10.1	95.3	56.2	72.5
Aliphatic compounds						1.6	1.3		1.0				

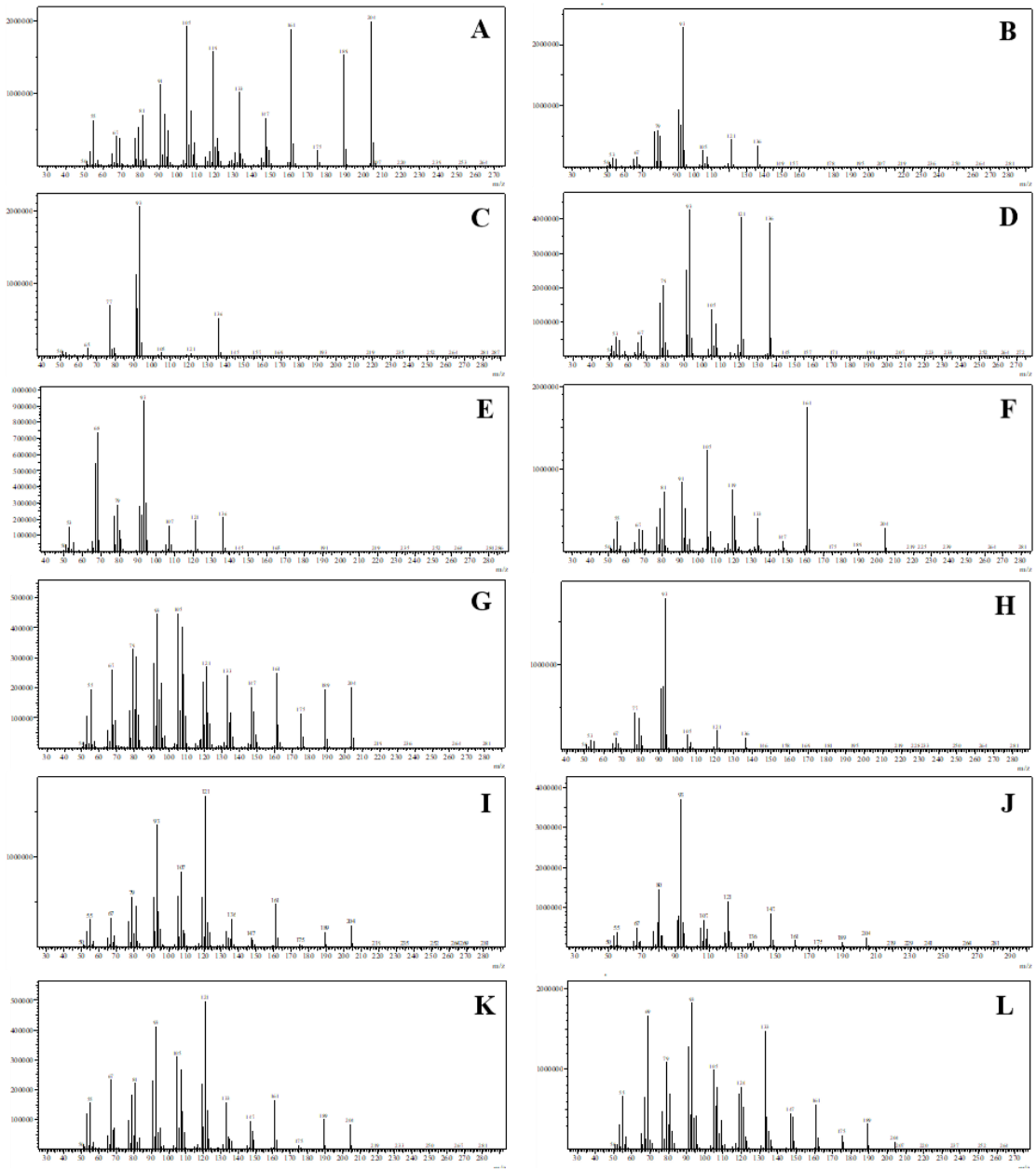

Figure 2. Mass spectra of major compounds identified in the leaf essential oils of *Mangifera indica* varieties: (A) Bicyclogermacrene (B) α-Gurjunene (C) α-Pinene (D)α-Phellandrene (E) E-Caryophyllene (F) Terpinolene (G) δ-3-Carene (H) Limonene (I) α-Humulene (J) Germacrene D (K) β-Selinene (L) Germacrene B

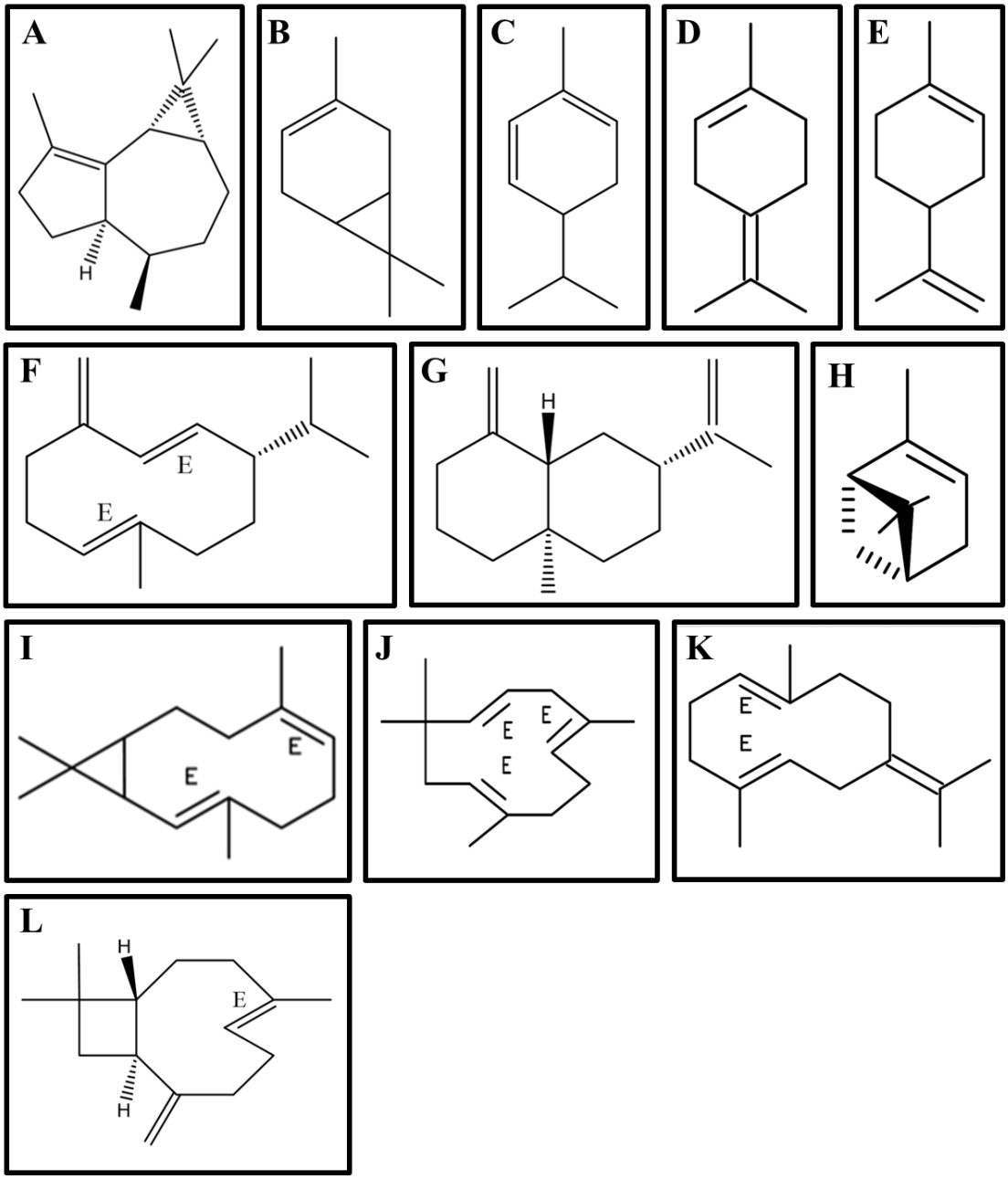

Figure 3. Structures of major volatile compounds identified in the leaf essential oils of *Mangifera indica* varieties: (A)α-Gurjunene (B)δ-3-Carene (C)α-Phellandrene (D)Terpinolene (E)Limonene (F)Germacrene D (G)β-Selinene (H)α-Pinene (I)Bicyclogermacrene (J)α-Humulene (K)Germacrene B (L)E-Caryophyllene

Table 6. Proximity matrix

Proximity Matrix

Case	Squared Euclidean Distance									
	1:1	2:10	3:2	4:3	5:4	6:5	7:6	8:7	9:8	10:9
1:1	.000	499.750	1.204E3	621.340	1.253E3	2.450E3	1.081E3	804.780	389.260	2.548E3
2:10	499.750	.000	2.157E3	517.970	1.553E3	2.665E3	1.255E3	1.074E3	653.170	1.776E3
3:2	1.204E3	2.157E3	.000	1.873E3	967.970	2.555E3	736.490	1.241E3	1.180E3	3.543E3
4:3	621.340	517.970	1.873E3	.000	1.585E3	2.307E3	1.538E3	864.480	607.780	1.917E3
5:4	1.253E3	1.553E3	967.970	1.585E3	.000	1.897E3	548.820	948.820	1.214E3	2.227E3
6:5	2.450E3	2.665E3	2.555E3	2.307E3	1.897E3	.000	2.318E3	1.898E3	2.306E3	3.496E3
7:6	1.081E3	1.255E3	736.490	1.538E3	548.820	2.318E3	.000	1.221E3	1.217E3	3.091E3
8:7	804.780	1.074E3	1.241E3	864.480	948.820	1.898E3	1.221E3	.000	589.120	2.157E3
9:8	389.260	653.170	1.180E3	607.780	1.214E3	2.306E3	1.217E3	589.120	.000	1.816E3
10:9	2.548E3	1.776E3	3.543E3	1.917E3	2.227E3	3.496E3	3.091E3	2.157E3	1.816E3	.000

This is a dissimilarity matrix

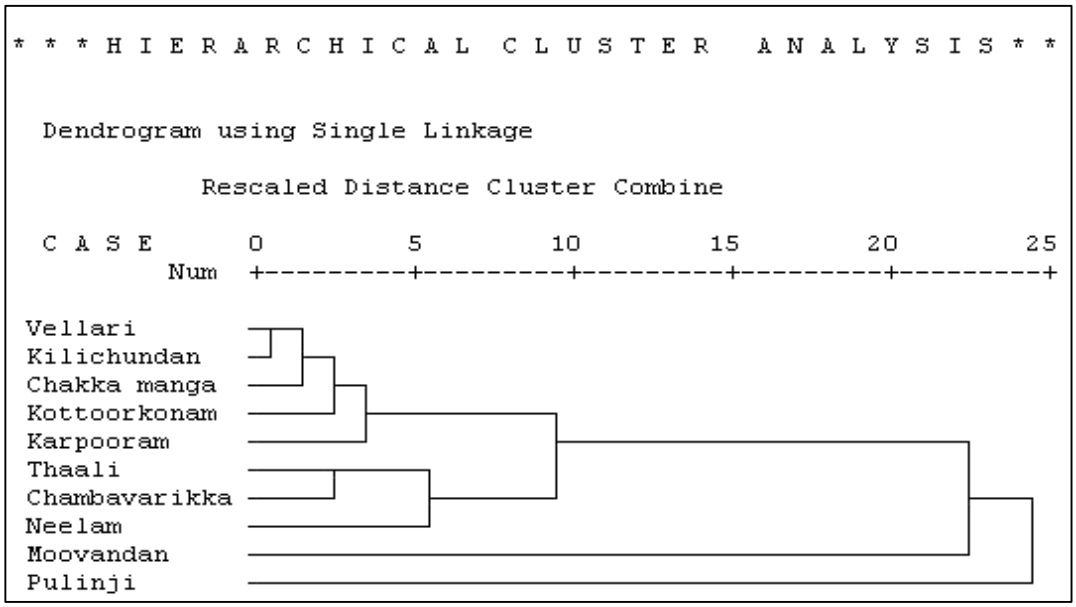

Figure 4. Dendrogram based on the leaf essential oil compositions of *Mangifera indica* varieties

4.3. GENETIC DIVERSITY ANALYSIS

4.3.1. Genomic DNA Isolation

Genomic DNA of the ten selected varieties of *M. indica* isolated from the leaves through CTAB method were used for the genetic diversity analysis. The isolated DNA quantified using Nanophotometer showed a maximum yield of 1209 ng/µL (Pulinji) and a minimum yield of 186 ng/µL (Kilichundan) (Table 7). The integrity of the genomic DNA was checked in 0.8 % agarose gel (Figure 5).

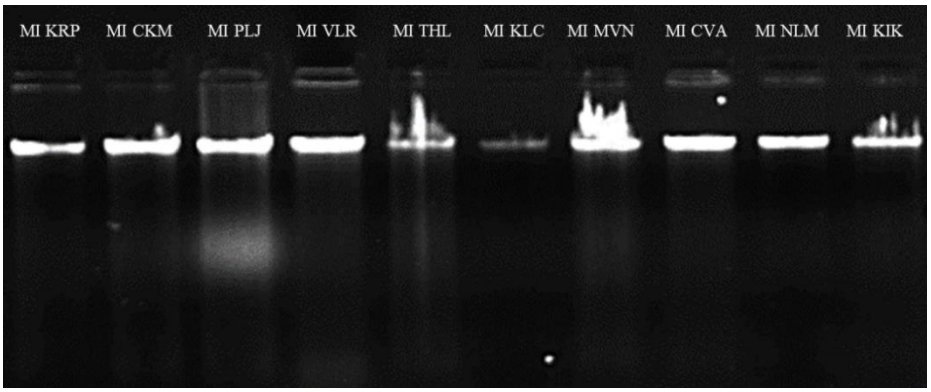

Figure 5. Genomic DNA isolated from the ten *Mangifera indica* varieties resolved under 0.8% agarose gel

Table 7. Quantity and Quality of the genomic DNA isolated from the *Mangifera indica* varieties

Sl. No.	Variety Code	Variety	Quantity (ng/µL)	Quality (A_{260}/A_{280})
1	MI KRP	Karpooram	583	2.0
2	MI CKM	Chakka manga	566	2.0
3	MI PLJ	Pulinji	1209	4.1
4	MI VLR	Vellari	217	1.7
5	MI THL	Thaali	295	1.9
6	MI KLC	Kilichundan	186	1.9
7	MI MVN	Moovandan	277	1.9
8	MI CVA	Chambavarikka	381	1.9
9	MI NLM	Neelam	465	1.9
10	MI KTK	Kottoorkonam	204	1.7

4.3.2. SCoT PCR Analysis

Among the 10 SCoT primers screened, the five primers (SCoT 21, SCoT 22, SCoT 32, SCoT 35, SCoT 45) that showed good and reliable bands were selected for the genetic diversity study of the ten *M. indica* varieties.

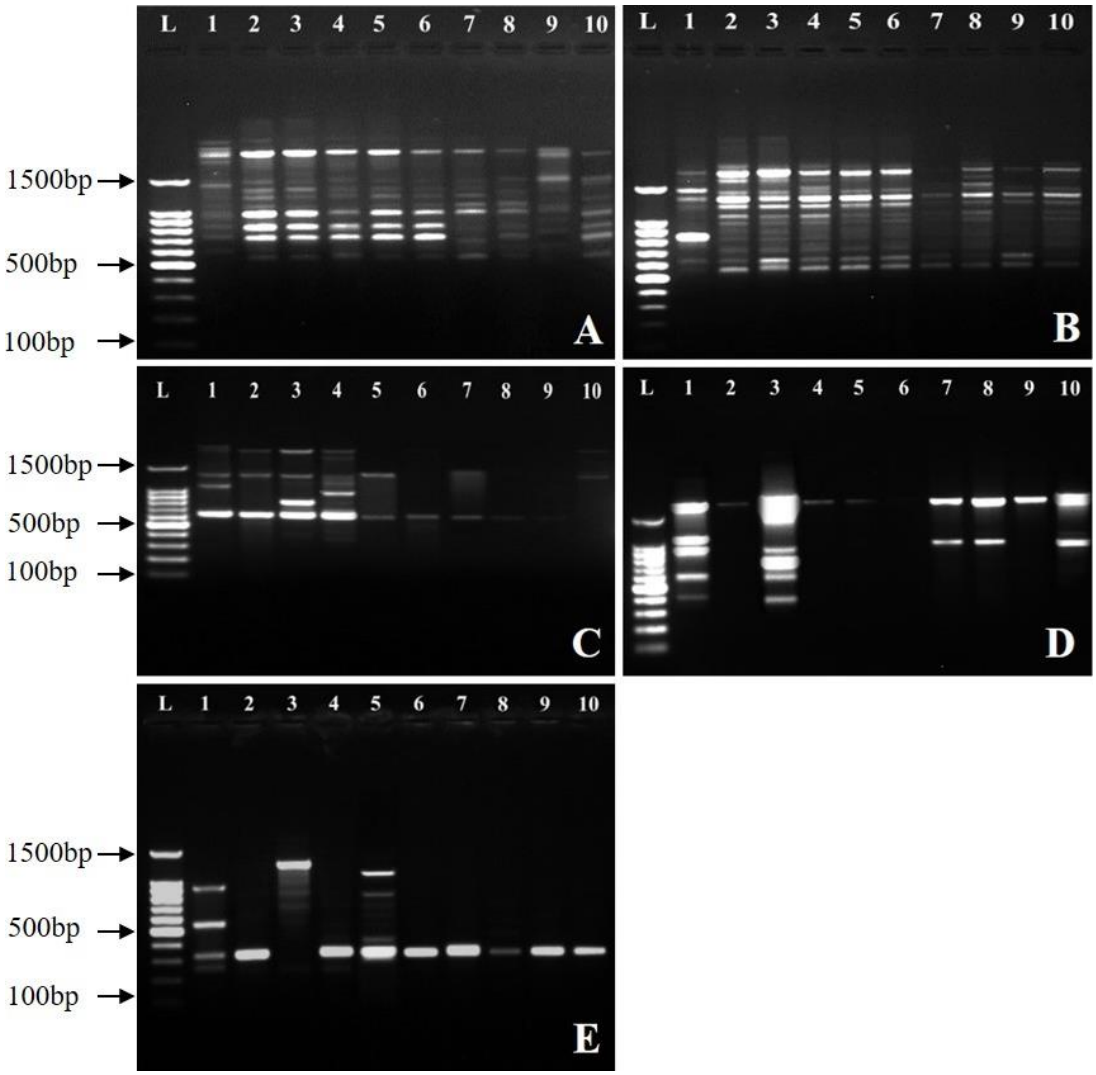

Plate 2. PCR amplified products of ten DNA templates (ten varieties) generated by SCoT primers**;** A-SCoT 21, B-SCoT 22, C-SCoT 32, D-SCoT 35, E-SCoT 45; Lane 1-MI KRP, Lane 2-MI CKM, Lane 3-MI PLJ, Lane 4-MI THL, Lane 5-MI MVN, Lane 6-MI CVA, Lane 7-MI VLR, Lane 8-MI KLC, Lane 9-MI NLM, Lane 10-MI KTR

4.3.3. Genetic Diversity Analysis Using SCoT Marker Polymorphisms

The five SCoT primers selected for the study generated a total of 69 bands for the ten varieties (Plate 2). Among them, fifty five were polymorphic which indicates an average of 82% polymorphism. While the number of polymorphic bands ranged from 8 (SCoT 22) to 14 (SCoT 32) with of 11 bands per primer on an average, the percentage polymorphism range from 47% to 100% across all the varieties (Table 8).

Table 8. Scorable polymorphic bands of each primer

Primer ID	Total Number of Bands (TNB)	Number of Polymorphic Bands (NPB)	Number of Monomorphic Bands (NMB)	Polymorphic Ratio (%)
SCoT 21	15	11	4	73
SCoT 22	17	8	9	47
SCoT 32	14	14	0	100
SCoT 35	10	9	1	90
SCoT 45	13	13	0	100

The Jaccard's similarity coefficients generated, ranged from a minimum of 0.11 to a maximum of 1.00. The highest genetic similarity 1.00 was found between the varieties Kilichundan, Moovandan, Neelam and Kottoorkonam (Table 9).

Table 9. Jaccard's similarity coefficient values

	MI KRP	MI CKM	MI PLJ	MI VLR	MI THL	MI KLC	MI MVN	MI CVA	MI NLM	MI KTK
MI KRP	1.00									
MI CKM	0.33	1.00								
MI PLJ	0.20	0.33	1.00							
MI VLR	0.43	0.43	0.56	1.00						
MI THL	0.18	0.18	0.42	0.50	1.00					
MI KLC	0.25	0.25	0.00	0.17	0.11	1.00				
MI MVN	0.25	0.25	0.00	0.17	0.11	1.00	1.00			
MI CVA	0.40	0.17	0.22	0.50	0.33	0.33	0.33	1.00		
MI NLM	0.25	0.25	0.00	0.17	0.11	1.00	1.00	0.33	1.00	
MI KTK	0.25	0.25	0.00	0.17	0.11	1.00	1.00	0.33	1.00	1.00

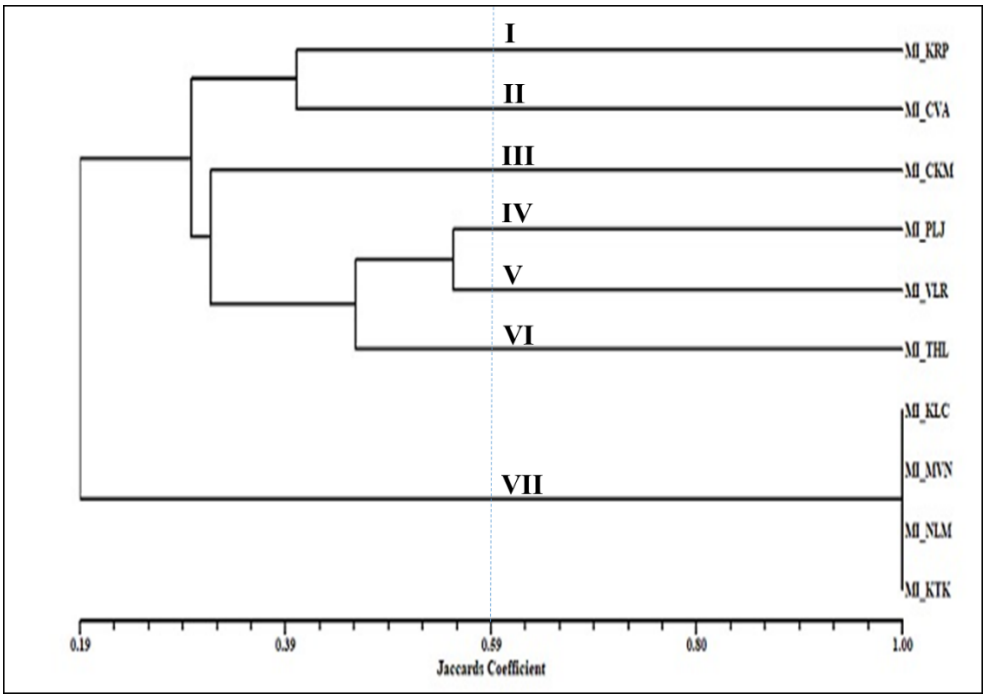

Figure 6. Dendrogram obtained using the banding patterns of the SCoT PCR amplified products of *Mangifera indica* genomic DNA

Table 10. Summary of genetic variation statistics

Locus	**na**	**ne**	**h**	**I**
ST21	2.0000	1.9431	0.4853	0.6784
ST22	2.0000	1.9284	0.4814	0.6745
ST32	2.0000	1.3621	0.2658	0.4361
ST35	2.0000	1.5660	0.3614	0.5473
ST45	2.0000	1.3950	0.2831	0.4570
Mean **St. Dev**	2.0000 0.0000	1.5659 0.1600	0.3552 0.0690	0.5382 0.0785

na = Observed number of alleles

ne = Effective number of alleles [Kimura and Crow (1964)]

h = Nei's (1973) gene diversity

I = Shannon's Information index [Lewontin (1972)]

The number of alleles per locus (na) were observed as 2.0000 and the effective number of alleles (ne) ranged from 1.3621 to 1.9431. While Shannon's information index (I) ranged from 0.4361 to 0.6784, Nei's gene diversity (h) ranged from 0.2658 to 0.4853.

5. DISCUSSION

5.1. MORPHOLOGICAL DIVERSITY

Extensive studies carried out on the morphological features of different varieties of *M. indica* across Kerala have been reported (Anila and Radha, 2006; Simi *et al.*, 2013). Primary morphological analysis of the leaves of selected *M. indica* varieties in the present study showed that their basic morphology varies considerably depending on the variety (Table 2, Plate 1). Though the shape of leaf lamina is lanceolate in all varieties, the leaf margins vary among them (entire and wavy). The length of the leaves range from an average of 19 cm (Neelam) to 40 cm (Chakka manga) and the breadth vary from an average of 5 cm (Vellari) to 11 cm (Chakka manga). While the young leaves are yellowish green with a brownish tinge, the mature leaves are dark green in colour. The data collected in the present study is comparable to the information gathered from the Kerala Agricultural University Mango Database and other related literatures (Naik, 1963).

5.2. PHYTOCHEMICAL DIVERSITY

Hydro-distillation of leaves of *M. indica* (10 local varieties) yielded 0.03% to 0.3% (v/w) essential oil. The average leaf oil yield of the varieties exempting Chakka manga leaf oil (0.3%) was 0.04%. Dzamic *et al.* (2010) reported that the leaf oil yield from Nigerian *M. indica* varieties were found to be 0.02% fresh weight which is comparable to the findings in present study. The high oil content of Chakka manga variety may be corroborated to the large size of their leaves.

5.2.1. Analysis of Leaf Essential Oil

A total of fifty five volatile compounds ranging from 92.2 % to 98.0 % were identified from all the ten varieties. Among them, sesquiterpenes were the predominant group in majority of the varieties, except Pulinji and Moovandan, where monoterpenes dominated. As per the reports from previous studies the predominance of both sesquiterpenes and monoterpenes could be seen in *M. indica* leaf oil as the region and variety varies. Dzamic *et al*. (2010) reported the presence of 70.3% sesquiterpenes in the leaf essential oil of mango varieties from Nigeria, where δ-3-carene (20.5%), α-gurjunene (19.2%), β-selinene (13.9%) and E-caryophyllene (13.7%) were the major

compounds. According to a similar study from Kenya, monoterpenes were the dominant class of compounds in *M. Indica* leaf essential oil where α-pinene (33.3 %), α-phellandrene (22.6 %) and limonene (13.2 %) were noted to be the compounds contributing to mango volatiles and aroma chemistry (Alwala *et al*., 2010).

Terpenes are the plant derived compounds behind the distinct aromas and flavours of various essential oils. Along with this, most of them possess specific health benefits also (Scott, 2005). Besides these terpene compounds, 3 varieties (Kottoorkonam, Thaali, Pulinji) showed the presence of aliphatic compounds also, though were not in considerable range (1.0% - 1.6%).

Bicyclogermacrene, α-gurjunene, α-pinene, α-phellandrene, E-caryophyllene, terpinolene, δ-3-carene, limonene, α-humulene, germacrene D, β-selinene and germacrene B were the major volatile compounds identified from the leaf essential oil of the selected 10 mango varieties (Figure 3).

The mango variety Kilichundan possess sesquiterpenes (69.8%) as its major volatile constituents where sesquiterpene hydrocarbon (63.0%) belongs to the greater part comparing to sesquiterpene oxygenated (6.8%). Bicyclogermacrene (21.9%), a sesquiterpene hydrocarbon is the major compound identified in Vellari variety followed by α-gurjunene (13.7%) α-pinene (11.0%) and germacrene B (11.0%).

Sesquiterpene hydrocarbons (90.5%) were the major volatile compounds in Neelam variety in which α-gurjunene (36.2%) contribute to the greater composition, followed by bicyclogermacrene (10.2%). Monoterpenes in this variety were of minimal quantity (1.6%).

Bicyclogermacrene (26.1%) followed by β-selinene (17.3%) were the prime volatiles in Kottoorkonam variety. These two volatiles belong to the sesquiterpene hydrocarbon group (64.7%) which constitute the major portion of volatile compounds in this variety. n-Nonanal (1.6%) is an aliphatic compound identified in this variety.

Among the 31 volatile compounds identified in the Thaali variety, E-caryophyllene (17.9%) and α-gurjunene (13.0%) were the major constituents. They belong to the sesquiterpene hydrocarbon (63.5%) class of compounds. 3E-Hexenyl

acetate (0.5%) and 2E-decenal (0.5%) were two aliphatic compounds identified in this variety.

While monoterpene hydrocarbons (58.2%) were the major class of compounds in Pulinji variety, sesquiterpene hydrocarbons and sesquiterpene oxygenated compounds are of 36.8% and 1.5% respectively. Terpinolene (39.8%), the prime compound of Pulinji variety showed a unique higher percentage in composition among all the varieties. It is observable that those varieties which did not yield terpinolene as their major volatile component had only traces or none of the compound. A study from Sri Lanka in which the volatile components of three mango cultivars were compared, is comparable with this observation (MacLeod and Pieris, 1984). An aliphatic compound identified in this variety is 3E-hexenyl acetate (1.3%).

The composition of monoterpenes (2.1%) were very low in Chambavarikka. E-Caryophyllene (22.0%), α-gurjunene (16.4%), α-humulene (14.6%), bicyclogermacrene (12.6%) and germacrene D (10.1%) were the major volatiles in this variety. E-Caryophyllene is the first known "dietary cannabinoid", a common constituent of food that has GRAS (Generally Recognized as Safe) status (Gertsch *et al*., 2008). This FDA approved food ingredient possess anti-inflammatory, anticancer, antibacterial, antimicrobial and pain relieving properties (Huang *et al*., 2012; Rufino *et al*., 2015; Fidyt *et al*., 2016).

Bicyclogermacrene (12.4%) is the prime volatile compound in the variety, Karpooram also. Other major compounds of this variety include δ-3-carene (12.3%) and α-gurjunene (10.0%).

Monoterpene class of compounds (86.2%) constitute the major composition of volatiles in Moovandan variety. Among them, limonene (28.3%) followed by α-pinene (23.4%) and α-phellandrene (25.9%) were the major ones with significant composition.

The sesquiterpene, bicyclogermacrene (32.1%) is the major volatile compound in the variety Chakka manga, where sesquiterpene class of compounds dominate the monoterpenes in total volatile composition.

Major class of volatiles in the leaves of the mango variety Vellari were sesquiterpenes (75.5%) where bicyclogermacrene (30.9%) dominate the most. α-Gurjunene (13.6%) and α-pinene (10.8%) were the other two dominant volatiles.

While α-pinene, viridiflorene and globulol were the compounds common to all varieties, the sesquiterpene, bicyclogermacrene was a major compound in seven varieties which was absent in the variety Thaali only. The variety Pulinji was distinct from other varieties with the predominance of terpinolene with a percentage of 39.8 which was not shown by any other varieties. The prevalence of monoterpenes in the variety Moovandan with a composition of 86.2% makes them unique in the odour. Though previous reports have shown δ-3-carene as a prominent compound contributing to the characteristic aroma of mango leaves (Nigam *et al*., 1962; Craveiro *et al*., 1980), the present study witnessed it as a major compound in the leaf oil of one variety (Karpooram) only. Another remarkable finding in the present analysis was that the compound camphor was not detected in the leaves of the variety Karpooram whose name denotes the camphoraceous odour of its fruits.

5.2.2. Statistical correlation of the varieties

The correlation established through the analysis of the distribution pattern of volatile chemicals in the previous section has been confirmed through statistical analysis. The varieties Vellari and Kilichundan, Thaali and Chambavarikka emerged as the very nearest neighbours among the ten selected *M. indica* varieties. This closeness in dendrogram was supported by the chromatogram profiles. While Chakka manga, Kottoorkonam and Karpooram were the other varieties sharing close similarities with Vellari and Kilichundan, Neelam was the next nearest neighbour to Thaali and Chambavarikka. Moovandan and Pulinji were the two varieties which were placed distinctly from other varieties and one another that could be traced to the predominance of monoterpenes and terpinolene in them respectively. Grouping of local mango varieties from Kerala on the basis of volatile phytochemical composition is not reported yet, which ruled out the chance of comparison of phytochemical relationship obtained among the varieties in present study.

5.3. GENETIC DIVERSITY

The present study used SCoT primers, an effective marker for comparing genetic diversity among 10 accessions of *M. indica*. Start codon targeted (SCoT) marker refers to the short-conserved region in plant genes flanking the translation initiation codon or start codon ATG (Collard and Mackill, 2009). SCoT markers are simple and less time-consuming and do not require prior sequence information, and generate polymorphism linked to functional genes and their corresponding traits (Xiong *et al*., 2011)

The present study using 5 selected SCoT primers generated 69 bands among the 10 accessions, of which 55 (82%) were polymorphic with an average of 11 bands per primer. Genetic relationships performed by using the Jaccard's similarity coefficients and dendrogram obtained using the banding patterns showed that the varieties Kilichundan, Moovandan, Neelam and Kottoorkonam possess the highest genetic similarity among the ten studied varieties, while the four appeared to be highly distinct from the variety Thaali with a similarity coefficient of 0.11 (Table 9, Figure 6). The UPGMA clustering algorithm based on similarity matrix grouped the varieties into 7 clusters at similarity index of 0.56. While the clusters III (Chakka maanga) VI (Thaali) includes only single variety, the rest were comprised of more than one variety. The cluster VII was the most distinct one among all which includes the most genetically similar varieties namely Kilichundan, Moovandan, Neelam and Kottoorkonam.

The percentage of polymorphism obtained in the present study is comparable to the previously reported studies. Genetic variation and relationships among 47 mango germplasm and 3 relative species from Guangxi province in China, were analyzed using SCoT markers (Luo *et al*., 2010). Using 33 selected SCoT primers, 273 bands were generated with an average of 8.27 bands per primer among the 50 accessions, of which 208 (76.19%) were polymorphic. Genetic relationships were estimated using the SM similarity coefficient generated values between different pairs of accessions, and the values varied from 0.531 to 0.923.

Luo *et al*. (2011) also compared the efficiency of ISSR and SCoT markers in analyzing the genetic diversity of mango germplasms (23 accessions) from Guangxi province of China. The study suggested that SCoT analysis (65.82 % polymorphism)

better represents the relationship in mango accessions than ISSR analysis (55.77% polymorphism).

Gajera *et al*. (2014), in a similar study analysed the variability on genetic constitution, in relation to fruit quality of local mango cultivars from western part of India using SCoT markers. Out of the total 117 loci, 96 comprising 79.57% were polymorphic, with an average of 5.05 polymorphic fragments per primer.

The morphological, phytochemical and genetic diversity analysis of *M. indica* varieties revealed that the varieties differ from each other in all means. The comparison of these diversities showed that the morphological and phytochemical diversity cannot be directly linked to the genetic diversity in the genomic DNA level. Different varieties could be affected by environmental conditions or may have variations in the expression levels that would contribute to the presence and absence of various phyto-constituents differently, rather than the genomic DNA contribution alone. This variations in the mRNA or protein levels could be responsible for the diversity in the volatile phytochemical composition among the studied varieties also.

6. SUMMARY

The study entitled **"**Phytochemical profiling and genetic diversity analysis of *Mangifera indica* Linn. varieties in Kerala**"** was carried out at Phytochemistry and Phytopharmacology Division of JNTBGRI, Palode, during 2019 – 2020 academic year. The study focused on the phytochemical and genetic diversity analysis of selected mango varieties from Kerala through volatile chemical profiling and SCoT marker polymorphisms.

Mature and tender leaves of ten local varieties (Kilichundan, Chambavarikka, Kottoorkonam, Pulinji, Chakka manga, Thaali, Moovandan, Neelam, Karpooram, Vellari) of *M. indica* were collected from JNTBGRI and nearby localities. Preliminary morphological analysis of the leaves of selected varieties revealed that they differ in terms of size and leaf margins comparatively. Volatile chemical profiling of the varieties were performed with the essential oils obtained from the leaves through hydrodistillation using a Clevenger-type apparatus. GC-MS analysis of the oils revealed that monoterpenes and sesquiterpenes occupy the major composition of leaf volatiles in mango. Besides these terpene compounds, 3 varieties (Kottoorkonam, Thaali, Pulinji) showed the presence of aliphatic compounds also. Among the 55 volatile compounds identified from the ten varieties, bicyclogermacrene, α-gurjunene, α-pinene, α-phellandrene, E-caryophyllene, terpinolene, δ-3-carene, limonene, α-humulene, germacrene D, β-selinene and germacrene B were the major ones with significant composition.

Hierarchical cluster analysis using the volatile phytochemical composition data revealed that the varieties Vellari and Kilichundan were the very nearest neighbours among the studied varieties. Moovandan and Pulinji were the two varieties that showed distant relation with the other varieties, in comparison.

The five SCoT Primers used for the genetic diversity analysis of *M. indica* varieties showed an average polymorphism of 82% among the ten studied varieties. The dendrogram construction of the varieties using Jaccard's similarity coefficients exhibited Kilichundan, Moovandan, Neelam and Kottoorkonam as the most genetically similar varieties among the ten.

The morphological, phytochemical and genetic diversities identified in the studied varieties were not directly correlatable. While the morphological characters could be greatly influenced by the surrounding environment, the volatile phytochemical diversities arose among the varieties could be a contribution of mRNA or protein level variations.

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8. APPENDICES

APPENDIX I

CTAB Buffer

Tris HCl – 100m*M* (pH 8)

EDTA – 20m*M* (pH 8)

NaCl – 1.4*M*

CTAB – 2%

APPENDIX II

TE Buffer

Tris – 10m*M*

EDTA – 1m*M*

APPENDIX III

TAE Buffer

Tris Base – 242g

Glacial Acetic Acid – 57.1 mL

0.5 M EDTA – 100mL

APPENDIX IV

Gel Loading Dye

Bromophenol Blue – 0.25%

Xylene Cyanol – 0.25%

Glycerol – 30%

Water – 69.5%

PHYTOCHEMICAL PROFILING AND GENETIC DIVERSITY ANALYSIS OF *Mangifera indica* Linn. VARIETIES IN KERALA

BONA SAJIMON

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ABSTRACT OF THESIS

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ABSTRACT

The study entitled **"**Phytochemical profiling and genetic diversity analysis of *Mangifera indica* Linn. varieties in Kerala**"** was carried out at the Phytochemistry and Phytopharmacology Division of Jawaharlal Nehru Tropical Botanic Garden and Research Institute, Palode, Thiruvananthapuram during the academic year 2019 – 2020. The objective of the study was to map and authenticate selected local varieties of *Mangifera indica* in Kerala through phytochemical profiling of leaf volatile chemicals and the genetic diversity analysis using SCoT marker polymorphisms.

Essential oils were obtained from the mature leaves of ten local mango varieties through hydrodistillation using a Clevenger-type apparatus and were analysed using GC-MS. A total of 55 volatile compounds ranging from 92.2 % to 98.0 % were identified from the ten varieties studied. Monoterpenes and sesquiterpenes were the major class of compounds among the identified volatiles. The prominent ones from this terpene classes include bicyclogermacrene, α-gurjunene, α-pinene, α-phellandrene, E-caryophyllene, terpinolene, δ-3-carene, limonene, α-humulene, germacrene D, β-selinene and germacrene B.

While the statistical analysis of the varieties using their phytochemical composition clustered Vellari and Kilichundan as the nearest neighbours, Moovandan and Pulinji emerged as two distinct varieties from others in comparison.

SCoT Primers proved to be a fine tool in studying the genetic diversity of *M. indica* varieties with an average polymorphism of 82% among the ten studied varieties. The varieties Kilichundan, Moovandan, Neelam and Kottoorkonam appeared as the ones with highest genetic similarity.

Though the phytochemical and genetic diversities observed among the varieties are not directly linkable, the analyses helped to establish fine distinction between the varieties.