DEVELOPMENT OF FUNCTIONAL SSR MARKERS FOR D -LIMONENE CONTENT AND ANALYSIS OF GENETIC POLYMORPHISM IN CARDAMOM (*Elettaria cardamom* Maton)

By

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(2013-09-112)

THESIS

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2018

DECLARATION

I hereby declare that the thesis entitled **"Development of functional SSR marker for D-Limonene content and analysis of genetic polymorphism in cardamom (*Elettaria cardamomum* Maton)"** is a bonafide record of research 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, fellowship or other similar title, of any other University or Society.

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കെ എസ് സി എസ് ടി ഇ - ജവഹർലാൽ നെഹ്റു ട്രോപിക്കൽ ബൊട്ടാണിക് ഗാർഡൻ ആന്റ് റിസർച്ച് ഇൻസ്റ്റിറ്റ്യൂട്ട്
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CERTIFICATE

This is to certify that this thesis entitled **"Development of functional SSR markers for D-Limonene content and analysis of genetic polymorphism in cardamom (*Elettaria cardamomum* Maton)"** is a record of research work done by **Ms. Reshma Retnakaran** (2013-09-112) under my guidance and supervision and that this has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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Reshma Retnakaran

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%	Percentage
µl	Microlitre
A_{260}	Absorbance at 260 nm wavelength
A_{280}	Absorbance at 280 nm wavelength
Ave_Het	Average hetrozygosity
bp	Base pair
cm	Centimetre
CTAB	Cetyltrimethylammonium bromide
DNA	Deoxyribo nucleic acid
EDTA	Ethylene diamine tetra acetic acid
EST	Expressed sequence tags
Exp_Het	Expected heterozygosity
G	Guanine
GC-MS	Gas Chromatography- Mass Spectrometry
g	Gram
h	Nei's heterozygosity

I	Shannon's information index
ISSR	Inter simple sequence repeats
M	Molar
mg	Milligram
min	Minute
ml	Millilitre
m*M*	Millimolar
mRNA	Messenger ribonucleic acid
MSA	Multiple Sequence Alignment
ng	Nanogram
□C	Degree Celsius
na	Number of alleles per locus
ne	Effective number of alleles
Obs_Het	Observed homozygosity
OD	Optical density
P	Percentage of polymorphic loci
PCR	Polymerase chain reaction

RAPD	Randam amplified polymorphic DNA
RFLPD	Restriction fragment length polymorphism
rpm	Revolution per minute
SCAR	Sequence characterised amplified region
SD	Standard Deviation
SRAP	Squence- related amplified polymorphisim
sp.	Species
SNP	Single Nucleotide Polymorphism
SSR	Simple sequence repeat
STR	Short tandem repeats
TBE	Tris-borate EDTA buffer
TE	Tris-EDTA buffer
Tm	Melting temperature
UPGMA	Unweighted pair group with arithmetic mean
UGT	UDP-glucuronosyltransferase
V	Volt
via	Through

1. INTRODUCTION

Elettaria cardamomum Maton, commonly known as green or true cardamom, is a large perennial, herbaceous, rhizomatous monocot belonging to Zingiberace family. Origin of the genus *Elettaria* is believed to be from evergreen rain forests of South India and Sri Lanka and later extended its boundary to other tropical countries.

Cardamom is one of the world's most expensive spice with sweet, burning taste and a very distinctive spicy odour. Plant grows upto a height of about 2 to 4 meters and forms a canopy. Flowers are beautiful and tiny and are usually white in colour with yellow or red strips over them. The colour of the fruit is green and turns golden yellow on ripening. The seeds from the capsules, which are harvested before they are fully ripe, constitute the spice known as cardamom.

The suitable growing condition of the cardamom is tropical, hot and wet climates. Small cardamom varieties are broadly classified into two depending upon their fruit size as *Elettaria cardamomum* var. major consisting of wild indigenous types and *Elettaria cardamomum* var. minor comprising the cultivated types *viz*., Malabar, Mysore and Vazhuka.

The world production of cardamom is around 36000 tonnes per annum. Kerala is the leading producer of cardamom in the country constituting 78% of the total production which is harvested from 56% of the total land area under cardamom cultivation in the country.

Small cardamom is affected by various major and minor diseases which have been identified as the major cause of crop loss. The higher altitude, with heavy rain fall and humid conditions permits the occurrence of many fungal, bacterial and viral diseases such as katte disease, nilgiri necrosis, and rhizome rot. Indian Institute of Spices Research (IISR) in Kozhikode, Indian Cardamom Research Institute (ICRI) in Myladumpara, and Cardamom Research Centre (CRC) in Pambadumpara are currently engaged in research focusing on the improvement of cardamom and promising new varieties.

The study aims to analyse the genetic diversity inherent in the cardamom genome using molecular (simple sequence repeats, SSR or microsatellite) markers and establish a correlation with D-Limonene, a component of cardamom essential oil possessing antioxidant activity.

D-Limonene, one of the constituent of cardamom oil is reported to have tumor suppressing effect against colon, mammary, liver, lung skin and stomach cancers in rodents (Samir *et al.,* 2015). The limonene possess antimicrobial and antioxidant properties (Desislave *et al.*, 2016).

Molecular markers are used to confess a polymorphism at the DNA level and have been playing an increasingly interesting part in plant biotechnology and molecular genetic studies. Microsatellite DNA markers has been widely used, due to its ease of handling by simple PCR, followed by a denaturing gel electrophoresis for the determination of allele size, and to the high degree of information provided by large number of alleles per locus.

Genomic libraries are used for developing microsatellite markers which belongs to either the transcribed region or non transcribed region of the genome (Jarne and Lagoda, 1996). Due to their high degree of variability, microsatellite sequences are suitable for distinguishing closely related genotypes. For studying variation in population genetics, microsatellite informative markers can be used.

Microsatellite markers have frequently been used for genetic diversity studies, because of their large number, distribution throughout the genome, high level of polymorphism, co-dominant inheritance, neutrality with respect to selection and easy automation of analytical procedures (Gupta and Varshney, 2000).

By screening diverse germplasm accessions, this study has the potential to analyze the genetic diversity with respect to limonene content and microsatellite markers. Correlation analysis was conducted to determine if the genetic variability as estimated by SSR markers as a good indicator for limonene content among the various germplasm accessions.

2. REVIEW OF LITERATURE

Small Cardamom, *Elettaria cardamomum* Maton belonging to the family Zingiberaceae. It is an under crop plant in forest lands and mostly grown in the hilly regions of South India at elevations of 800- 1300 m above msl. According to reliable reports, cardamom cultivation started in India at 1803 and until then, cardamom pods were harvested from forests (Ravindran and Madhusoodanan *et al.,* 2002). Cardamom belongs to the genus *Elettaria* and species *cardamomum* (Maton). The generic name *Eletteria* was derived from its Tamil root 'Elettari' which means cardamom seeds (Sasikumar *et al.*, 1999). Ecosystem diversity is very limited in cardamom and majority of the diversity in cardamom comes from varietal diversity (Madhusoodhanan *et al.*, 1994).

Large cardamom commonly known as 'bada elachi' in Hindi (*Amomum subulatum* Roxburg) (Scitaminae: Zingiberaceae) is indigenous to moist deciduous and evergreen forests of sub Himalayan tracts. The presence of wild species locally known as 'churumpa' viz. A. *delbatum,* A. *aromaticum,* A. *kingir,* A. *lingriformi,* A. *corynostachum* etc. and the tremendous variability within the cultivated species support the view of its origin in Sikkim (Subba *et al.*, 1984). It is the most important cash crop of Sikkim from where it spread to North Eastern States and parts of West Bengal and neighbouring countries; Nepal and Bhutan (Singh *et al.,* 1978; Gupta *et al.,* 1983). Sikkim has the largest area under cultivation (91%) with the highest production (87.5%) in India (John *et al.*, 1984). Large cardamom is a shade loving crop and is better established under humid and cold conditions at an altitude of 900 to 2000 m above MSL and within a temperature range of 10 to 30°C under uniformly distributed annual rainfall of 2000 to 3500 mm (Biswas *et al.*, 1988).

2.1 PLANT MORPHOLOGY

Small cardamom is a perennial herb having subterranean rhizomes and several leafy aerial shoots (tillers) rising from the rhizome that grows up to 1.5 to 2 m height. Leaves are lanceolate and distichous in nature. Top portion of leaf

is dark green and bottom portion is light green in colour. Inflorescence emerges from the foot of leafy shoot and flowers are seen on inflorescences which can be of three different types such as erect, prostrate and semi-erect which varies with different varieties of cardamom. Flowers are racemic and zygomorphic nature. Bisexual flowers with inferior ovary are usually white in colour with violet lines characterized with a charismatic labellum. Fruit is a trilocular capsule and green in colour (Anandaraj and Sudharshan, 2010). Capsules are 20 to 25 mm in length, oval to globose and contain 30 to 55 seeds. It is a shade loving plant and 40 to 50 percent shade promote its vegetative growth and development (Kumar *et al.*, 2015)

Three varieties of cardamom are recognized based on their panicles nature (Sastri *et al.*, 1952) which include Mysore, Malabar and Vazhukka. Panicle of var. Malabar is of prostrate nature whereas var. Mysore is erect. Vazhukka is a cross-breed of the Malabar and Mysore varieties and its panicle is semi-erect in nature. Malabar var. plants are medium in size and attain 2-3 m height on maturity whereas var. Mysore plants are robust and attain about 3-4 m. Vazhukka var. has been considered to be a natural hybrid of both Malabar and Mysore varieties and exhibits characteristics intermediate to both (Ravindran and Madhusoodanan, 2002)

2.1.2 Use of Cardamom

Cardamom is one of the world's ancient, expensive and highly valued spice. Its fruits are oblong, gray, each containing many seeds. It is effective against cardiac disorders, renal and vesicular calculi, dyspepsia, debility, anorexia, asthma, bronchitis, halitosis, gastrointestinal disorders including indigestion and flatulence. It possessed antioxidant, antihypertensive, gastro protective, antispasmodic, antibacterial, antiplatelet aggregation and anticancer properties (Verma *et al.*, 2009). It works as a laxative and soothes colic, wind, dyspepsia and nausea, even during pregnancy. It warms the stomach and also helps recovering from heart burn. In aromatherapy it is used as a massage oil or diluted in the bath,

cardamom oil comforts the digestive system, coughs and is used as a general tonic (Nirmala *et al.*, 2000).

Its aphrodisiac property makes it useful in medicines fighting stress, obesity and loss of appetite (Lwasa *et al.*, 2007). It is effective to remove fats and as a cure for urinary and skin infections in Ayurvedic medicine. The ancient Egyptians chewed it as a tooth cleaner and to aid in digestion. The seeds are regarded as carminative, stomachic, desiccant, resolvent, digestive and anti-emetic and ingested for the treatment of gastrointestinal disorders (Farah *et al.*, 2005) Cardamom essential oil has proven to inhibit fungus growth and marked antispasmodic, analgesic, and anti–inflammatory activities (Rehaman and Al-Zuhair, 2000). The seeds are used in spicy mixtures like curries, beverages such as tea and coffee, baked foods, confectionaries, meat products, biscuits, custards, wines and liqueurs (Lwasa *et al.*, 2007).

2.1.3 Phytochemical studies

Cardamom is used as a flavouring material in three forms; whole capsule, decorticated seeds and ground seed powder. Essential oil of cardamom is the source of its aroma and flavour. Researches so far were concerned mainly with the composition of the oil. As early as 1908 there were reports that cardamom oil contained terpinene, sabinene, limonene, 1, 8-cineole, α- terpineol, α- terpinyl acetate, terpinen- 4-yl formate, acetate and terpinen-4-ol (Guenther *et al.*, 1975). The volatile oil is extracted from the seeds and the husks hardly give 0.2 per cent oil. Even though the public perception about good quality cardamom is the greenish capsule, the appearance of the capsule has little to do with the recovery of volatile oil (Sarath kumar *et al.*, 1985).

2.2 D- LIMONENE

D- Limonene (1-methyl-4-(1-methylethenyl) cyclohexane), a bioactive component in the cardamom essential oil was reported to posses chemo preventive property towards colon cancer, lung, liver mammary, skin and stomach cancers in rodents (Acharya *et al.*, 2010) (Asamoto and Ota, 2002). Hence better

understanding of genes and pathways associated with the biosynthesis of the active compounds in cardamom essential oil might be beneficial for therapeutic purposes as well as selection of superior genotypes.

D-limonene is a monocyclic monoterpene with a lemon-like odour and one among the major constituent in several citrus oils (orange, lemon, mandarin, lime, and grapefruit). It is widely used as a flavour and fragrance additive in perfumes, soaps, foods, chewing gum, and beverages due to its pleasant citrus fragrance..

D-limonene has well-established chemopreventive activity against many sorts of cancers. Evidence from a phase I clinical trial shows a partial response in a patient with breast cancer and stable disease for more than six months in three patients with colorectal cancer. D-limonene induces phase I and phase II carcinogen-metabolizing enzymes (cytochrome p450), which metabolize carcinogens to less toxic forms and prevent the interaction of chemical carcinogens with DNA. D-limonene has been shown to enhance gastrointestinal UDP- glucuronosyltransferase (UGT) activity in rats (Van der *et al.*, 2004). It also inhibits tumor cell proliferation, acceleration of the rate of tumor cell death and/or induction of tumor cell differentiation. Furthermore, d-limonene inhibits protein isoprenylation.

The antimicrobial activity of the oil of black pepper is due to the carbohydrates sabinene, cymene, limonene, linalool and bisabolol. In coriander oil the components responsible for the antimicrobial activity are sabinene, cymene, limonene, linalool, borneol, geraniol, thymol and citral. In cumin oil the antimicrobial activity is due to cymene, limonene and linalol, in cardamom oil - to sabinene, cymene, limonene, linalool, geraniol and citral *(*Asbahani *et al.*, 2015).

2.3 GENETIC DIVERSITY

Bio-diversity refers to the "variations" existing within the living world, while Rao and Hodgkin (2002) describe genetic diversity as the sum total of genetic characteristics within any species or genus. Many scientific studies remarked the importance of genetic diversity in providing genetic resistance against different biotic and abiotic stresses (Hughes *et al.*, 2004; Hajjar *et al.*,

2008). Hajjar *et al.*, 2008 showed that as genetic diversity increases, pest and disease management tends to improve and hence provides opportunity for further improvement of the species. Genetic diversity is essential for the rapid genetic improvement of crop species (Trethowan and Kazi, 2008).

India, the centre of diversity of small cardamom is one of the most important economical spices, along with saffron and vanilla. Cardamom var. minor acclaimed, as the 'Queen of spices', is the true cardamom belonging to the family Zingiberaceae under natural order Scitaminae. The monocot genus consists of relatively six to seven species distributed in India, Sri Lanka, Malaya and Indonesia (Hooker *et al.*, 1894; Holttum *et al.,* 1950; Madhusoodanan *et al.*, 2002). Small cardamom occurs in the wild gaps of evergreen mountain monsoon forests of Western Ghats in South India and in the western high lands of Sri Lanka (Wardini and Thomas, 1999).

Microsatellites occur in plant genomes are widely studied for analyzing the genetic variations. They are abundant in non-coding genomic regions, but also detected in coding regions through studies using microsatellites from expressed sequence tags (EST-SSRs) (Ranade *et al.,* 2014). The significance of EST-SSR as a molecular tool in genetic studies is well known (Ellis and Burke, 2007) and demonstrated in population studies and analysis of genetic diversity in many species like *Populus* (Xinye *et al.,* 2009). Recently, it was reported that EST-SSRs of turmeric and ginger showed considerable genetic variation in cardamom (Anjali *et al.* 2015).

Genetic diversity analysis *via* molecular markers paved way for genetic mapping and marker-assisted selection in breeding (Lapitan *et al.*, 2007). George *et al.* (2006) stated the preliminary assessment of the utility of ISSR analysis for detecting polymorphism in spices. Venugopal and Prasanth, 2004 reported on the analysis of genetic diversity for quantitative characters in cardamom germplasm. The improvement of crop genetic resources is reliant on the continuous infusions of wild relatives and traditional varieties with the help of modern breeding

techniques. All these procedures require the assessment of diversity at some level, to select resistant and highly productive varieties for raising a potential population (Mondini *et al.*, 2009).

DNA based molecular marker technologies, such as simple sequence repeat (SSR), amplified fragment length polymorphism (AFLP), sequence-related amplified polymorphism (SRAP) and single nucleotide polymorphisms (SNPs), have several advantages including abundant, independent from the environment, suitability for early and rapid evaluation, and having non-tissue specific characteristics. Among them, SSRs, also known as microsatellites or short tandem repeats (STR), are widely present in eukaryotic genomes (Sharma et al., 2007) and very useful for a number of reasons including co-dominant inheritance, high polymorphism, high variability and suitability for automated allele sizing and cross-species transferability (Jian-Wei Zong *et al.*, 2015).

2.4 PRIMER DESIGNING

For a PCR reaction to be successful it requires a good primer for the exponential amplification process. Designing a good primer is very essential for carrying out an effective PCR reaction. Primer specificity, denying mis-priming and efficiency for precise amplification are important parameters in PCR primer design. Primers having a melting temperature in the range of 52-58°C usually yield best results. Primers showing an increased melting temperature have a tendency for secondary annealing and the GC content of the sequence provides a reasonable hint about the melting temperature of the same (Patricia *et al.*, 2009).

According to the set of rules recommended by Innis and Gelfand (1990), the designed primers should have a length of 17-28 bases, with 50-60% (G+C) combination, ending with G,C,GC or CG (at 3') and Tm ranges between 55–80°C. It should be devoid of three or more Cs or Gs at the 3'-ends that promotes mismatches, and also lack 3'-end complementarity and self-complementarity (Innis and Gelfand, 1990). Reducing intra-molecular or inter-molecular homology

while designing primers minimise the formation of hairpins and primer dimerization (Abd-Elsalam *et al.,* 2003).

Designing primers with last two nucleotides as AA or TT reduce the possibility for dimerization of the primers, is a practical strategy for upholding the stability of the hybridised 3 prime end (Innis and Gelfand, 1999). The primer pairs selected should be able to extend the newly synthesizing strand on either side in the direction of the parent strand to cover the specified target region (Kampke *et al.*, 2001).

2.5 MOLECULAR ANALYSIS

2.5.1 DNA Isolation

Isolation of genomic DNA is the first step in most molecular biology experiments. The decisive factors when selecting an extraction method are quantity, quality and purity of isolated DNA. Techniques of molecular biology require DNA of varying purity and quality. Currently, there are many methodologies and isolation kits for the extraction of genomic DNA with optimal properties (Oza *et al.*, 2008). The buffer containing nonionic detergents such as cetyltrimethylammonium bromide (CTAB) is often used for DNA isolation, followed by a series of steps for the purification of DNA from contaminants using organic solvents or salt precipitation (Bossinger et al., 2006).

Isolation of DNA from plant tissues is problematic in comparison with DNA isolation from animal tissues because of the rigid cell wall that surrounds the plant cells. For DNA extraction from cells of animal tissues, only buffer containing detergents and proteinase K are necessary. For DNA isolation from plant tissues, participation of carbohydrates and enzymes ensuring lysis of cell wall is necessary (Manen *et al.,* 2005). The presence of polysaccharides, polyphenols and other organic compounds may interfere with DNA isolation process (Cota-Sánchez *et al.,* 2006). In techniques of nucleic acids isolation there are two basic steps – degradation of cell membranes and purification of nucleic acids from contaminants of cellular content.

Traditional methods like phenol/chloroform extraction are still in use because of its consistency in the production of high-quality DNA (Hillis *et al.*, 1996). Proteins, lipids, carbohydrates, and cell debris are removed through extraction of the aqueous phase with the organic mixture of phenol and chloroform (Sambrook and Russel, 2001; Chomczynski and Sacchi, 2006). The DNA pellet recovered at the end is usually kept dissolved with TE buffer or sterile distilled water (Buckingham and Flaws, 2007).

The extraction of DNA from ancient and forensic material demands surface decontamination, by cleaning with 0.5% sodium hypochlorite solution following mechanical removal of the outer surface under strictly DNA-free circumstances (Zink *et al.*, 2005). Currently numerous attractive DNA extraction kits are commercially available and these systems are gradually becoming popular because of their ease and comfort of use, limited labour, and the ability to reliably produce high-quality DNA.

The potential benefits of DNA-level analysis embrace the fact that the DNA can be prepared from minor quantities of sample at any phase of life cycle and that it is relatively stable (Onyango *et al.*, 2010).

2.5.2 Polymerase Chain Reaction

PCR is a scientific technique in molecular biology developed for amplifying a single piece or a number of copies of DNA across many orders of magnitude and producing thousands to millions of copies of a particular DNA sequence. It was developed in 1984 by the American biochemist, Kary Mullis. He got the Nobel Prize and also the Japan Prize for developing PCR in 1993 (Bartlett *et al.*, 2003). However the main principle of replicating a single copy of DNA using two primers had already been described by Gobind Khorana in 1971.

PCR is currently a standard and often indispensable technique used in medical and biological research labs because it is rapid, simple and inexpensive (Saiki *et al.*, 1985). The technique amplifies specific DNA fragments from minute

quantities of source DNA material, even when that source DNA is of relatively poor quality.

The major three steps involved in PCR technique are denaturation, annealing, and extension. In step one; the DNA is denatured at high temperatures from 90-97^{0}C. In step two, primers anneal to the DNA template strands to prime extension. In step three, extension occurs at the end of the annealed primers to create a complementary copy of DNA strand. This effectively doubles the DNA quantity through the third steps within the PCR cycle. To amplify a section of DNA using PCR, the sample is first heated so the DNA denatures, or separates into two pieces of single-stranded DNA. Next, an enzyme called 'Taq polymerase' will synthesis two new strands of DNA, using the original strands as templates. This method ends up in the duplication of the original DNA, with each of the new molecules containing one old and one new strand of DNA. Then each of these strands can be used to create two new copies, and so on, and so on (Ochman *et al.,* 1988).

The annealing phase happens at a lower temperature, 50-60°C. This allows the primers to hybridize to their respective complementary sequences in template strand, a very useful tool in forensic chemistry. The newly-formed DNA strand of primer attached to template is then used to create identical copies of the original template strands desired. The enzyme, Taq polymerase adds available nucleotides to the end of the annealed primers and the extension of the primers occurs at approx 72°C for 2-5 minutes.

DNA polymerase I cannot be used to elongate the primers as one would expect because it is not stable at the high temperatures required for PCR. The beauty of the PCR cycle and process is that it is very fast compared to other techniques and each cycle doubles the number of copies of the desired DNA strand. After 25-30 cycles, whoever is doing the PCR process on a DNA sample will have number of copies of the original DNA sample to conduct

experimentation. Assuming the maximum amount of time for each step, 30 cycles would only take 6 hours to complete.

As the process of denaturation, annealing, and polymerase extension is continued the primers repeatedly bind to both the original DNA template and complementary sites in the newly synthesized strands and are extended to produce new copies of DNA. The end result is an exponential increase in the total number of DNA fragments that include the sequences between the PCR primers, which are finally represented at a theoretical abundance of 2n, where n, is the number of cycles (Gibbs and Arnheim, 1990).

In molecular biology, qRT- PCR, also known as quantitative real time polymerase chain reaction is a laboratory technique based on the PCR, which is used to amplify and simultaneously quantify a targeted DNA molecule. Traditionally, PCR is performed in a tube and when the reaction is complete the products of the reaction (the amplified DNA fragments) are analyzed and visualized by gel electrophoresis. However, Real-Time PCR allows the analysis of the products while the reaction is actually in progress. This is achieved by using various fluorescent dyes which react with the amplified product and can be measured by an instrument. This also facilitates the quantification of the DNA.

PCR is helping in the investigation and diagnosis of increasing number of diseases. It has also long been a standard method in all laboratories that carry out research on or with nucleic acids. Even competitive techniques equivalent to DNA chips typically need amplification of DNA by means of PCR as an essential preliminary step. By allowing the determination and quantification of changes in gene expression, these techniques have provided a greater understanding of disease processes and now serve as a foundation for diagnostics and basic science research (Faoud and Cristiana, 2008). One of the important application of PCR in medical diagnosis, is the analysis of mutations that occur in many genetic diseases such as, cystic fibrosis, sickle cell anaemia, phenylketonuria, muscular dystrophy,

etc. (Prescott *et al.*, 2008). In genetic engineering PCR offers rapid screening of bacterial colonies for the correct DNA vector constructs (Pavlov *et al.*, 2006).

PCR methods have also succeeded in the discovery of unknown genes for an organism from the homologous sequences of known genes from similar organisms (Rose *et al.*, 1998). The prerequisite of knowledge about primers that are complementary to both the termini of the template DNA is one of the major limitations of conventional PCR.

2.6 MOLECULAR MARKERS

Molecular markers are important tools for dissection and analysis of genomes for better understanding of genome architecture. This helps breeders to incorporate important agronomical traits in breeding programmes by marker assisted selection (Christiansen *et al.*, 2002). Among the molecular markers, microsatellite (Simple Sequence Repeat) markers are widely accepted for genetic diversity studies because of their reproducibility and codominant nature (Zane *et al.*, 2002). They show allelic variation and can be used for germplasm studies, linkage mapping and breeding studies. Molecular markers include PCR based and hybridisation based markers.

2.6.1 PCR based Molecular markers

PCR is a precise scientific technique generally used in molecular biology and genetics for the exponential amplification of a specific segment of DNA (Joshi and Deshpande, 2010). The development of Polymerase Chain Reaction marked the commencement of the era of PCR based molecular markers (Mullis *et al.*, 1986).

2.6.1.1 Random Amplified Polymorphic DNA (RAPD)

RAPD technique is simple, fast, does not require any DNA sequence information and is able to identify genetic variation within and between populations (Govarthanan *et al.*, 2011). This PCR based procedure detects nucleotide sequence polymorphisms by using arbitrary chosen primers usually 8-

10 bp long. The choice of a compatible primer and optimization of PCR conditions are the significant aspects in RAPD analysis (Blixt *et al.*, 2003). RAPD is considered to have a crucial role in cultivar identification (DNA typing), assessment of genetic variability and relationships management of genetic resources and biodiversity, studies of phylogenetic relationships and in genome mapping (Hasibe *et al.*, 2009).

2.6.1.2 Amplified Fragment Length Polymorphism (AFLP)

To overcome the constraints of reproducibility linked with RAPD, AFLP technology for DNA fingerprinting was established by the Dutch company, Keygene (Zabeau and Vos, 1992). First employed by Vos *et al.*1995, the procedure of AFLP begins with the digestion of whole genomic DNA with two restriction enzymes. The technique dominates over RFLP and RAPD by combining the supremacy of RFLP with the flexibility of PCR based technology by ligating primer recognition sequence (adaptors) to the restricted DNA (Lynch and Walsh, 1998).

For the DNA of any organism, it is possible to construct AFLP markers, without an initial investment in primer/probe development and sequence analysis. As described by (Vos *et al.* 1995), amplification occur only when the primers anneal to fragments having the adaptor sequence plus the complementary base pairs to the additional nucleotides called selective nucleotides. AFLP succeeded in consolidating the advantage of PCR based technique in terms of efficacy, high throughput and amenability to automation with the specificity and robustness of RFLP based techniques (Vos *et al.*, 1995).

2.6.1.3 Simple Sequence Repeats (SSR)

Microsatellites are multiple copies of tandemly arranged simple sequence repeats (SSRs) having a size ranging from 1 to 6 base pairs (Tautz, 1989; Litt and Luty, 1989). Because of their multi-allelic nature, reproducibility, codominant inheritance, high abundance and extensive genome coverage, SSR markers offer a

wide range of applications in plant breeding and genetics (Gupta and Varshney, 2000).

One of the significant features of microsatellite markers that make them the ideal candidates for genetic analysis is their extreme polymorphic nature, *i.e.*, across different genotypes, a large number of allelic variants are possible (Akkaya *et al.*, 1992; Powell *et al.*, 1996). Cardle *et al.*, 2000 reported that SSRs happen frequently as once in almost every 6 kb in plant genomes.

In addition to molecular mapping, EST-SSRs also offer opportunity for gene discovery when linked with a trait of interest (Thiel *et al.*, 2003). Enormous datasets of ESTs are being generated with the recently increased emphasis on functional genomics, and it is now possible to identify and develop EST–SSR markers at a large scale in a time and cost-effective manner using bioinformatics approaches (Scott *et al.*, 2000; Kantety *et al.*, 2002; Varshney *et al.*, 2002). Compared to those markers generated from genomic sequences, EST-SSR markers have more conserved region and therefore show increased transferability between species (Portis *et al.*, 2007; Varshney *et al.*, 2005).

Bioinformatics has great potential for being an attractive alternative for molecular approaches, subsequently, databases available for a large number of genome sequences, can be mined for SSR development (Gu *et al.*, 1998; Kantety *et al.*, 2002; Robinson *et al.*, 2004; Varshney *et al.*, 2002). In addition to the reduction in developmental costs it also permits development of a large number of markers in a short span of time.

Even though the development of EST-SSR markers was reported in other plant species such as wheat (Gupta *et al.*, 2003), cucumber (Hu *et al.*, 2010) medicago (Gupta and Prasad, 2009) etc, no EST based SSR markers of cardamom have been reported. Microsatellite markers are widely used in population genetic studies as they offer high ubiquity, relative ease of screening and also the requirement of minor quantity of low quality DNA for PCR amplification (Hutchinson *et al.*, 2003).

The course of discovery, isolation and design of suitable flanking primers which imparts time consumption, expensive and technical demand are the major limitations reflected in the application of microsatellite markers (Benali *et al.*, 2012).

2.6.1.4 Inter Simple Sequence Repeats (ISSR)

Inter Simple Sequence Repeat marker was reported as a new fingerprinting method in 1994 by Zietkiewicz *et al.* and is useful for taxonomic, phylogenetic and mapping studies. ISSRs are around 100-3000 bp long DNA sequences situated between oppositely aligned and neighbouring SSR regions (Bracci *et al.*, 2011). Primers used for the amplification of ISSR regions will be microsatellite sequences.

2.6.1.5 Single Nucleotide Polymorphism (SNP)

The single base differences occurring between DNA of different individuals is termed as Single Nucleotide Polymorphisms (SNPs). Once identified, SNPs can be converted into usable genetic markers that support several assays in molecular biology aided for clinical purpose and evolutionary studies (Gut *et al.*, 2001; Kwok *et al.,* 2001).

SNPs present within the coding regions, can result either in non-synonymous mutations with alternation in amino acid sequence, or synonymous mutations that causes no change in the amino acid sequence (Sunyaev *et al.*, 1999). Sunyaev and co-workers estimated that about 20% of common non-synonymous SNPs will have deleterious effects on protein structure based on the location of SNPs mapped onto 3D-structures and comparative homology analyses (Sunyaev *et al.*, 2001). Real-Time PCR is the most common application which is currently in use for detection of SNPs (Kwok, 2002). The increased interest towards the assay is because of the requirement of only a small amount of purified DNA.

2.6.1.6 Sequence Characterised Amplified Region (SCAR)

Sequence Characterised Amplified Region (SCAR) marker is a modified version of RAPD marker system. The basic idea of changing dominant RAPD marker to codominant SCAR marker is to overcome the difficulties caused by the laborious procedures of RAPD (Yang et al., 2013). Sequence obtained from the cloned amplified product of RAPD marker is used to design primers for SCAR and this oligonucleotide primer pairs amplify DNA fragment at a single loci (Paran and Michelmore, 1993).

2.6.2 Hybridisation based markers

In this technique, genomic DNA from different genotypes is cleaved by specific restriction endonucleases to produce DNA fragments and variation in length of these fragments is analysed.

2.6.2.1 Restriction Fragment Length polymorphism (RFLP)

Restriction Fragment Length Polymorphism (RFLP) markers were used as molecular marker for plants after identifying DNA sequence polymorphism in adeno-virus and human genome mapping. Due to mutation, one or more nucleotides in the DNA of individuals of same species may differ. If this difference in nucleotides occurs at restriction sites, DNA fragments of different size will be produced by restriction enzymes. These fragments are separated by gel electrophoresis and transferred by southern blotting to facilitate the hybridisation of target DNA to the labelled probe. Finally autoradiography is employed for visualisation (Semagn *et al.*, 2006).

3. MATERIALS AND METHOD

The research work was carried out at Biotechnology and Bioinformatics division of Jawaharlal Nehru Tropical Botanic Garden and Research Institute (JNTBGRI), Palode during the period 2017- 2018.

3.1 SAMPLE COLLECTION

Elettaria cardamomum Maton was used for the present study to analyse the genetic diversity of thirty different accessions and to develop a functional molecular marker in relation to its D- Limonene content. Samples were collected from different locations of Idukki and from the cardamom germplasm conservatory of JNTBGRI, Palode (Table 1).

Table 1: List of Cardamom accessions used for the study

Sl No.	Accession Nos.	Local Name	Location
1	C65	PV2	Pampadumpara, Idukki
2	C51	Kanniyelam (Feral)	Maniyaramkudi, Idukki
3	T7	Wild	Therakudi
4	C70	Unknown	Kaki, Periyar
5	C53	Unknown	Bonacadu
6	C61	ICRI 2 (RV)	Myladumpara, Idukki
7	C103	Pathumuriyan	Vacacity, Idukki
8	C116	Kothu mysore	Chemmanar, Idukki
9	C107	Njellani	Vacacity, Idukki
10	C108	Njellani	Vacacity, Idukki
11	C104	Nadan	Chemmanar, Idukki
12	V8	Wild	Variyam
13	C106	Elam Raja	Mukkadi, Idukki

14	C105	Njellani	Mukkadi, Idukki
15	C112	Njellani	Kadamankudi, Idukki
16	C109	Green bold	Vallakkadavu, Idukki
17	C118	Thiruthaali	Chemmanar, Idukki
18	C120	Palakkudi	Vangalappara, Idukki
19	C113	Palakkudi	Vangalappara, Idukki
20	C117	Vazhukka mysore	Chemmanar, Idukki
21	LC	Large cardamom	Vallakkadavu, Idukki
22	C111	Njellani	Kadamankudi, Idukki
23	C64	PV1	Pampadumpara, KAU
24	C24	Wild	Pandimotta
25	C110	Green bold	Vallakkadavu, Idukki
26	ER	Elam Raja	Mukkadi, Idukki
27	NJ1	Njellani	Vacacity, Idukki
28	NJ2	Njellani	Vacacity, Idukki
29	NJ3	Njellani	Vacacity, Idukki
30	NJ4	Njellani	Vangalappara, Idukki

3.2 DIVERSITY ANALYSIS

3.2.1 DNA Isolation

The genomic DNA of thirty different cardamom accessions were isolated using Origin Plant Genomic DNA purification kit. Leaf samples were collected and wiped with sterile distilled water and subjected to DNA isolation with the following procedure;

Before the commencement of isolation, the buffer PW and GD were prepared by adding 60 ml of ethanol (96-100%) as instructed on the bottle and shaken thoroughly.

- 100mg of fresh leaf sample was weighed and grinded to fine powder using liquid nitrogen.
- 700 µl 65°C pre- heated GP1 buffer along with 0.1% β-Mercaptoethanol was added to the powdered sample and vortexed for 10- 20 s to disperse all clumps. The sample was then incubated at 65°C for 20 mins and mixed by inverting the tube several times.
- 700µl chloroform was added, mixed by inverting the tube and centrifuged for 5 min at 12,000 rpm.
- The aqueous supernatant was transferred to a new tube and 700 µl buffer GP2 was added, mixed by inverting the tube for several times.
- The whole mixture was then transferred into the Spin Column CB3 placed in a collection tube. Closed the CB3 lid and centrifuged for 30 s at 12,000 rpm. The filtrate was then discarded and the spin column CB3 was placed back into the collection tube.

  (When the sample volume exceeded 700µl, successive aliquots were centrifuged in the same column.)
- 500 µl Buffer GD was added and centrifuged at 12000 rpm for 30 s, the filtrate was discarded and placed the Spin Column CB3 back into the collection tube.
- 600 µl Buffer PW was added to the Spin Column CB3, and repeated centrifugation for 30 s at 12,000 rpm, discarded the flow-through and placed the Spin Column CB3 back into the collection tube.
- The above step was repeated for one more time.
- Placed the Spin Column CB3 in the collection tube and kept for centrifugation at 12,000 rpm for 2 min, the flow through was discarded. The lid of CB3 column was kept opened at room temperature for a while to dry the membrane completely.

- Spin Column CB3 was placed into a fresh 1.5 ml tube, and 50-200µl TE buffer was added directly onto the CB3 membrane, incubated 2-5 min at room temperature (15-25°C), and then eluted DNA via centrifugation at 12,000 rpm for 2 min.

3.2.2 Agarose Gel Electrophoresis

The integrity of the isolated DNA samples were analyzed using Agarose Gel Electrophoresis. The technique separates biomolecules based on their charge/ size under the influence of an electric field. Samples were added to the well close to the anode. The DNA which was negatively charged due to their phosphate backbone repels from anode and moves towards cathode. Larger nucleic acids have a harder time moving through the gel matrix, thus the nucleic acid will get separated by size.

3µl of DNA aliquots from each sample was loaded in 0.8% agarose gel. The gel was run at 110V with 1X tank TBE buffer in horizontal gel electrophoresis unit for about 3 hours. The genomic DNA bands in the gel were visualized by Ethidium bromide staining under the gel documentation system.

3.2.3 Biophotometer analysis

The quality and quantity of the DNA samples were analyzed by using biophotometer ((Eppendorf India Limited). The instrument was calibrated with 50µl sterile water as blank. Biophotometer required a sample size of 1µl DNA diluted with 49µl sterile distilled water for measuring the optical density and to identify the specified parameters. The instrument provided analytical data on,

- The absorbance of nucleic acid at 260 and 280 nm wavelength
- Concentration of DNA in the sample (µg/µl)
- Purity of DNA sample (260/ 280)

3.2.4 Diversity analysis using available primer

For the analysis of genetic polymorphism and differentiation, already available cardamom specific SSR markers in JNTBGRI was used (https://doi.org/10.2174/1574893611666161128123827) (Table 2)

Table2: Cardamom specific SSR markers

Sl. No.	Primer	Sequence
1	CaSSR 41F	GGAGGAGGAAGAAGGAGAAGAG
	CaSSR 41R	GAGCGAGAAGAAGAGGAGGAG
2	CaSSR 42F	ACCGTCTTGTCGTAGGTCTTGT
	CaSSR 42R	GGAAGGAGAGGATGGAGAGGTA
3	CaSSR 46F	TGTTGTTACTGTTGCTGTTCCC
	CaSSR 46R	GCTAACTCAATGTTTTCCCTGC
4	CaSSR 49F	CGAGGCAGAAATACAGATGATG
	CaSSR 49R	GCTGCTGATTCTTAGGCTTTGT
5	CaSSR 52F	ACTGGAGAGGGTATGGATGAGA
	CaSSR 52R	AAAAGCAACGAAAAGACGAGAG
6	CaSSR 53F	TTCGTCGCAGGATAGGCTT
	CaSSR 53R	CGATAAGATGGAGAAGGAGGC

3.2.4 PCR amplification of genomic DNA with the designed primers

The newly synthesized primer pairs were optimized for PCR parameters such as the annealing temperature and concentration of reagents. The annealing

temperature was standardized using gradient PCR. DNA samples were then subjected to PCR amplification with the designed non redundant primers. The reaction mixture consisted of the following items: (Table 3)

Table3: PCR reaction mix

Reagents	**Volume**
Water	6.2µl
Taq PCR smart mix	7.5 µl
Forward primer (25 nM)	0.15µl
Reverse primer (25 nM)	0.15 µl
Template DNA	1 µl
Total volume	**15.0 µl**

Amplification was carried out on Agilent Technologies thermal cycler (Agilent technologies, Malaysia) using the following temperature profile: Reactions began with a 2 min initial denaturation at 94 °C, followed by 35 cycles with 30 sec at 94 °C, then 1 min at specific annealing temperature and 2 min extension at 72 °C, a final extension step covering 7 min at 72 °C marked the end of the reaction. The amplified products were resolved in 3 % agarose gel with ethidium bromide and were analysed using gel documentation system (UVP, UK). A 1000 bp DNA ladder was loaded along with the samples to compare the size of resultant bands.

3.2.6 Diversity analysis with POPGENE

Diversity analysis was done using POPGEN software following the UPGMA method. The gel scoring data was converted to software acceptable format and statistical analysis was performed.

3.3 D- LIMONENE ESTIMATION AND MOLECULAR ANALYSIS

3.3.1 Sample preparation for oil extraction

Fresh cardamom capsules collected from different locations of Idukki were used as the samples for limonene estimation. Four varieties used for oil extraction were Palakudi, Pathumuriyan, Green bold, and Njellani. High yielding varieties such as Green bold and Njellani are the recent popular cultivars whereas Pathumuriyan and Palakudi are not widely in use due to their low productivity.

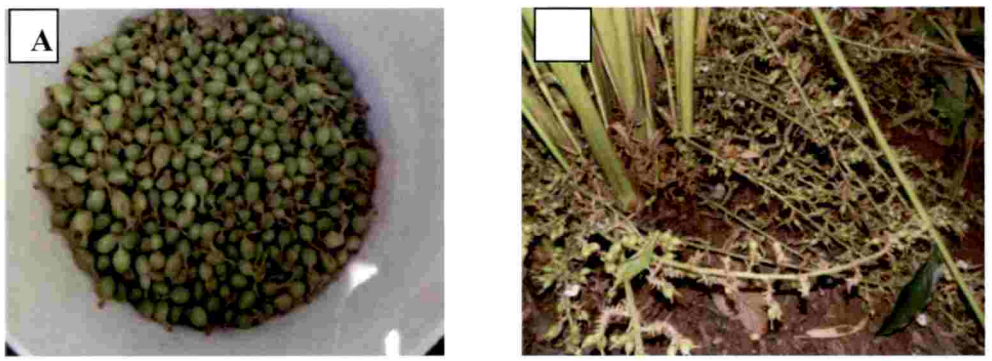
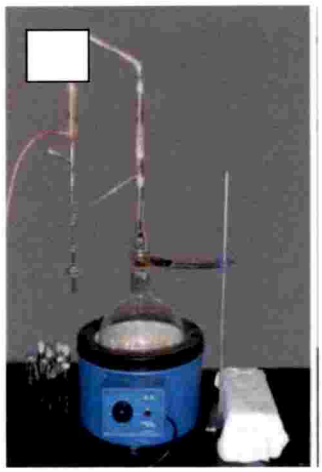


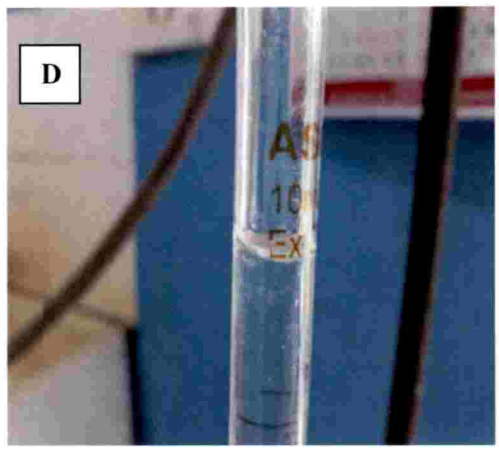
Plate 1: (A) Freshly collected cardamom Capsules; (B) Cardamom plant bearing capsules

Freshly collected capsules were washed under running tap water and let it dried to drain off the water. Each sample was weighed 65g and grinded using a blender to rupture the pods.(Plate:1)

3.3.2 Extraction of oil

Clevenger- type apparatus, a vertical hydro distillation unit has been used for essential oil extraction. It consists of a hot plate, boiling flask, biomass flask, still head, condenser and a receiver. The grinded capsules were soaked in water and boiled until the oil gets separated from water. The essential oil which was clearly distinguishable from water was then collected from the receiver. The samples were hydro- distilled for 3 hours and the collected oil was dried over anhydrous sodium sulphate ($NaSO_4$) and stored at 4□C until analyzed.(Plate:2)





The extracted essential oils were analyzed by Gas Chromatography–Mass Spectrometry (GC- MS). GC- MS analysis was performed on a Shimadzu (Tokyo, Japan) Make GCMS-TQ8030 with non polar Rxi 5Sil MS capillary column, full scan mode, injector mode- split with 50:1 split ratio, quadra pole mass selective detector (MSD), injection temperature 220□C, GC- MS interface temperature 220□C, injection volume of essential volume was 1µl. Helium was employed as carrier gas, at a pressure of 57.5KPa and its flow rate was 1ml/min. Mass spectra were detected at 70eV. Temperature programming was set as follows: column temperature was started from 60 °C (held for 2 min) and linearly increased by 3°C/min to 250°C (held for 4 min). Total GC running time was 69.33 min.

3.3.4 Designing of Primer

Using the keyword "Limonene synthase", a set of EST sequences was downloaded in FASTA format from the dbEST database available in NCBI Genbank (http://www.ncbi.nlm.nih.gov/dbEST/index.html). dbEST is a division of Genbank that contains sequence data and other information on single pass cDNA sequences or expressed sequence tags. Cardamom specific limonene sequence was not available in NCBI Genbank, so limonene specific gene sequences of various plant species were selected for the synthesis of functional SSR primers. By using Multiple Sequence Alignment (MSA) tool in Clustal

Omega, sequences were aligned. The sequences were uploaded to the WEBSAT software for identifying the SSR regions residing within the sequences. However, no SSRs were detected within the target region. So for, validating the results of GC-MS, gene specific primers for limonene were designed with the conserved regions obtained from Multiple Sequence Alignment (MSA). Efficiency of the newly designed primers was verified by using the software Oligo analyzer. The GC content of the derived primers were around 35-55% and melting temperature was about 45-58°C. Details of the primers synthesized were represented in table 4

Table 4: **Gene specific primers**

Sl. No.	Primer	Sequence	No. of bases	GC (%)	Tm (°C)
1	LMP F	GATGATATTTACGATGTCTATGGTAC	26	34.62	53.23
	LMP R	GAATTGATTTCGGCACATCGCCTC	24	50	57.38
2	LMS1 F	ATAAGCCAGTCCAATGCCTT	20	45	49.73
	LMS1 R	TTTGTCCTCCAAGGGAATGG	20	50	51.78
3	LMS2 F	GCAGTCATTGAATAGCAAC	19	42	46.77
	LMS2 R	CCTTCTTCCACATCTGTCTC	20	50	51.78
4	LMK1 F	GAACAGAGCGGCCAGAAA	18	55.56	50.32
	LMK1 R	GATAGCCCACGCGCATATAA	20	50	51.78
5	LMK2 F	TTTCGCCAGAACGGCTTTA	19	47.37	48.98
	LMK2 R	CATCGCCGCGTTTCATTTC	19	52.63	51.09

4. RESULTS

This chapter includes the results of the project work entitled "Development of functional SSR markers for D- Limonene content and analysis of genetic polymorphism in cardamom (*Elettaria cardamomum* Maton)" carried out at the Biotechnology and Bioinformatics Division of JNTBGRI, Palode during the academic year 2017- 2018.

4.1 GENETIC DIVERSITY ANALYSIS

Morphological observations like plant height, number of tillers and number of leaves per tiller were recorded prior to the molecular experimentation procedures. The morphological data documented is represented in table 5

4.1.1 DNA isolation

DNA samples were isolated from the thirty accession used for the study with Origin Plant Genomic DNA Kit. Isolated samples were resolved in 0.8% agarose gel with ethidium bromide for visual observation (Plate 3). The quality and quantity of the isolated materials estimated with biophotometer is represented in table 6

Table 5: Morphological data collected from the thirty different cardamom accessions.

S.No.	Acession no.	Plant variety	Plant height	No. of tillers	No. of leaves per tillers
1	C_{65}	PV2	7	6	17,14,15
2	C_{51}	Kanniyelam	5.10	5	13,11,14
3	T_7	Wild	7.1	7	16,18,14
4	C_{70}	Unknown	4.8	6	14,12,11
5	C_{53}	Unknown	6	6	13,16,17
6	C_{61}	ICRI-2	8	10	16,12,13
7	C_{103}	Pathumuriyan	13.2	13	19,13,17
8	C_{116}	Kothu mysore	8.8	9	8,9,11
9	C_{107}	Njellani	12.4	37	12,25,18
10	C_{108}	Njellani	10.5	22	27,21,23
11	C_{104}	Nadan	10.4	7	12,14,13
12	V_8	Wild	7.1	8	15,11,13
13	C_{106}	Elam Raja	10.9	22	16,15,19
14	C_{105}	Njellani	13.4	35	22,21,17
15	C_{112}	Njellani	12.3	42	17,12,20
16	C_{109}	Green bold	9.7	25	10,13,20
17	C_{118}	Thiruthaali	9.5	40	18,9,10
18	C_{120}	Palakkudi	10.8	35	11,17,15
19	C_{113}	Palakkudi	12	60	18,21,20
20	C_{117}	Vazhukka mysore	9.2	36	21,17,24
21	LC	Large cardamom	5	11	9, 8, 9
22	C_{111}	Njellani	9.10	56	14,17,10
23	C_{64}	PV1	6.4	3	12,13,10
24	C_{24}	Wild	6	5	9,11,14
25	C_{110}	Green bold	11.7	32	26,23,19
26	ER	Elam Raja	10	31	16,17,18
27	NJ_1	Njellani	10.8	13	19,17,16
28	NJ_2	Njellani	11.3	32	14,18,16
29	NJ_3	Njellani	10.3	18	27,20,19
30	NJ_4	Njellani	9.5	40	9,8,20

Table 6: Quality and quantity of isolated DNA samples

Sl. no.	Sample (accession no.)	Concentration (µg/µl)	purity (260/280)	concentration (ng/µl)
1	C_{65}	0.31	1.9	310
2	C_{51}	0.273	1.87	270
3	T_7	0.08	1.97	80
4	C70	0.35	1.79	350
5	C53	0.03	1.76	306
6	C61	0.04	1.63	402
7	C103	0.32	1.7	320
8	C116	0.20	1.93	200
9	C107	0.21	1.91	210
10	C108	0.16	1.89	160
11	C104	0.23	1.83	230
12	V8	0.15	1.8	150
13	C106	0.15	1.96	150
14	C105	0.18	1.93	180
15	C112	0.25	1.87	250
16	C109	0.13	1.91	130
17	C118	0.27	1.86	270
18	C120	0.13	1.88	130
19	C113	0.16	1.86	160
20	C117	0.31	1.94	310
21	LC	0.42	1.91	420
22	C111	0.21	1.90	210
23	C64	0.26	1.92	260
24	C24	0.25	1.87	250
25	C110	0.12	1.85	120
26	ER	0.22	1.90	220
27	NJ1	0.22	1.83	220
28	NJ2	0.17	1.90	170
29	NJ3	0.21	1.88	210
30	NJ4	0.21	1.90	210

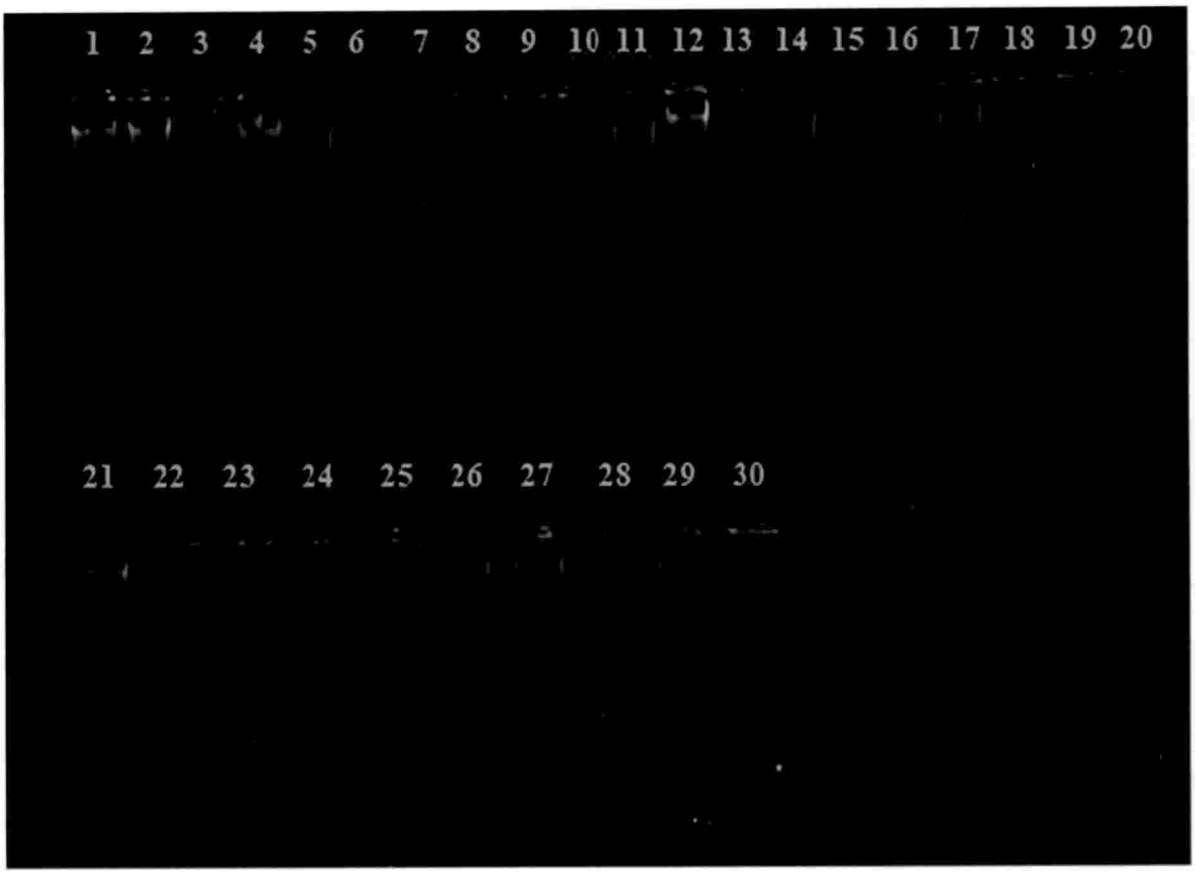


Plate 3: Genomic DNA isolated from 30 cardamom accessions.

Lane representation:

1. C_{65} (PV2)
2. C_{51} (Kanniyelam)
3. T_7 (Wild)
4. C_{70} (Unknown)
5. C_{53} (Unknown)
6. C_{61} (ICRI-2)
7. C_{103} (Pathumuriyan)
8. C_{116} (Kothu mysore)
9. C_{107} (Njellani)
10. C_{108} (Njellani)
11. C_{104} (Nadan)
12. V_8 (Wild)
13. C_{106} (Elam Raja)
14. C_{105} (Njellani)
15. C_{112} (Njellani)
16. C_{109} (Green bold)
17. C_{118} (Thiruthali)
18. C_{120} (Palakkudi)
19. C_{113} (Palakkudi)
20. C_{117} (Vazhukka mysore)
21. LC (Large cardamom)
22. C_{111} (Njellani)
23. C_{64} (PV1)
24. C_{24} (Wild)
25. C_{110} (Green bold)
26. ER (Elam Raja)
27. NJ_1 (Njellani)
28. NJ_2 (Njellani)
29. NJ_3 (Njellani)
30. NJ_4 (Njellani)

4.1.2 Primers used for diversity analysis

Gradient PCR was performed at varying temperatures for optimizing the annealing temperature of the primers used for diversity analysis. The six SSR primers along with their optimized annealing temperature is represented by table 7. Amplified DNA sequences were observed by 3% agarose gel electrophoresis (Plate: 4-9).

Table 7: Annealing temperature of Primers used for diversity analysis

Primers	CaSSR 41	CaSSR 42	CaSSR 46	CaSSR 49	CaSSR 52	CaSSR 53
Annealing Temperature	50 ^{0}C	50 ^{0}C	52 ^{0}C	44 ^{0}C	58 ^{0}C	56 ^{0}C

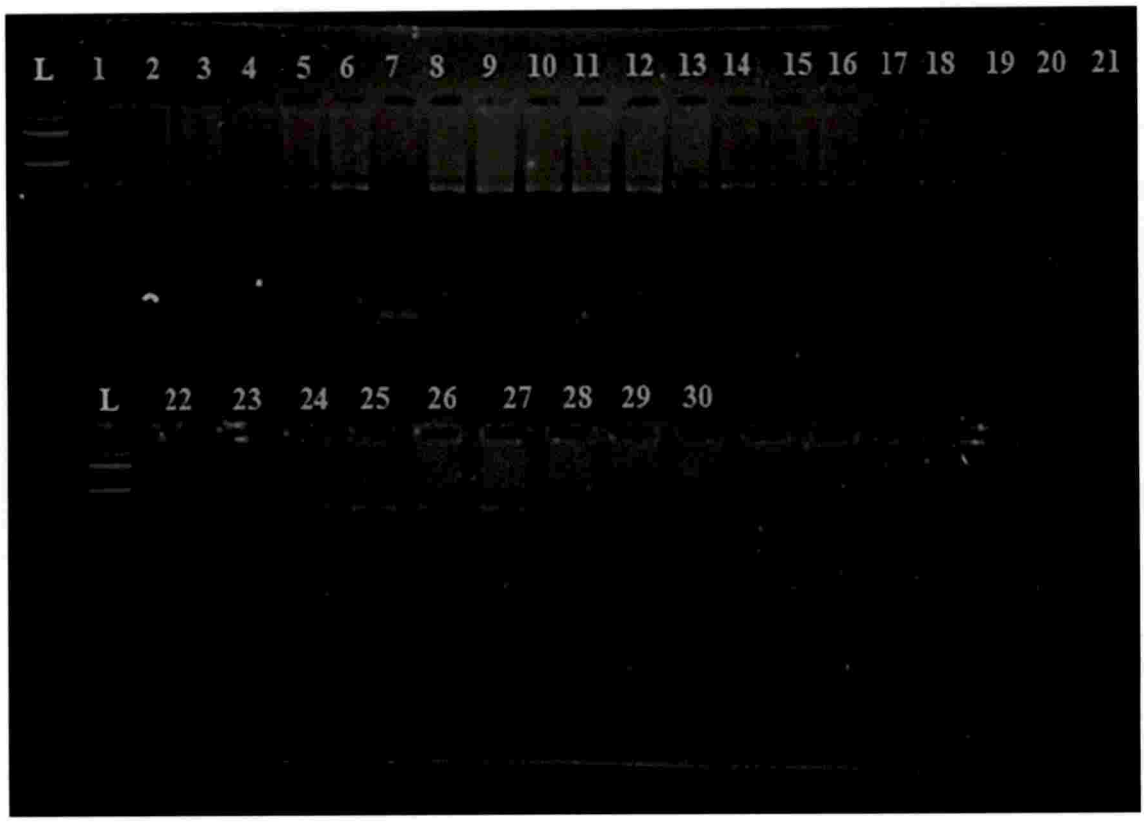


Plate 4: SSR profile obtained for primer CaSSR 41 (Product size: 310bp)

Lane representation:

L- Ladder

1. C_{65} (PV2)
2. C_{51} (Kanniyelam)
3. T_7 (Wild)
4. C_{70} (Unknown)
5. C_{53} (Unknown)
6. C_{61} (ICRI-2)
7. C_{103} (Pathumuriyan)
8. C_{116} (Kothu mysore)
9. C_{107} (Njellani)
10. C_{108} (Njellani)
11. C_{104} (Nadan)
12. V_8 (Wild)
13. C_{106} (Elam Raja)
14. C_{105} (Njellani)
15. C_{112} (Njellani)
16. C_{109} (Green bold)
17. C_{118} (Thiruthali)
18. C_{120} (Palakkudi)
19. C_{113} (Palakkudi)
20. C_{117} (Vazhukka mysore)
21. LC (Large cardamom)
22. C_{111} (Njellani)
23. C_{64} (PV1)
24. C_{24} (Wild)
25. C_{110} (Green bold)
26. ER (Elam Raja)
27. NJ_1 (Njellani)
28. NJ_2 (Njellani)
29. NJ_3 (Njellani)
30. NJ_4 (Njellani)

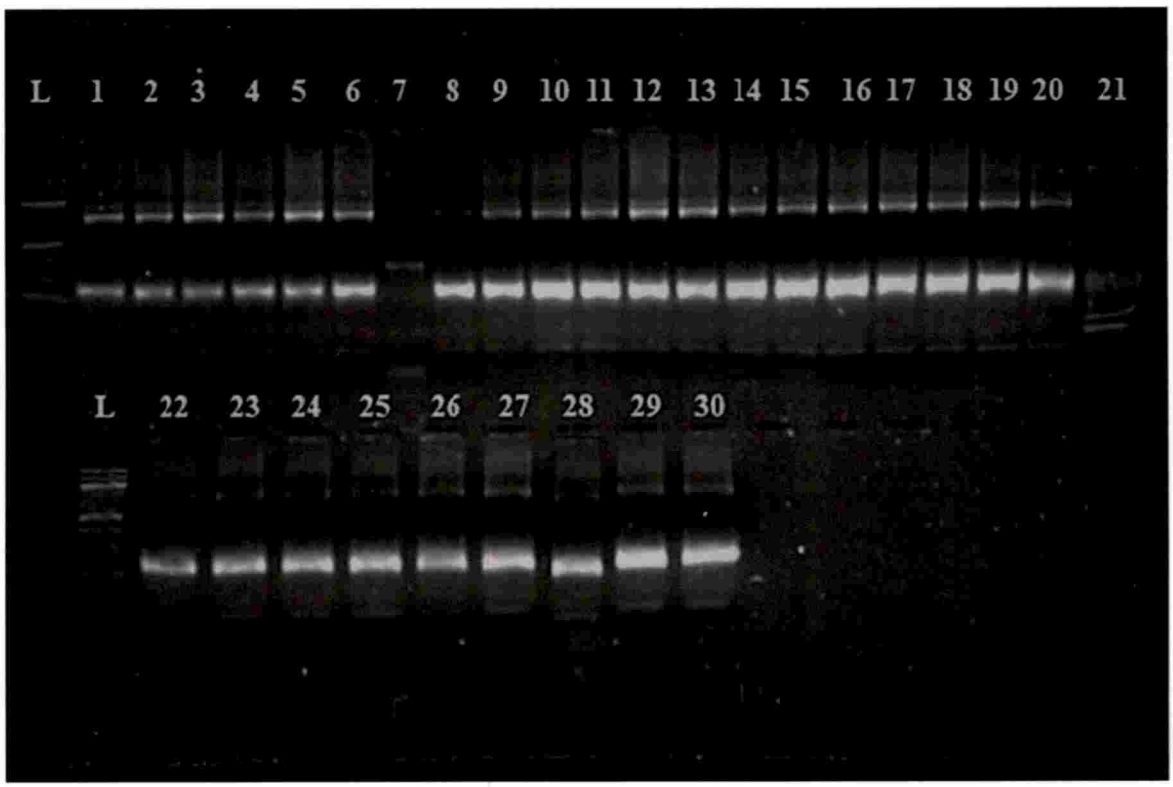


Plate 5: SSR Profile obtained for CaSSR 42 (Product size: 271b)

Lane representation:

L- Ladder

1. C_{65} (PV2)
2. C_{51} (Kanniyelam)
3. T_7 (Wild)
4. C_{70} (Unknown)
5. C_{53} (Unknown)
6. C_{61} (ICRI-2)
7. C_{103} (Pathumuriyan)
8. C_{116} (Kothu mysore)
9. C_{107} (Njellani)
10. C_{108} (Njellani)
11. C_{104} (Nadan)
12. V_8 (Wild)
13. C_{106} (Elam Raja)
14. C_{105} (Njellani)
15. C_{112} (Njellani)
16. C_{109} (Green bold)
17. C_{118} (Thiruthali)
18. C_{120} (Palakkudi)
19. C_{113} (Palakkudi)
20. C_{117} (Vazhukka mysore)
21. LC (Large cardamom)
22. C_{111} (Njellani)
23. C_{64} (PV1)
24. C_{24} (Wild)
25. C_{110} (Green bold)
26. ER (Elam Raja)
27. NJ_1 (Njellani)
28. NJ_2 (Njellani)
29. NJ_3 (Njellani)
30. NJ_4 (Njellani)

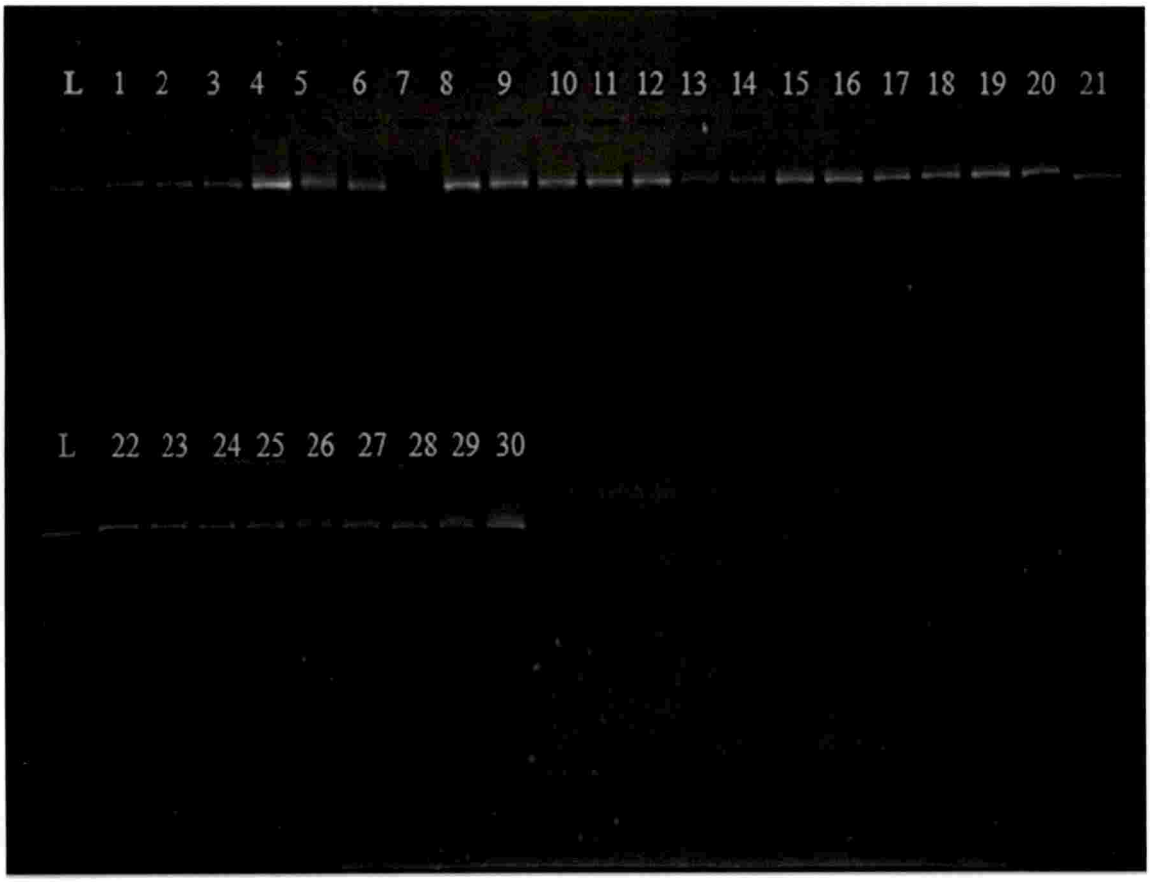


Plate 6: SSR Profile obtained for CaSSR 46 (Product size: 184bp)

Lane representation:

L- Ladder
1. C_{65} (PV2)
2. C_{51} (Kanniyelam)
3. T_7 (Wild)
4. C_{70} (Unknown)
5. C_{53} (Unknown)
6. C_{61} (ICRI-2)
7. C_{103} (Pathumuriyan)
8. C_{116} (Kothu mysore)
9. C_{107} (Njellani)
10. C_{108} (Njellani)
11. C_{104} (Nadan)
12. V_8 (Wild)
13. C_{106} (Elam Raja)
14. C_{105} (Njellani)
15. C_{112} (Njellani)
16. C_{109} (Green bold)
17. C_{118} (Thiruthali)
18. C_{120} (Palakkudi)
19. C_{113} (Palakkudi)
20. C_{117} (Vazhukka mysore)
21. LC (Large cardamom)
22. C_{111} (Njellani)
23. C_{64} (PV1)
24. C_{24} (Wild)
25. C_{110} (Green bold)
26. ER (Elam Raja)
27. NJ_1 (Njellani)
28. NJ_2 (Njellani)
29. NJ_3 (Njellani)
30. NJ_4 (Njellani)

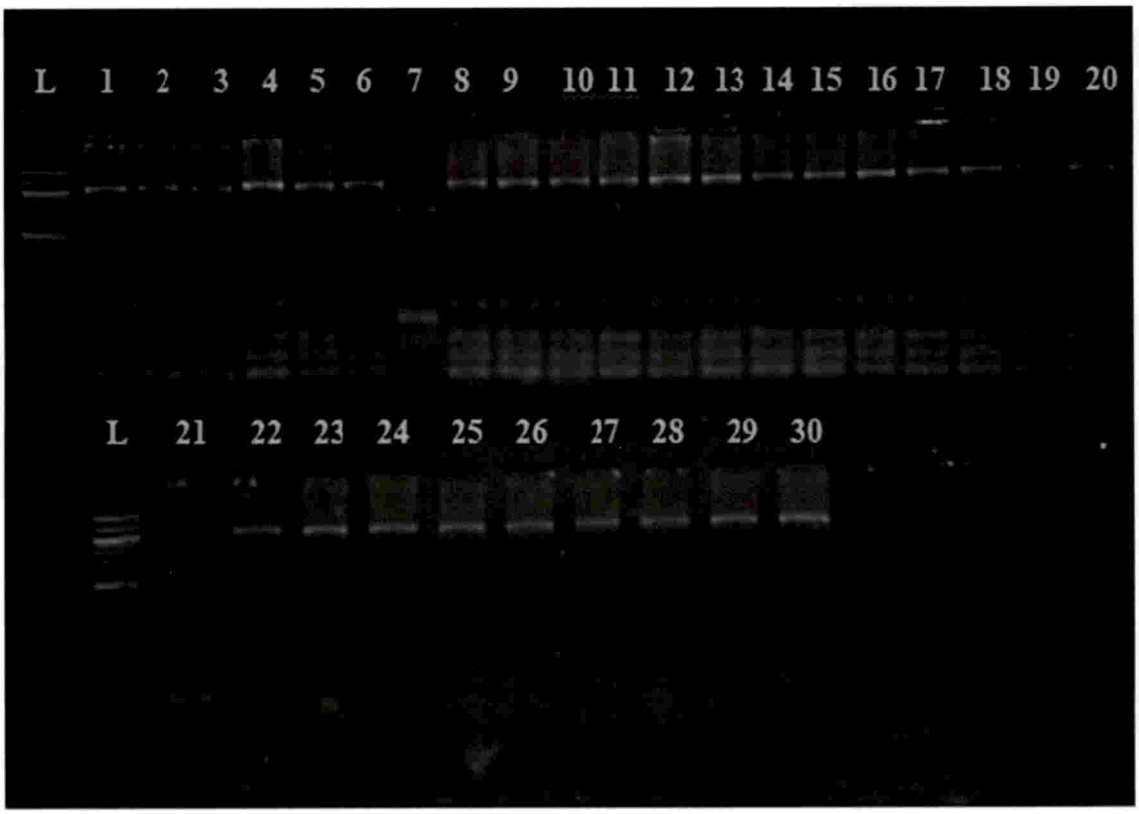


Plate 7: SSR Profile obtained for CaSSR 49 (Product size: 210 bp)

Lane representation:

L- Ladder

1. C_{65} (PV2)
2. C_{51} (Kanniyelam)
3. T_7 (Wild)
4. C_{70} (Unknown)
5. C_{53} (Unknown)
6. C_{61} (ICRI-2)
7. C_{103} (Pathumuriyan)
8. C_{116} (Kothu mysore)
9. C_{107} (Njellani)
10. C_{108} (Njellani)
11. C_{104} (Nadan)
12. V_8 (Wild)
13. C_{106} (Elam Raja)
14. C_{105} (Njellani)
15. C_{112} (Njellani)
16. C_{109} (Green bold)
17. C_{118} (Thiruthali)
18. C_{120} (Palakkudi)
19. C_{113} (Palakkudi)
20. C_{117} (Vazhukka mysore)
21. LC (Large cardamom)
22. C_{111} (Njellani)
23. C_{64} (PV1)
24. C_{24} (Wild)
25. C_{110} (Green bold)
26. ER (Elam Raja)
27. NJ_1 (Njellani)
28. NJ_2 (Njellani)
29. NJ_3 (Njellani)
30. NJ_4 (Njellani)

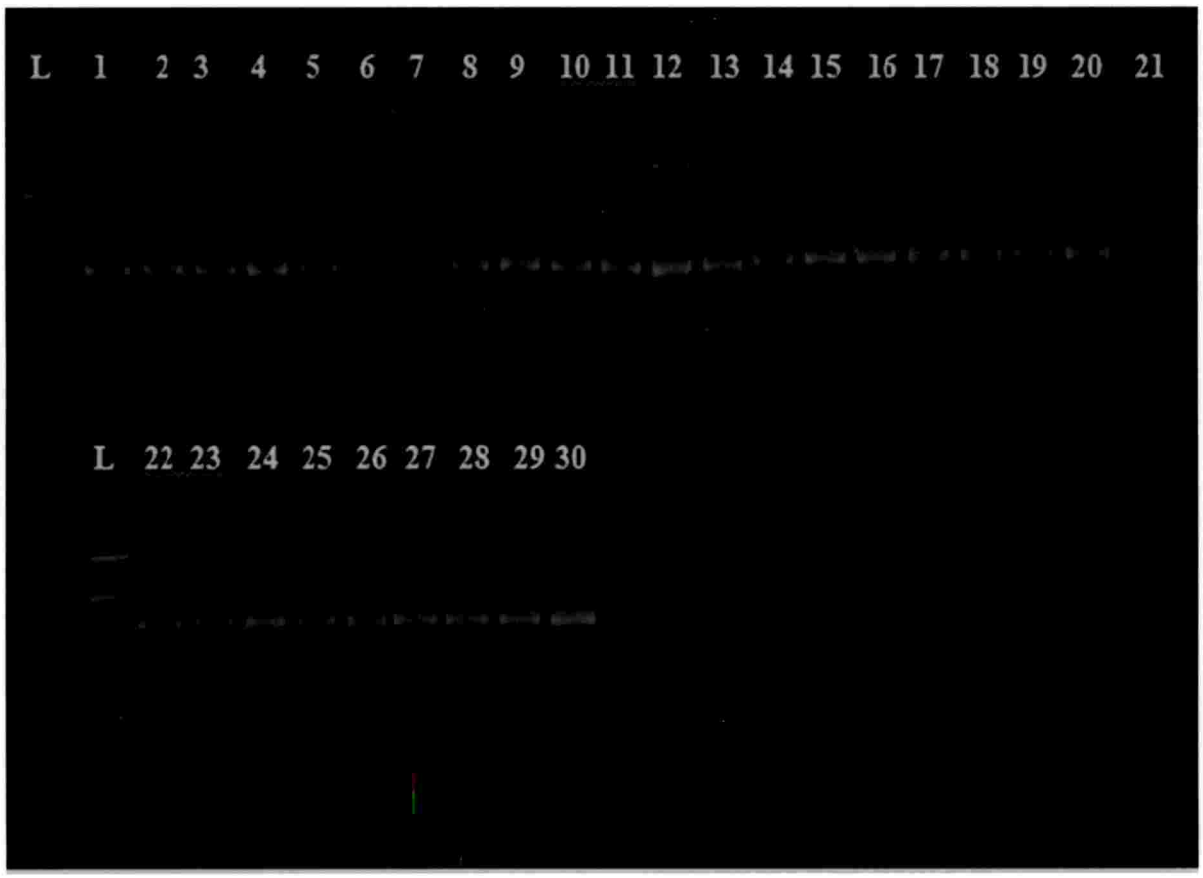


Plate 8: SSR Profile obtained for CaSSR 52 (Product size: 332bp)

Lane representation:

L - Ladder

1. C_{65} (PV2)
2. C_{51} (Kanniyelam)
3. T_7 (Wild)
4. C_{70} (Unknown)
5. C_{53} (Unknown)
6. C_{61} (ICRI-2)
7. C_{103} (Pathumuriyan)
8. C_{116} (Kothu mysore)
9. C_{107} (Njellani)
10. C_{108} (Njellani)
11. C_{104} (Nadan)
12. V_8 (Wild)
13. C_{106} (Elam Raja)
14. C_{105} (Njellani)
15. C_{112} (Njellani)
16. C_{109} (Green bold)
17. C_{118} (Thiruthali)
18. C_{120} (Palakkudi)
19. C_{113} (Palakkudi)
20. C_{117} (Vazhukka mysore)
21. LC (Large cardamom)
22. C_{111} (Njellani)
23. C_{64} (PV1)
24. C_{24} (Wild)
25. C_{110} (Green bold)
26. ER (Elam Raja)
27. NJ_1 (Njellani)
28. NJ_2 (Njellani)
29. NJ_3 (Njellani)
30. NJ_4 (Njellani)

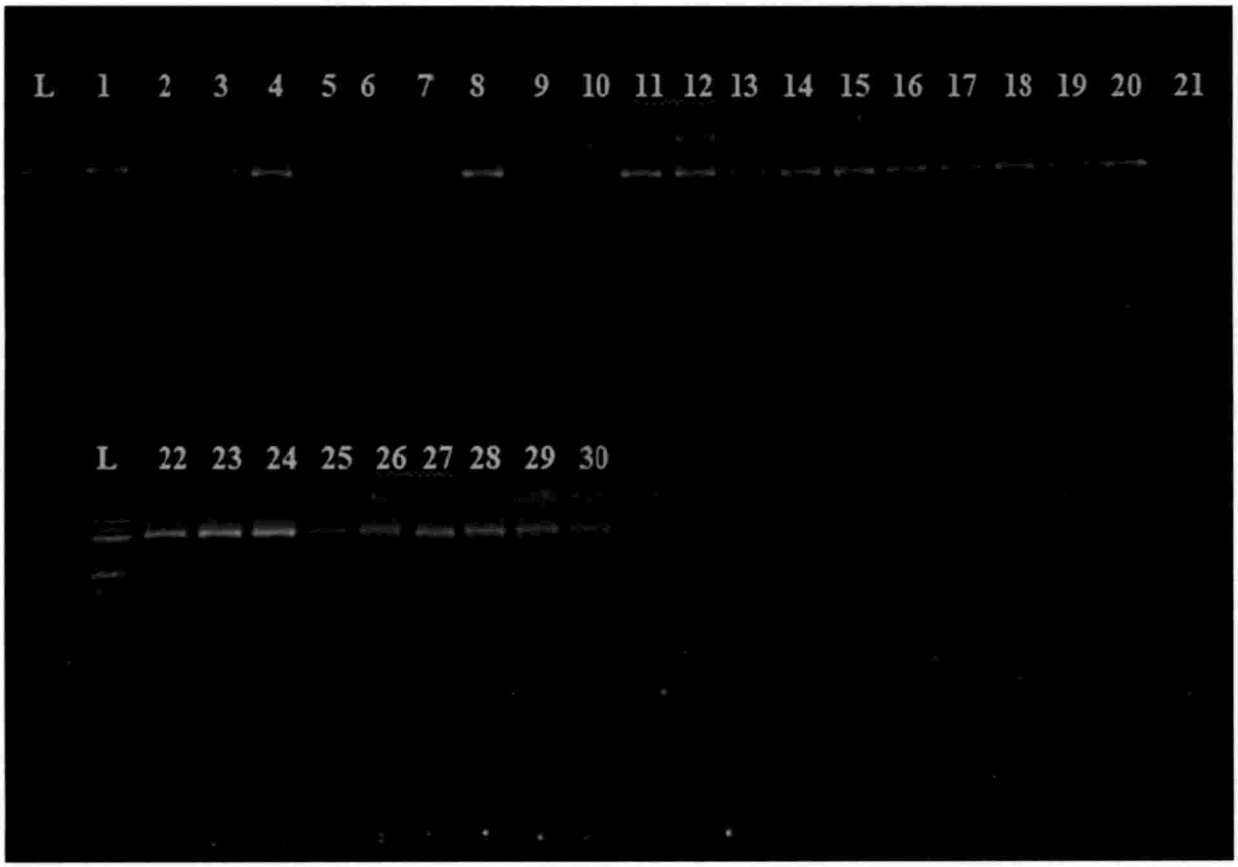


Plate 9: SSR Profile obtained for CaSSR 53 (Product size: 311bp)

Lane representation:

L- Ladder

1. C_{65} (PV2)
2. C51 (Kanniyelam)
3. T7 (Wild)
4. C70 (Unknown)
5. C53 (Unknown)
6. C61 (ICRI-2)
7. C103 (Pathumuriyan)
8. C116 (Kothu mysore)
9. C107 (Njellani)
10. C108 (Njellani)
11. C104 (Nadan)
12. V8 (Wild)
13. C106 (Elam Raja)
14. C105 (Njellani)
15. C112 (Njellani)
16. C109 (Green bold)
17. C118 (Thiruthali)
18. C120 (Palakkudi)
19. C113 (Palakkudi)
20. C117 (Vazhukka mysore)
21. LC (Large cardamom)
22. C111 (Njellani)
23. C64 (PV1)
24. C24 (Wild)
25. C110 (Green bold)
26. ER (Elam Raja)
27. NJ1 (Njellani)
28. NJ2 (Njellani)
29. NJ3 (Njellani)
30. NJ4 (Njellan)

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4.2 SSR PROFILE AND GENETIC DATA ANALYSIS

Genetic data analysis of the 30 accessions was subjected to microsatellite analysis and their summary of genetic variation and genetic distance were statistically generated using POPGENE (Yeh and Boyle, 1997). Results are represented in Table 8

4.3 ESTIMATION OF SSR POLYMORPHISM

The observed number of alleles per locus (na) ranged from 1.00 to 1.33, effective number of alleles (ne) also ranged from 1.00 to 1.33, Shannon's information index (I) ranged from 0.00 to 0.23, observed heterozygosity (Obs_Het) ranged from 0.00 to 0.33, expected heterozygosity (Exp_Het) ranged from 0.00 to 0.33, Nei's heterozygosity (h) ranged from 0.00 to 0.16, average heterozygosity (Ave_Het) ranged from 0.01 to 0.09 and percentage of polymorphic loci (P) ranged from 0.00 to 33.33.

4.4 GENETIC RELATIONSHIPS AND CLUSTER ANALYSIS

The pair- wise genetic similarity and distance matrix were prepared on the basis of SSR data (Table 9). The genetic similarity values varied from 0.10 to 1.00 with a mean value of 0.854.

Cluster analysis was performed on the SSR data using POPGENE software following the UPGMA method and the dendrogram were constructed through MEGA7 showing overall genetic relatedness among the individuals (Figure 1). The accession studied were clustered into two main group, one with 29 accessions and other with one accession, which is an out group sample (Large cardamom). The first group has again divided into two subgroup and the remaining 29 samples are mixed in the cluster. The dendrogram revealed a complex distribution of pattern.

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Table 8: Summary of genetic variation statistics for 30 accessions analyzed using 6 SSR primer pairs

Sl.No.	Accession no.	na*	ne*	I*	Obs_Het	Exp_Het*	Nei**	Ave_Het	Number of polymorphic loci	% of polymorphic loci (P)
1	C65	1.0000	1.0000	0.0000	0.0000	0.0000	0.0000	0.0667	0	0.00
2	C51	1.0000	1.0000	0.0000	0.0000	0.0000	0.0000	0.0733	0	0.00
3	T7	1.0000	1.0000	0.0000	0.0000	0.0000	0.0000	0.0800	0	0.00
4	C70	1.0000	1.0000	0.0000	0.0000	0.0000	0.0000	0.0667	0	0.00
5	C53	1.0000	1.0000	0.0000	0.0000	0.0000	0.0000	0.0667	0	0.00
6	C61	1.0000	1.0000	0.0000	0.0000	0.0000	0.0000	0.0667	0	0.00
7	C103	1.0000	1.0000	0.0000	0.0000	0.0000	0.0000	0.0917	0	0.00
8	C116	1.1667	1.1667	0.1155	0.1667	0.1667	0.0833	0.0667	1	16.67
9	C107	1.2000	1.2000	0.1386	0.2000	0.2000	0.1000	0.0733	1	16.67
10	C108	1.2000	1.2000	0.1386	0.2000	0.2000	0.1000	0.0733	1	16.67
11	C104	1.1667	1.1667	0.1155	0.1667	0.1667	0.0833	0.0667	1	16.67
12	V8	1.2000	1.2000	0.1386	0.2000	0.2000	0.1000	0.0800	1	16.67
13	C106	1.1667	1.1667	0.1155	0.1667	0.1667	0.0833	0.0667	1	16.67
14	C105	1.1667	1.1667	0.1155	0.1667	0.1667	0.0833	0.0667	1	16.67
15	C112	1.1667	1.1667	0.1155	0.1667	0.1667	0.0833	0.0667	1	16.67
16	C109	1.1667	1.1667	0.1155	0.1667	0.1667	0.0833	0.0667	1	16.67
17	C118	1.1667	1.1667	0.1155	0.1667	0.1667	0.0833	0.0667	1	16.67
18	C120	1.1667	1.1667	0.1155	0.1667	0.1667	0.0833	0.0667	1	16.67
19	C113	1.1667	1.1667	0.1155	0.1667	0.1667	0.0833	0.0667	1	16.67
20	C117	1.1667	1.1667	0.1155	0.1667	0.1667	0.0833	0.0667	1	16.67
21	LC	1.1667	1.1667	0.1155	0.1667	0.1667	0.0833	0.0667	1	16.67
22	C111	1.1667	1.1667	0.1155	0.1667	0.1667	0.0833	0.0667	1	16.67
23	C64	1.3333	1.3333	0.2310	0.3333	0.3333	0.1667	0.0667	2	33.33
24	C24	1.3333	1.3333	0.2310	0.3333	0.3333	0.1667	0.0667	2	33.33
25	C110	1.2000	1.2000	0.1386	0.2000	0.2000	0.1000	0.0733	1	16.67
26	ER	1.1667	1.1667	0.1155	0.1667	0.1667	0.0833	0.0667	1	16.67
27	NJ1	1.2000	1.2000	0.1386	0.2000	0.2000	0.1000	0.0733	1	16.67
28	NJ2	1.0000	1.0000	0.0000	0.0000	0.0000	0.0000	0.0111	0	0.00
29	NJ3	1.2000	1.2000	0.1386	0.2000	0.2000	0.1000	0.0733	1	16.67
30	NJ4	1.2000	1.2000	0.1386	0.2000	0.2000	0.1000	0.0733	1	16.67
	Min	**1.00**	**1.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.01**	**0**	**0.00**
	Max	**1.33**	**1.33**	**0.23**	**0.33**	**0.33**	**0.16**	**0.09**	**2**	**33.33**

Table 9: Nei's original measures of genetic identity and genetic distance (Nei, 1972) estimated from the SSR analysis

pop ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	****	0.91	0.91	1.00	1.00	1.00	0.00	0.98	0.89	0.89	0.98	0.89	0.98	0.98	0.98
2	0.10	****	0.80	0.91	0.91	0.91	0.00	0.88	0.97	0.97	0.88	0.76	0.88	0.88	0.88
3	0.10	0.22	****	0.91	0.91	0.91	0.00	0.88	0.76	0.76	0.88	0.76	0.88	0.88	0.88
4	0.00	0.10	0.10	****	1.00	1.00	0.00	0.98	0.89	0.89	0.98	0.89	0.98	0.98	0.98
5	0.00	0.10	0.10	0.00	****	1.00	0.00	0.98	0.89	0.89	0.98	0.89	0.98	0.98	0.98
6	0.00	0.10	0.10	0.00	0.00	****	0.00	0.98	0.89	0.89	0.98	0.89	0.98	0.98	0.98
7	0.00	0.00	0.00	0.00	0.00	0.00	****	0.10	0.12	0.12	0.10	0.12	0.10	0.10	0.10
8	0.02	0.13	0.13	0.02	0.02	0.02	2.22	****	0.93	0.93	1.03	0.93	1.03	1.03	1.03
9	0.12	0.03	0.29	0.12	0.12	0.12	2.11	0.07	****	1.03	0.93	0.80	0.93	0.93	0.93
10	0.12	0.03	0.29	0.12	0.12	0.12	2.11	0.07	-0.03	****	0.93	0.80	0.93	0.93	0.93
11	0.02	0.13	0.13	0.02	0.02	0.02	2.22	-0.03	0.07	0.07	****	0.93	1.03	1.03	1.03
12	0.12	0.29	0.29	0.12	0.12	0.12	2.11	0.07	0.21	0.21	0.07	****	0.93	0.93	0.93
13	0.02	0.13	0.13	0.02	0.02	0.02	2.22	-0.03	0.07	0.07	-0.03	0.07	****	1.03	1.03
14	0.02	0.13	0.13	0.02	0.02	0.02	2.22	-0.03	0.07	0.07	-0.03	0.07	-0.03	****	1.03
15	0.02	0.13	0.13	0.02	0.02	0.02	2.22	-0.03	0.07	0.07	-0.03	0.07	-0.03	-0.03	****
16	0.02	0.13	0.13	0.02	0.02	0.02	2.22	-0.03	0.07	0.07	-0.03	0.07	-0.03	-0.03	-0.03
17	0.02	0.13	0.13	0.02	0.02	0.02	2.22	-0.03	0.07	0.07	-0.03	0.07	-0.03	-0.03	-0.03
18	0.02	0.13	0.13	0.02	0.02	0.02	2.22	-0.03	0.07	0.07	-0.03	0.07	-0.03	-0.03	-0.03
19	0.02	0.13	0.13	0.02	0.02	0.02	2.22	-0.03	0.07	0.07	-0.03	0.07	-0.03	-0.03	-0.03
20	0.02	0.13	0.13	0.02	0.02	0.02	2.22	-0.03	0.07	0.07	-0.03	0.07	-0.03	-0.03	-0.03
21	0.45	0.72	0.72	0.49	0.49	0.49	2.22	0.42	0.66	0.66	0.42	0.31	0.42	0.42	0.42
22	0.02	0.13	0.13	0.02	0.02	0.02	2.22	-0.03	0.07	0.07	-0.03	0.07	-0.03	-0.03	-0.03
23	0.06	0.08	0.19	0.05	0.06	0.06	2.15	-0.00	-0.00	-0.00	-0.00	0.11	-0.00	-0.00	-0.00
24	0.06	0.08	0.19	0.05	0.06	0.06	2.15	-0.00	-0.00	-0.00	-0.00	0.11	-0.00	-0.00	-0.00
25	0.12	0.03	0.29	0.12	0.12	0.12	2.11	0.07	-0.03	-0.03	0.07	0.21	0.07	0.07	0.07
26	0.02	0.13	0.13	0.02	0.02	0.02	2.22	-0.03	0.07	0.07	-0.03	0.07	-0.03	-0.03	-0.03
27	0.12	0.03	0.29	0.12	0.12	0.12	2.11	0.07	-0.03	-0.03	0.07	0.21	0.07	0.07	0.07
28	0.34	0.67	0.67	0.34	0.34	0.34	0.00	0.29	0.59	0.59	0.29	0.59	0.29	0.29	0.29
29	0.12	0.03	0.29	0.12	0.12	0.12	2.11	0.07	-0.03	-0.03	0.07	0.21	0.07	0.07	0.07
30	0.12	0.03	0.29	0.12	0.12	0.12	2.11	0.07	-0.03	-0.03	0.07	0.21	0.07	0.07	0.07

pop ID	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1	0.98	0.98	0.98	0.98	0.98	0.61	0.98	0.94	0.94	0.89	0.98	0.89	0.70	0.89	0.89
2	0.88	0.88	0.88	0.88	0.88	0.49	0.88	0.93	0.93	0.97	0.88	0.97	0.51	0.97	0.97
3	0.88	0.88	0.88	0.88	0.88	0.49	0.88	0.82	0.82	0.76	0.88	0.76	0.51	0.75	0.75
4	0.98	0.98	0.98	0.98	0.98	0.61	0.98	0.94	0.94	0.89	0.98	0.89	0.70	0.89	0.89
5	0.98	0.98	0.98	0.98	0.98	0.61	0.98	0.94	0.94	0.89	0.98	0.89	0.70	0.89	0.89
6	0.98	0.98	0.98	0.98	0.98	0.61	0.98	0.94	0.94	0.89	0.98	0.89	0.70	0.89	0.89
7	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.11	0.11	0.12	0.10	0.12	0.00	0.12	0.12
8	1.03	1.03	1.03	1.03	1.03	0.65	1.03	1.00	1.00	0.93	1.03	0.93	0.75	0.93	0.93
9	0.93	0.93	0.93	0.93	0.93	0.52	0.93	1.00	1.00	1.03	0.93	1.03	0.55	1.03	1.03
10	0.93	0.93	0.93	0.93	0.93	0.52	0.93	1.00	1.00	1.03	0.93	1.03	0.55	1.03	1.03
11	1.03	1.03	1.03	1.03	1.03	0.65	1.03	1.00	1.00	0.93	1.03	0.93	0.75	0.93	0.93
12	0.93	0.93	0.93	0.93	0.93	0.72	0.93	0.89	0.89	0.80	0.93	0.80	0.55	0.80	0.80
13	1.03	1.03	1.03	1.03	1.03	0.65	1.03	1.00	1.00	0.93	1.03	0.93	0.75	0.93	0.93
14	1.03	1.03	1.03	1.03	1.03	0.65	1.03	1.00	1.00	0.93	1.03	0.93	0.75	0.93	0.93
15	1.03	1.03	1.03	1.03	1.03	0.65	1.03	1.00	1.00	0.93	1.03	0.93	0.75	0.93	0.93
16	****	1.03	1.03	1.03	1.03	0.65	1.03	1.00	1.00	0.93	1.03	0.93	0.75	0.93	0.93
17	-0.03	****	1.03	1.03	1.03	0.65	1.03	1.00	1.00	0.93	1.03	0.93	0.75	0.93	0.93
18	-0.03	-0.03	****	1.03	1.03	0.65	1.03	1.00	1.00	0.93	1.03	0.93	0.75	0.93	0.93
19	-0.03	-0.03	-0.03	****	1.03	0.65	1.03	1.00	1.00	0.93	1.03	0.93	0.75	0.93	0.93
20	-0.03	-0.03	-0.03	-0.03	****	0.65	1.03	1.00	1.00	0.93	1.03	0.93	0.75	0.93	0.93
21	-0.00	0.42	0.42	0.42	0.42	****	0.65	0.60	0.60	0.52	0.65	0.52	0.50	0.52	0.52
22	-0.03	-0.03	-0.03	-0.03	-0.03	0.42	****	1.00	1.00	0.93	1.03	0.93	0.75	0.93	0.93
23	-0.00	-0.00	-0.00	-0.00	-0.00	0.50	-0.00	****	1.08	1.00	1.00	1.00	0.66	1.00	1.00
24	-0.00	-0.00	-0.00	-0.00	-0.00	0.50	-0.00	-0.07	****	1.00	1.00	1.00	0.67	1.00	1.00
25	0.07	0.07	0.07	0.07	0.07	0.66	0.07	-0.00	-0.00	****	0.93	1.03	0.55	1.03	1.03
26	-0.03	-0.03	-0.03	-0.03	-0.03	0.42	-0.03	-0.00	-0.00	0.07	****	0.93	0.75	0.93	0.93
27	0.07	0.07	0.07	0.07	0.07	0.66	0.07	-0.00	-0.00	-0.03	0.07	****	0.55	1.03	1.03
28	0.29	0.29	0.29	0.29	0.29	0.70	0.29	0.40	0.40	0.59	0.29	0.59	****	0.55	0.55
29	0.07	0.07	0.07	0.07	0.07	0.66	0.07	-0.00	-0.00	-0.03	0.07	-0.03	0.59	****	1.03
30	0.07	0.07	0.07	0.07	0.07	0.66	0.07	-0.00	-0.00	-0.03	0.07	-0.03	0.589	-0.03	****

*****Nei's genetic identity (above diagonal) and genetic distance (below diagonal)**

S4.4

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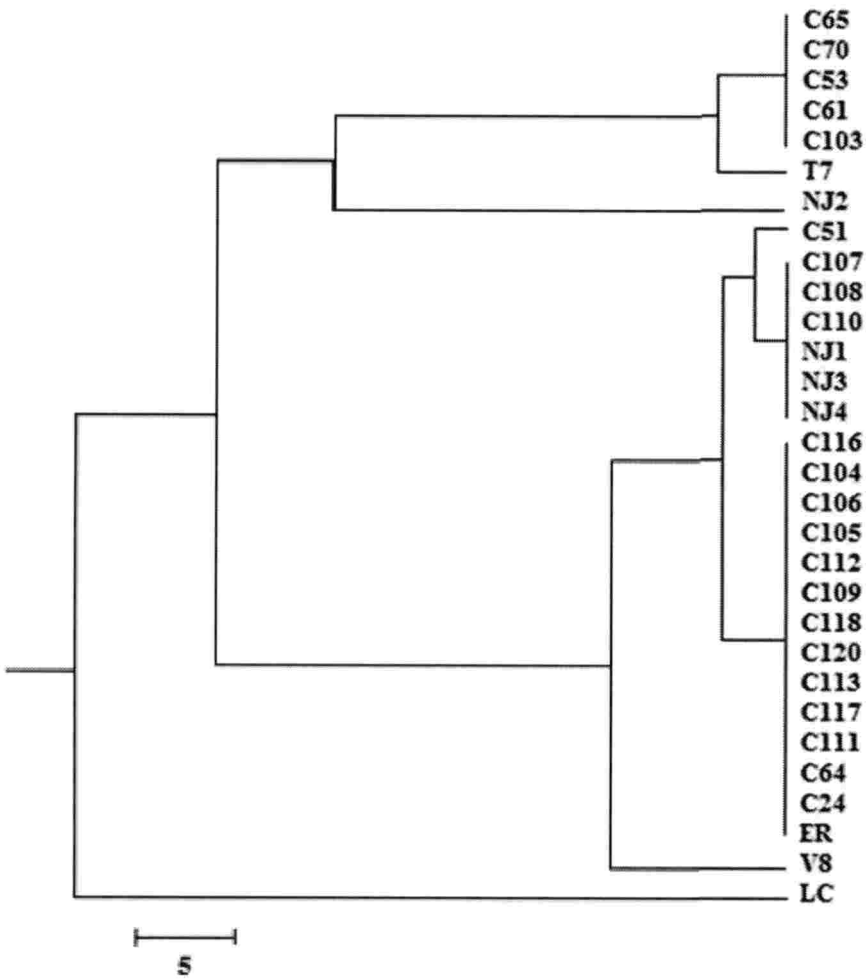

Figure 1: Dendrogram based on genetic distance assessed using 6 SSR primers in 30 cardamom accessions denoted by the local name.

4.5 GC- MS Analysis

GC- MS analysis of essential oil of cardamom revealed the presence of around 45 components in selected four samples (Palakkudi, Pathumuriyan, Green bold and Njellani) of cardamom. The limonene percentages were identified from their fragmentation pattern by mass spectrometry using NIIST library. The percentage of the components in oil were identified by their retention time, retention indices as well as by comparison of their mass spectra (Table 10-13). By comparing the retention time and area percentage of D-limonene of each sample, it is identified that Njellani and Green bold has highest amount of limonene compared with other two (Table 14). Spectrogram obtained for the four samples Palakkudi, Pathumuriyan, Green bold and Njellani were represented as figure 2, 3, 4, 5 respectively.

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Table 10: Essential oil components of the sample Palakkudi

Peak#	Ret.Time	Area%	Height%	Name
1	**9.230**	**0.31**	**0.48**	**D-Limonene**
2	9.340	2.28	3.54	Eucalyptol
3	10.864	0.79	1.01	5-ISOPROPYL-2-METHYLBICYCLO[3.1.0]HEXAN-2-OL
4	12.112	3.38	4.13	LINALOOL L
5	15.129	0.36	0.33	3-Cyclohexene-1-methanol, .alpha.,.alpha.,4-trimethyl-
6	15.575	3.29	4.14	3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)-
7	15.940	0.27	0.29	Benzenemethanol, .alpha.,.alpha.,4-trimethyl-
8	16.329	31.01	25.58	3-Cyclohexene-1-methanol, .alpha.,.alpha.,4-trimethyl-
9	16.929	0.43	0.57	1-Hexanol, 2-ethyl-, acetate
10	17.335	0.47	0.59	6-Octenal, 2,3-epoxy-3,7-dimethyl-
11	17.636	0.27	0.30	2,3-Pinanediol
12	17.806	0.46	0.60	Oxiranecarboxaldehyde, 3-methyl-3-(4-methyl-3-pentenyl)-
13	18.748	2.37	2.40	(2E)-3,7-DIMETHYL-2,6-OCTADIEN-1-OL
14	21.120	0.34	0.29	2-METHYLBICYCLO[2.2.1]HEPTAN-2-OL
15	21.383	0.40	0.36	Ocimenyl acetate
16	21.786	1.06	1.21	2,6-Octadienoic acid, 3,7-dimethyl-, methyl ester, (Z)-
17	22.913	27.78	30.28	1-METHYL-1-(4-METHYL-3-CYCLOHEXEN-1-YL)ETHYL ACETATE
18	24.253	4.28	5.04	2,6-Octadien-1-ol, 3,7-dimethyl-, acetate, (E)-
19	24.601	0.52	0.37	(Z)-Dec-4-enyl ethyl carbonate
20	26.442	1.00	0.69	1-Methyl-4-(1-acetoxy-1-methylethyl)-cyclohex-2-enol
21	27.062	1.16	1.29	Epoxy-.alpha.-terpenyl acetate
22	27.503	1.99	2.09	Epoxy-.alpha.-terpenyl acetate
23	28.706	0.47	0.61	.beta.-Selinene
24	29.710	0.95	1.06	1-ISOPROPYL-7-METHYL-4-METHYLENE-1,2,3,4,4A,5,6,8A-OCTAHYDRONAPHTHALENE
25	30.066	1.35	1.23	Hydroxy-.alpha.-terpenyl acetate
26	31.253	0.84	0.70	Hydroxy-.alpha.-terpenyl acetate
27	31.686	3.96	3.64	1,6,10-Dodecatrien-3-ol, 3,7,11-trimethyl-
28	33.646	1.85	1.42	1-Methyl-4-(1-acetoxy-1-methylethyl)-cyclohex-2-enol
29	34.772	0.95	0.84	1-Methyl-4-(1-acetoxy-1-methylethyl)-cyclohex-2-enol
30	34.905	1.72	1.27	Hydroxy-.alpha.-terpenyl acetate
31	36.294	0.71	0.66	Terpinyl formate
32	37.496	0.50	0.46	2,6,10-Dodecatrien-1-ol, 3,7,11-trimethyl-
33	40.316	0.89	0.83	Acetic acid, 1-[2-(2,2,6-trimethyl-bicyclo[4.1.0]hept-1-yl)-ethyl]-vinyl ester

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34	50.696	0.47	0.48	Retinal, 9-cis-
35	56.046	0.55	0.61	Heneicosane
36	61.399	0.57	0.61	Tetratetracontane

Table 11: Essential oil components of the sample Pathumuriyan

Peak#	Ret.Time	Area%	Height%	Name
1	**9.234**	**0.10**	**0.15**	**D-Limonene**
2	9.344	3.30	4.74	Eucalyptol
3	10.874	0.19	0.24	5-ISOPROPYL-2-METHYLBICYCLO[3.1.0]HEXAN-2-OL
4	12.114	2.84	3.34	LINALOOL L
5	13.140	0.31	0.38	4-ISOPROPYL-1-METHYL-3-CYCLOHEXEN-1-OL
6	13.919	0.25	0.31	4-ISOPROPYL-1-METHYL-3-CYCLOHEXEN-1-OL
7	14.326	0.12	0.16	METHYL 2-HYDROXYHEXANOATE
8	15.134	0.44	0.41	3-Cyclohexene-1-methanol, .alpha.,.alpha.,4-trimethyl-
9	15.585	7.73	8.89	3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)-
10	16.275	17.44	17.50	3-Cyclohexene-1-methanol, .alpha.,.alpha.,4-trimethyl-
11	16.865	0.15	0.19	2-Cyclohexen-1-ol, 3-methyl-6-(1-methylethyl)-, trans-
12	16.929	0.29	0.33	1-Hexanol, 2-ethyl-, acetate
13	18.123	0.28	0.29	2,6-Octadienal, 3,7-dimethyl-, (Z)-
14	18.646	0.15	0.22	LINALYL ACETATE
15	18.747	4.01	3.38	(2E)-3,7-DIMETHYL-2,6-OCTADIEN-1-OL
16	19.443	0.70	0.65	2,6-Octadienal, 3,7-dimethyl-
17	21.203	0.18	0.09	4-Decenoic acid, methyl ester
18	21.389	0.23	0.28	Ocimenyl acetate
19	21.784	0.60	0.61	2,6-Octadienoic acid, 3,7-dimethyl-, methyl ester, (Z)-
20	22.923	48.25	46.19	1-METHYL-1-(4-METHYL-3-CYCLOHEXEN-1-YL)ETHYL ACETATE
21	22.960	0.14	0.12	.mu.3-(Tetracarbonylcobaltosilylidine)-cyclo-tris(tricarbonylcobalt)
22	23.100	0.99	1.35	1H-Indene, 1-(2,3-dihydro-1H-inden-1-ylidene)-2,3-dihydro-
23	24.243	3.67	3.88	2,6-Octadien-1-ol, 3,7-dimethyl-, acetate, (E)-
24	26.493	0.49	0.32	3-Cyclohexene-1-methanol, .alpha.,.alpha.,4-trimethyl-, propanoate
25	27.058	0.43	0.44	Epoxy-.alpha.-terpenyl acetate
26	27.501	0.89	0.82	Epoxy-.alpha.-terpenyl acetate
27	30.068	0.28	0.22	Hydroxy-.alpha.-terpenyl acetate
28	31.257	0.21	0.17	Hydroxy-.alpha.-terpenyl acetate
29	31.675	0.87	0.64	1,6,10-Dodecatrien-3-ol, 3,7,11-trimethyl-
30	33.640	0.71	0.41	1-Methyl-4-(1-acetoxy-1-methylethyl)-

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				cyclohex-2-enol
31	34.778	0.31	0.25	1-Methyl-4-(1-acetoxy-1-methylethyl)-cyclohex-2-enol
32	34.901	0.50	0.32	Hydroxy-.alpha.-terpenyl acetate
33	36.310	0.28	0.17	Terpinyl formate
34	40.313	0.17	0.16	Acetic acid, 1-[2-(2,2,6-trimethyl-bicyclo[4.1.0]hept-1-yl)-ethyl]-vinyl ester
35	50.701	0.68	0.63	Retinal, 9-cis-
36	55.257	0.22	0.18	2,5-Furandione, 3-(dodecenyl)dihydro-
37	56.054	0.57	0.54	Heneicosane
38	58.780	0.12	0.13	Heneicosane
39	60.683	0.23	0.23	1-Heptacosanol
40	61.405	0.68	0.67	Hexacosane

Table 12: Essential oil components of the sample Njellani

Peak#	Ret.Time	Area%	Height%	Name
1	7.236	0.77	1.21	4(10)-Thujene
2	7.770	0.56	0.85	1,6-Octadiene, 7-methyl-3-methylene-
3	7.841	0.12	0.17	METHYL 2-HYDROXY-3-METHYLPENTANOATE
4	8.254	0.16	0.22	Octanal
5	9.074	1.00	0.98	Benzene, methyl(1-methylethyl)-
6	**9.305**	**2.76**	**2.72**	**D-Limonene**
7	9.418	32.30	27.05	2-Oxabicyclo[2.2.2]octane, 1,3,3-trimethyl-
8	10.868	0.62	0.55	5-ISOPROPYL-2-METHYLBICYCLO[3.1.0]HEXAN-2-OL
9	12.125	2.99	3.37	LINALOOL L
10	13.135	0.40	0.46	2-Cyclohexen-1-ol, 1-methyl-4-(1-methylethyl)-, trans-
11	13.916	0.31	0.35	4-ISOPROPYL-1-METHYL-3-CYCLOHEXEN-1-OL
12	15.134	0.45	0.35	3-Cyclohexene-1-methanol, .alpha.,.alpha.,4-trimethyl-
13	15.620	9.55	8.89	3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)-
14	16.327	16.84	11.86	3-Cyclohexene-1-methanol, .alpha.,.alpha.,4-trimethyl-
15	16.716	0.11	0.10	Decanal
16	16.927	0.61	0.50	1-Hexanol, 2-ethyl-, acetate
17	17.335	0.11	0.12	6-Octenal, 2,3-epoxy-3,7-dimethyl-
18	17.804	0.12	0.14	6-Octenal, 2,3-epoxy-3,7-dimethyl-
19	18.114	0.50	0.52	2,6-Octadienal, 3,7-dimethyl-, (Z)-
20	18.781	4.59	3.54	Geraniol
21	19.441	0.93	0.91	2,6-Octadienal, 3,7-dimethyl-
22	19.883	0.14	0.12	1,4-dihydroxy-p-menth-2-ene
23	20.106	0.15	0.13	Bicyclo[2.2.1]heptan-2-ol, 1,7,7-trimethyl-, acetate, (1S-endo)-

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24	21.183	0.23	0.18	[1,1'-Bicyclopropyl]-2-octanoic acid, 2'-hexyl-, methyl ester
25	21.391	0.60	0.51	Ocimenyl acetate
26	21.795	0.88	0.69	2,6-Octadienoic acid, 3,7-dimethyl-, methyl ester, (Z)-
27	22.923	19.41	16.43	1-METHYL-1-(4-METHYL-3-CYCLOHEXEN-1-YL)ETHYL ACETATE
28	23.115	2.79	17.08	3-Cyclohexene-1-methanol, .alpha.,.alpha.,4-trimethyl-, acetate

Table 13: Essential oil components of the sample Green bold

Peak#	Ret.Time	Area%	Height%	Name
1	6.052	0.89	2.56	2-Pinene
2	7.275	3.90	8.73	4(10)-Thujene
3	7.431	0.34	0.93	2(10)-Pinene
4	7.801	1.08	2.54	1,6-Octadiene, 7-methyl-3-methylene-
5	**9.240**	**1.73**	**1.96**	**D-Limonene**
6	9.325	0.94	2.27	Benzene, 1,2,3,4-tetramethyl-
7	9.587	35.75	20.71	2-Oxabicyclo[2.2.2]octane, 1,3,3-trimethyl-
8	10.914	1.25	2.43	5-ISOPROPYL-2-METHYLBICYCLO[3.1.0]HEXAN-2-OL
9	12.184	2.62	4.68	LINALOOL L
10	15.655	3.59	5.54	3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)-
11	16.449	12.03	9.47	2-(4-METHYL-3-CYCLOHEXEN-1-YL)-2-PROPANOL
12	18.841	1.53	2.04	2,6-Octadien-1-ol, 3,7-dimethyl-, (Z)-
13	19.494	0.63	1.02	2,6-Octadienal, 3,7-dimethyl-
14	23.098	18.47	12.92	1-METHYL-1-(4-METHYL-3-CYCLOHEXEN-1-YL)ETHYL ACETATE
15	24.336	1.60	2.72	Geranyl acetate
16	26.519	0.85	0.95	1-Methyl-4-(1-acetoxy-1-methylethyl)-cyclohex-2-enol
17	27.132	0.98	1.73	Epoxy-.alpha.-terpenyl acetate
18	27.592	1.76	2.67	Epoxy-.alpha.-terpenyl acetate
19	28.764	1.07	1.90	Naphthalene, decahydro-4a-methyl-1-methylene-7-(1-methylethenyl)-, [4aR-(4a.alpha.,7.alpha.,8a.beta.)]-
20	30.142	1.07	1.25	Hydroxy-.alpha.-terpenyl acetate
21	31.333	0.75	0.84	Hydroxy-.alpha.-terpenyl acetate
22	31.762	2.08	3.25	1,6,10-Dodecatrien-3-ol, 3,7,11-trimethyl-
23	33.763	1.69	2.12	Cyclohexene, 2-ethenyl-1,3,3-trimethyl-
24	34.883	1.11	1.24	2,4-Pentanedione, 3-tricyclo[3.3.1.13,7]dec-1-yl-
25	35.041	1.27	2.02	1,2,4,5-TETRAMETHYL-6-METHYLENESPIRO[2.4]HEPTANE
26	36.396	1.02	1.51	2-Norbornanol, 5,5-dimethyl-6-methylene-

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Table 14: Total D- Limonene percentage of 4 samples

Variety	Limonene %
Njellani	2.76%
Green bold	1.73%
Palakkudi	0.31%
Pathumuriyan	0.1%

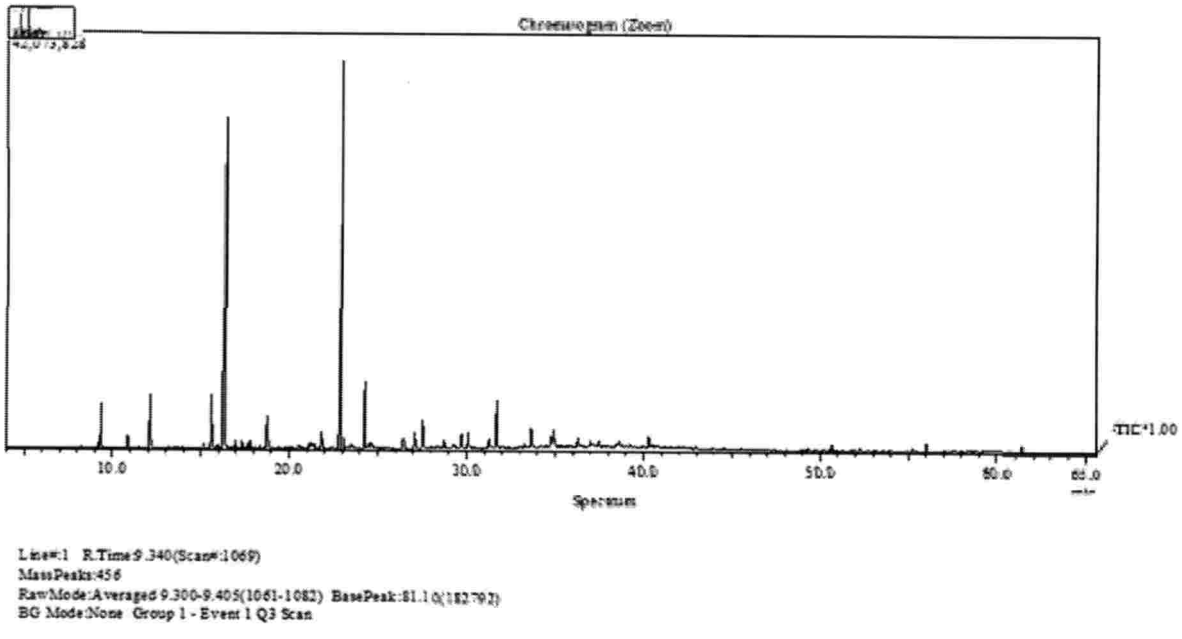

Figure 2: Spectrogram of Palakkudi

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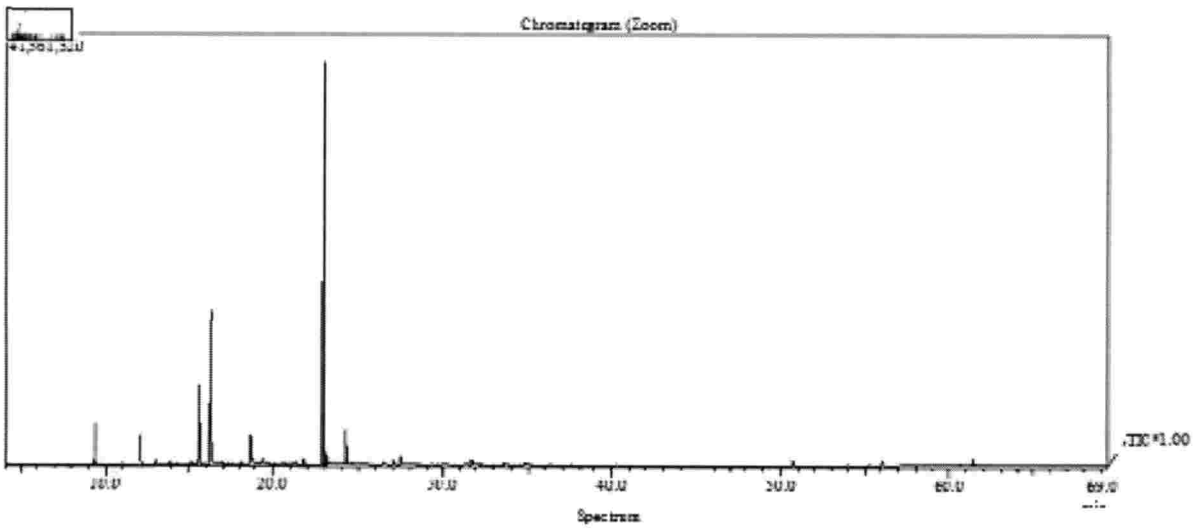

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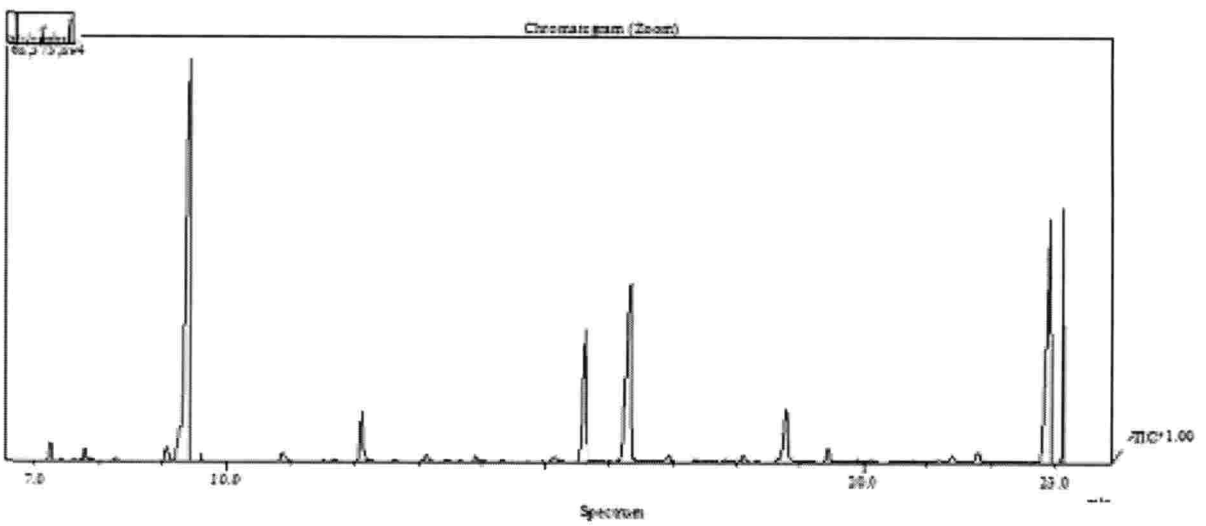
RawMode:Averaged 9.195-9.250(1040-1051) BasePeak:68.05(15237)
BG Mode:None Group 1 - Event 1 Q3 Scan

Figure 3: Spectrogram of Pathumuriyan



Line#:1 R.Time:22.925(Scan#:3786)
MassPeaks:367
RawMode:Averaged 22.920-22.930(3785-3787) BasePeak:121.10(6945384)
BG Mode:Calc. from Peak Group 1 - Event 1 Q3 Scan

Figure 4: Spectrogram of Njellanai

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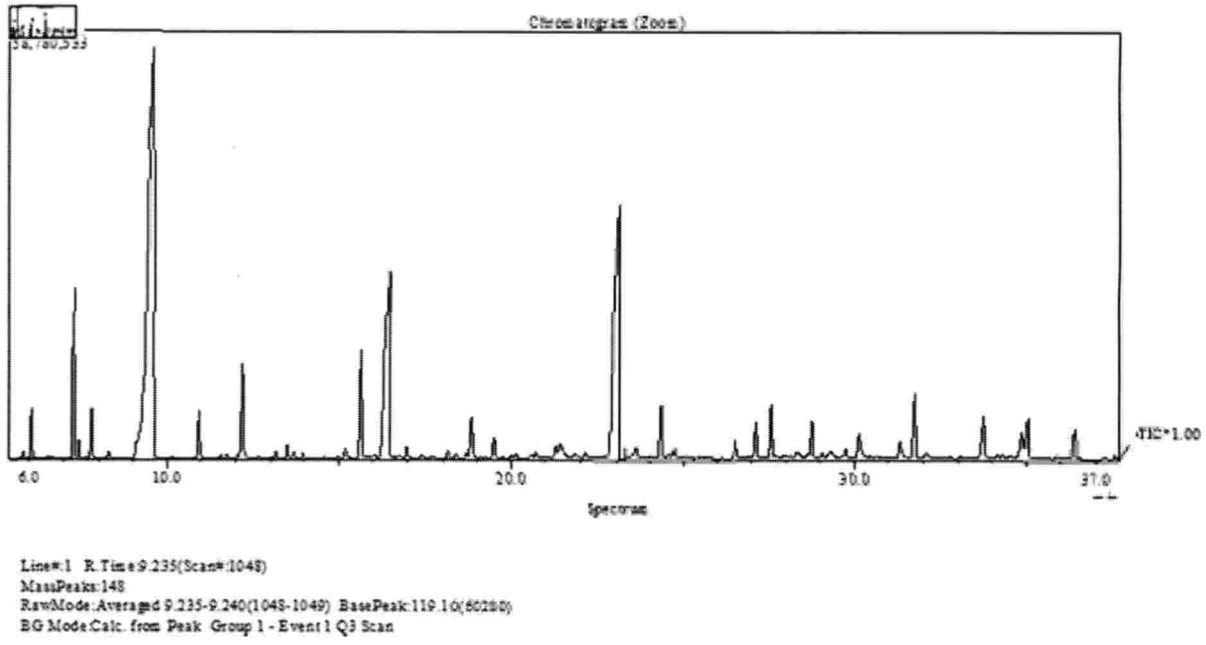

Figure 5: Spectrogram of Greenbold

4.6 PRIMER DESIGNING AND ANALYSIS

No SSR were detected within the target sequence. So validating the results of GC-MS gene specific primers for limonene was designed using various bioinformatics tool. The efficiency of the newly designed primers was verified by using the software Oligo analyzer. The newly synthesised primers were GC rich and devoid of self-dimerization. Gradient PCR for the primers were performed at varying temperatures optimized the annealing temperature for analysis. The primers along with the optimized annealing temperature used for the study is represented by Table15. Amplified sequences were observed by 3% agarose gel electrophoresis (Plate: 10-14).

Table 15: Gene specific limonene primers

Sl. No.	Primer	Sequence	No. of bases	GC (%)	Tm (°C)	Annealing temperature
1	LMP F	GATGATATTTACGATGTCTATGGTAC	26	34.62	53.23	54
	LMP R	GAATTGATTTCGGCACATCGCCTC	24	50	57.38	
2	LMS1 F	ATAAGCCAGTCCAATGCCTT	20	45	49.73	46
	LMS1 R	TTTGTCCTCCAAGGGAATGG	20	50	51.78	
3	LMS2 F	GCAGTCATTGAATAGCAAC	19	42	46.77	46
	LMS2 R	CCTTCTTCCACATCTGTCTC	20	50	51.78	
4	LMK1 F	GAACAGAGCGGCCAGAAA	18	55.56	50.32	50
	LMK1 R	GATAGCCCACGCGCATATAA	20	50	51.78	
5	LMK2 F	TTTCGCCAGAACGGCTTTA	19	47.37	48.98	54
	LMK2 R	CATCGCCGCGTTTCATTTC	19	52.63	51.09	

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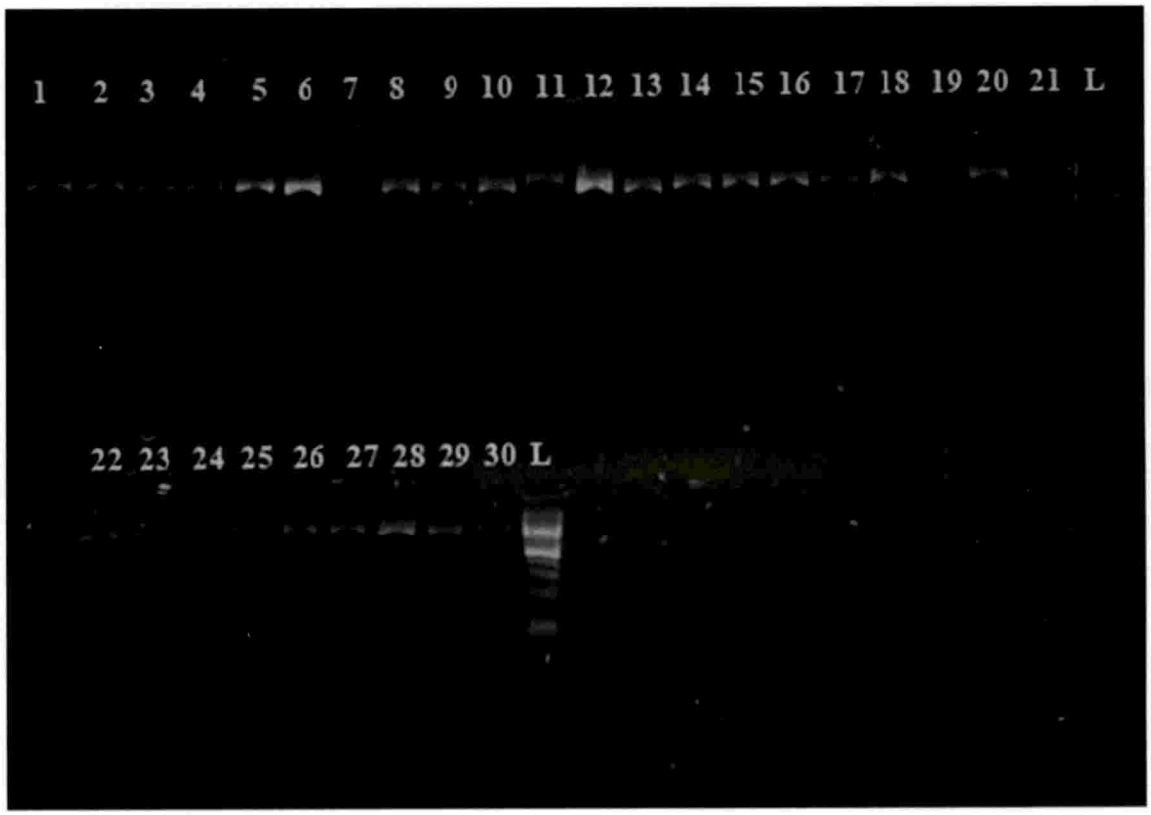

Plate 10: PCR profile obtained for primer S1

Lane representation:

L- Ladder

1. C_{65} (PV2)
2. C51 (Kanniyelam)
3. T7 (Wild)
4. C70 (Unknown)
5. C53 (Unknown)
6. C61 (ICRI-2)
7. C103 (Pathumuriyan)
8. C116 (Kothu mysore)
9. C107 (Njellani)
10. C108 (Njellani)
11. C104 (Nadan)
12. V8 (Wild)
13. C106 (Elam Raja)
14. C105 (Njellani)
15. C112 (Njellani)
16. C109 (Green bold)
17. C118 (Thiruthali)
18. C120 (Palakkudi)
19. C113 (Palakkudi)
20. C117 (Vazhukka mysore)
21. LC (Large cardamom)
22. C111 (Njellani)
23. C64 (PV1)
24. C24 (Wild)
25. C110 (Green bold)
26. ER (Elam Raja)
27. NJ1 (Njellani)
28. NJ2 (Njellani)
29. NJ3 (Njellani)
30. NJ4 (Njellan)

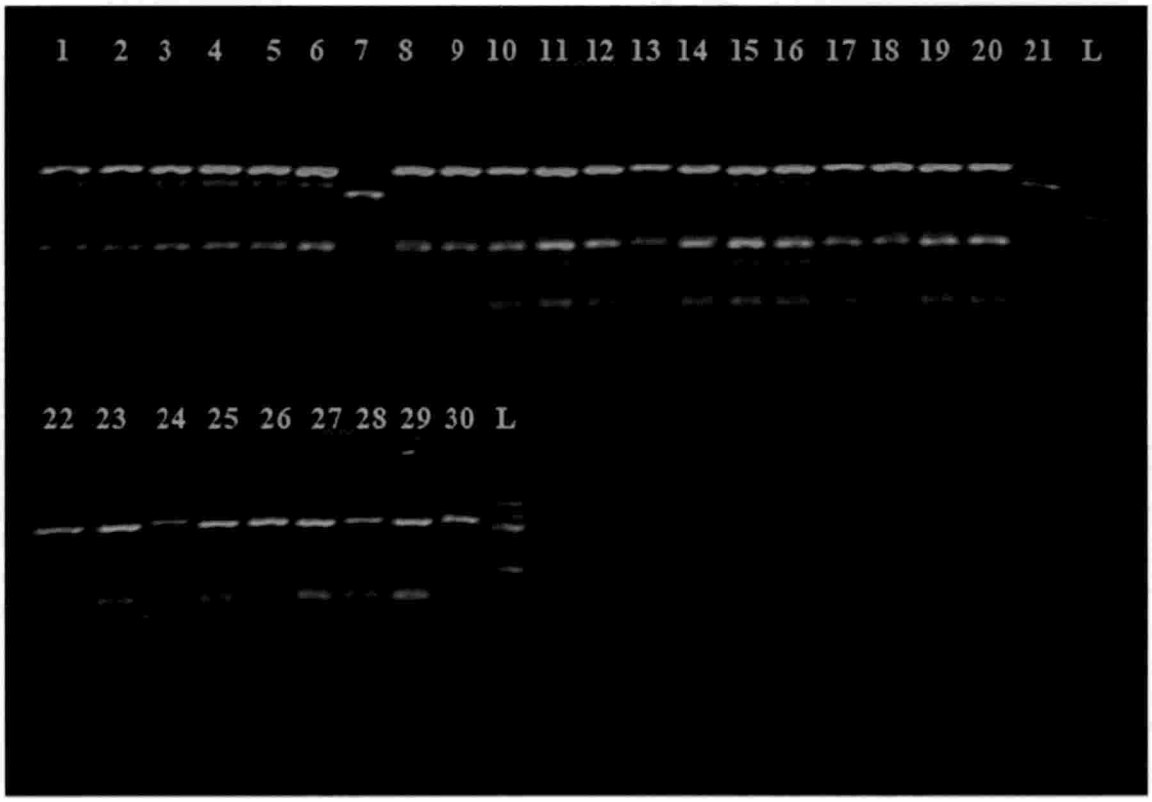

Plate 11: PCR profile obtained for primer S2

Lane representation:

L- Ladder

1. C_{65} (PV2)
2. C51 (Kanniyelam)
3. T7 (Wild)
4. C70 (Unknown)
5. C53 (Unknown)
6. C61 (ICRI-2)
7. C103 (Pathumuriyan)
8. C116 (Kothu mysore)
9. C107 (Njellani)
10. C108 (Njellani)
11. C104 (Nadan)
12. V8 (Wild)
13. C106 (Elam Raja)
14. C105 (Njellani)
15. C112 (Njellani)
16. C109 (Green bold)
17. C118 (Thiruthali)
18. C120 (Palakkudi)
19. C113 (Palakkudi)
20. C117 (Vazhukka mysore)
21. LC (Large cardamom)
22. C111 (Njellani)
23. C64 (PV1)
24. C24 (Wild)
25. C110 (Green bold)
26. ER (Elam Raja)
27. NJ1 (Njellani)
28. NJ2 (Njellani)
29. NJ3 (Njellani)
30. NJ4 (Njellan)

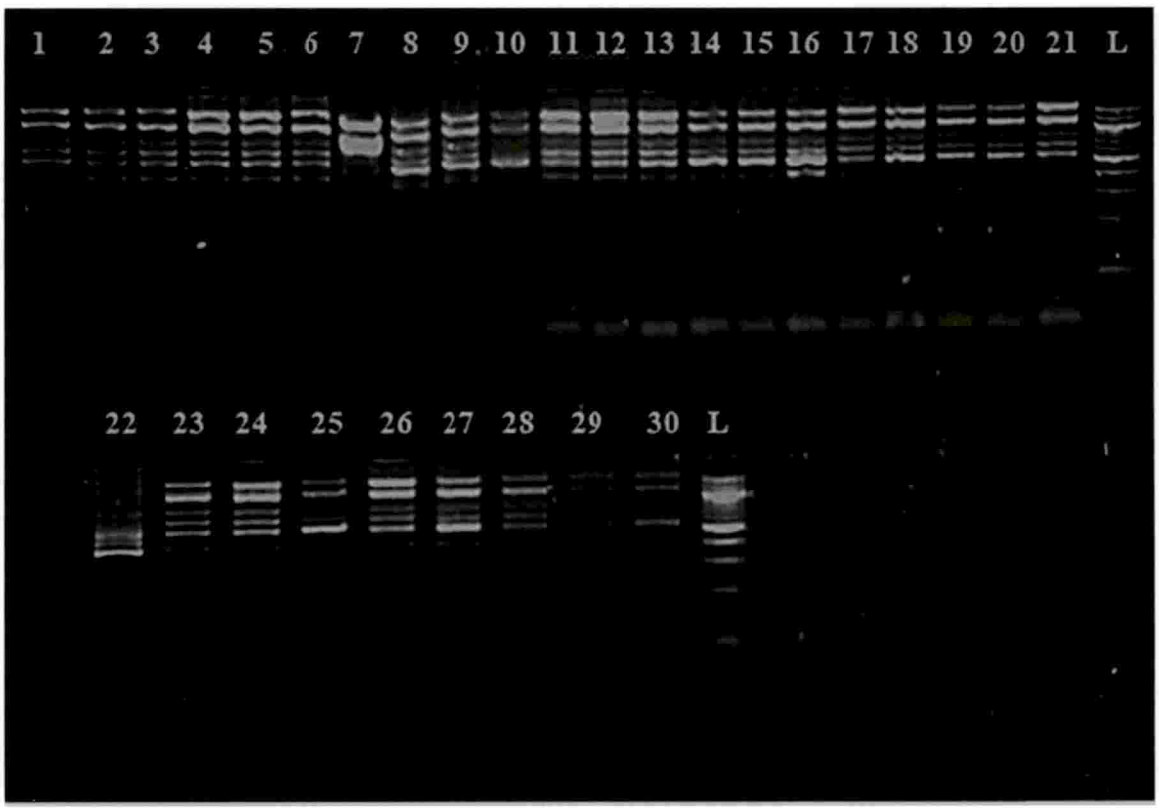

Plate 12: PCR profile obtained for primer K1(1030)

Lane representation:

L- Ladder

1. C_{65} (PV2)
2. C51 (Kanniyelam)
3. T7 (Wild)
4. C70 (Unknown)
5. C53 (Unknown)
6. C61 (ICRI-2)
7. C103 (Pathumuriyan)
8. C116 (Kothu mysore)
9. C107 (Njellani)
10. C108 (Njellani)
11. C104 (Nadan)
12. V8 (Wild)
13. C106 (Elam Raja)
14. C105 (Njellani)
15. C112 (Njellani)
16. C109 (Green bold)
17. C118 (Thiruthali)
18. C120 (Palakkudi)
19. C113 (Palakkudi)
20. C117 (Vazhukka mysore)
21. LC (Large cardamom)
22. C111 (Njellani)
23. C64 (PV1)
24. C24 (Wild)
25. C110 (Green bold)
26. ER (Elam Raja)
27. NJ1 (Njellani)
28. NJ2 (Njellani)
29. NJ3 (Njellani)
30. NJ4 (Njellan)

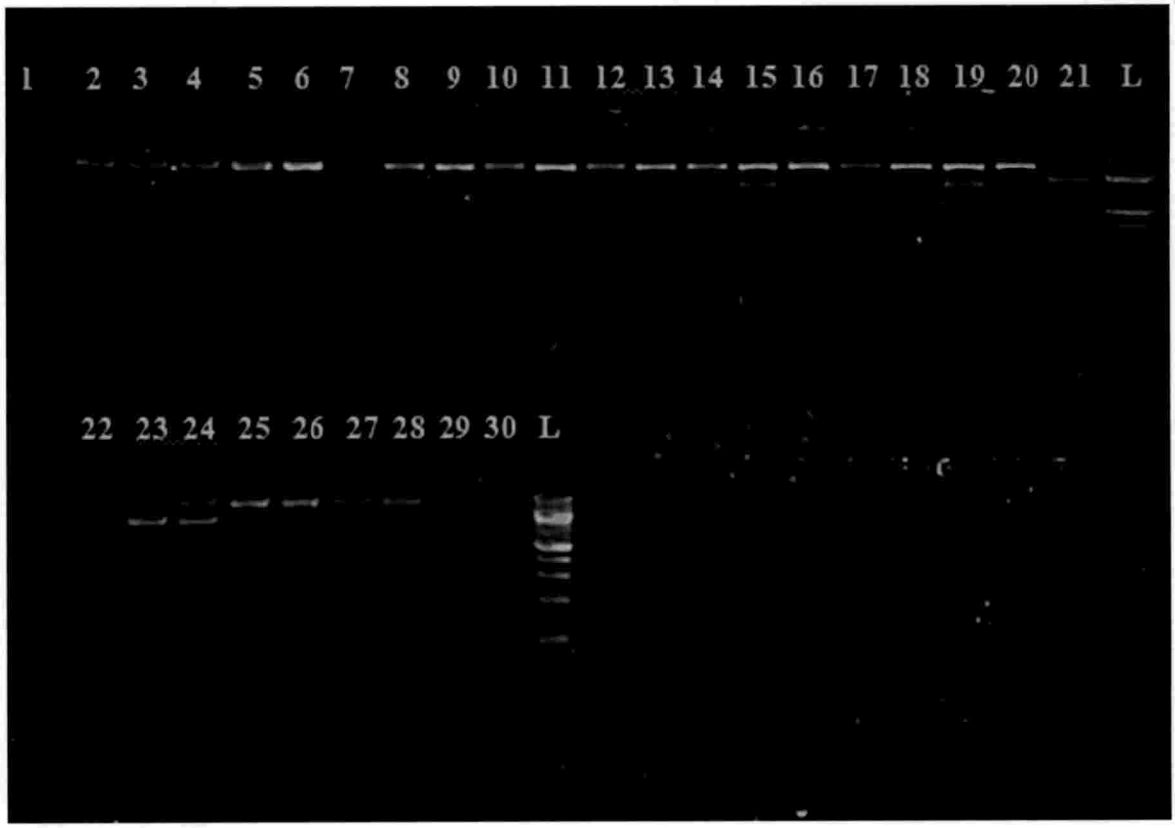

Plate 13: PCR profile obtained for primer K2 (1036bp)

Lane representation:

L- Ladder

1. C_{65} (PV2)
2. C51 (Kanniyelam)
3. T7 (Wild)
4. C70 (Unknown)
5. C53 (Unknown)
6. C61 (ICRI-2)
7. C103 (Pathumuriyan)
8. C116 (Kothu mysore)
9. C107 (Njellani)
10. C108 (Njellani)
11. C104 (Nadan)
12. V8 (Wild)
13. C106 (Elam Raja)
14. C105 (Njellani)
15. C112 (Njellani)
16. C109 (Green bold)
17. C118 (Thiruthali)
18. C120 (Palakkudi)
19. C113 (Palakkudi)
20. C117 (Vazhukka mysore)
21. LC (Large cardamom)
22. C111 (Njellani)
23. C64 (PV1)
24. C24 (Wild)
25. C110 (Green bold)
26. ER (Elam Raja)
27. NJ1 (Njellani)
28. NJ2 (Njellani)
29. NJ3 (Njellani)
30. NJ4 (Njellan)

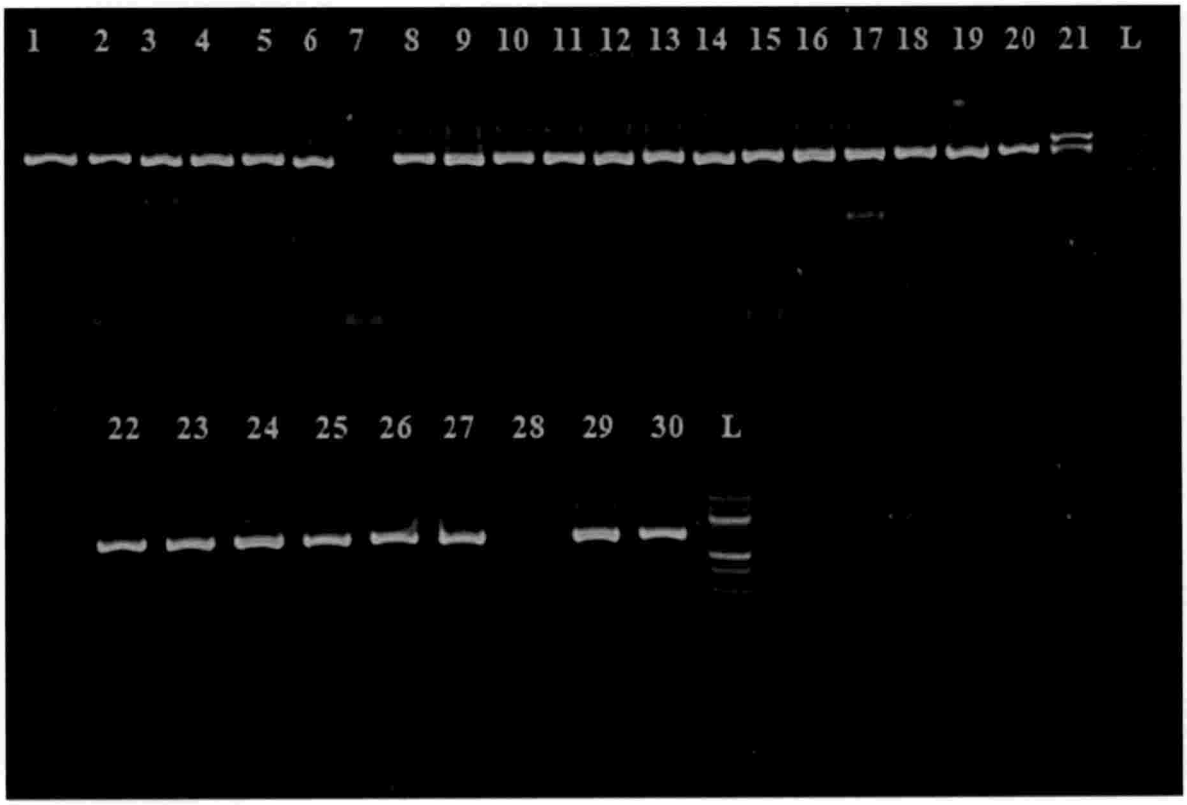

Plate 14: PCR profile obtained for primer P(490)

Lane representation:

L- Ladder
1. C_{65} (PV2)
2. C51 (Kanniyelam)
3. T7 (Wild)
4. C70 (Unknown)
5. C53 (Unknown)
6. C61 (ICRI-2)
7. C103 (Pathumuriyan)
8. C116 (Kothu mysore)
9. C107 (Njellani)
10. C108 (Njellani)
11. C104 (Nadan)
12. V8 (Wild)
13. C106 (Elam Raja)
14. C105 (Njellani)
15. C112 (Njellani)
16. C109 (Green bold)
17. C118 (Thiruthali)
18. C120 (Palakkudi)
19. C113 (Palakkudi)
20. C117 (Vazhukka mysore)
21. LC (Large cardamom)
22. C111 (Njellani)
23. C64 (PV1)
24. C24 (Wild)
25. C110 (Green bold)
26. ER (Elam Raja)
27. NJ1 (Njellani)
28. NJ2 (Njellani)
29. NJ3 (Njellani)
30. NJ4 (Njellan)

5. DISCUSSION

The significance of EST-SSR as a molecular tool in genetic studies is well known (Ellis and Burke, 2007) and demonstrated in population studies and analysis of genetic diversity in many species like *Populus* (Xinye *et al.,* 2009). Recently, it was reported that EST-SSRs of turmeric and ginger showed considerable genetic variation in cardamom (Anjali *et al.*, 2015).

In the present study microsatellites were used to analyze the genetic polymorphism in cardamom. The genetic polymorphism and differentiation analysis of the accessions were done by using already available cardamom specific SSR markers in JNTBGRI (https://doi.org/10.2174/1574893611666161128123827). By comparing the results, the sample Pathumuriyan (C_{103}) has shown a great variation in the banding pattern. The summary of genetic variation and genetic distance were statistically generated using POPGENE (Yeh & Boyle, 1997). The pair-wise genetic similarity and distance matrix were prepared on the basis of SSR data. The genetic similarity values varied from 0.10 to 1.00 with a mean value of 0.854. Cluster analysis was performed on the SSR data following UPGMA method and dendrogram were constructed through MEGA7 showing overall genetic retardness among the individuals.

For the estimation of D-Limonene, essential oil was extracted from freshly collected capsules of cardamom using clevenger- type apparatus through hydro-distillation (Sattar *et al.,* 1989). Essential oil of *Elettaria cardamomum* Maton has antimicrobial, anticarcinogenic, anti-inflammatory and antioxidant activities (Kubo *et al.*, 1991; Vijayan *et al.*, 2002 & Al Tahir *et al.*, 1997). Four samples was used for the analysis of essential oil and was carried out on GC-MS at NIIST (National Institute for Interdisciplinary Science and Technology), Trivandrum.

In reference to polymorphism analysis, the D-Limonene content was estimated from the sample Pathumuriyan (C_{103}) along with two other currently

popular varieties (Njellani and Green bold) and one non- popular cultivar Palakkudi. The essential oil of cardamom revealed the presence of around 45 components. The limonene percentage was identified from their fragmentation pattern by Mass Spectrometry using NIIST library. By comparing the retention time and area percentage of D- Limonene of each 4 sample, the sample Pathumuriyan has got the lowest D- Limonene content of 0.1% indicating some sort of genetic variation related to the D- Limonene synthesis.

In order to validate the GC- MS results, limonene specific functional SSR markers were developed. The advent of DNA marker technology has revolutionized the field of genetics by changing the pace and precision of genetic analysis (Cullis *et al.*, 2002; Dodgson *et al.*, 1997; Rafalski and Tingey, 1993). Microsatellites or Simple Sequence Repeats (SSRs) are one of the most widely used molecular markers in plant breeding, agricultural genetics, mapping, marker assisted selection, and genetic diversity studies (Powell *et al.*, 1996; Gupta & Varshney, 2000). They are stretches of DNA consisting of tandemly arranged units of 1–6 bp in length (Thiel *et al.*, 2003), characterized by the relative abundance, hyper variable, locus specific, codominant, and multiallelic nature (Thiel *et al.*, 2003).

Cardamom specific limonene sequence was not available in NCBI Genebank, so limonene specific gene sequences of various plant species was selected for designing degenerative primers. PCR amplification of the thirty different accessions was done with the newly synthesised primers in which the sample Pathumuriyan has shown a distinct variation in its gel banding pattern. Since the primers custom synthesized were based on the D-Limonene, the differential banding pattern obtained in the accession Pathumuriyan can be considered as a genetic variation associated with genes specific for the synthesis of D- limonene.

6. SUMMARY

The research work entitled "Development of functional SSR markers for D-Limonene content and analysis of genetic polymorphism in cardamom (*Elettaria cardamomum* Maton)" was carried out at Biotechnology and Bioinformatics Division of Jawaharlal Nehru Tropical Botanic Garden and Research Institute (JNTBGRI), Palode during 2017-18. The objective of the study was to analyse the genetic polymorphism and to identify SSR marker with respect to D-Limonene content, a component in cardamom oil having tumor suppressing effect.

Cardamom is one of the world's ancient, expensive and highly valued spices. It's a native of tropical ever green forests in Western Ghats where presumably rich and untapped genetic diversity of the species still resides. The world production of cardamom is around 36000 tonnes per annum. Kerala is the leading producer of cardamom in the country constituting 78% of the total production from 56% of the total land area under cardamom cultivation in the country.

D- Limonene is a bioactive component in the cardamom essential oil and it was reported to posses chemo preventive property towards colon cancer, lung, liver mammary, skin and stomach cancers in rodents. The percentage of limonene is varies in different accessions.

Thirty accessions of cardamom used for this study which were collected from different geographical locations of Idukki and also from the cardamom germplasm conservatory of JNTBGRI which included wild collection, landraces and released varieties. Genomic DNA samples were isolated from the thirty accessions used for the study with Origin Plant Genomic DNA Kit. Isolated DNA samples were resolved in 0.8% agarose gel with ethidium bromide for visual observation. The genetic polymorphism and differentiation analysis of the thirty different accessions of cardamom done with the already available cardamom specific SSR markers in JNTBGRI resulted drastic variation in the banding

pattern of the variety Pathumuriyan (C_{103}) collected from vacacity region of Idukki. This was a remarkable indication of the existence of genetic variation within the species.

The summary of genetic variation and genetic distance were statistically generated using POPGENE. The statistic data which includes the observed number of alleles per locus (na) ranged from 1.00 to 1.33, effective number of alleles (ne) also ranged from 1.00 to 1.33, Shannon's information index (I) ranged from 0.00 to 0.23, observed heterozygosity (Obs_Het) ranged from 0.00 to 0.33, expected heterozygosity (Exp_Het) ranged from 0.00 to 0.33, Nei's heterozygosity (h) ranged from 0.00 to 0.16, average heterozygosity (Ave_Het) ranged from 0.01 to 0.09 and percentage of polymorphic loci (P) ranged from 0.00 to 33.33.

The pair- wise genetic similarity and distance matrix were prepared on the basis of SSR data. The genetic similarity values varied from 0.10 to 1.00 with a mean value of 0.854. Cluster analysis was performed on the SSR data following UPGMA method and dendrogram were constructed through MEGA7 showing overall genetic retardness among the individuals. The accession studied were clustered into two main group, one with 29 accessions and other with one accession, which is an out group sample (Large cardamom). The first group has again divided into two subgroup and the remaining 29 samples are mixed in the cluster. The dendrogram revealed a complex distribution of pattern.

In reference to polymorphism analysis, the D-Limonene content was estimated from that particular sample Pathumuriyan (C_{103}) along with two other currently popular varieties (Njellani and Green bold) and one non- popular cultivar Palakkudi. The D-Limonene percentage in each of the 4 samples was analyzed by using GC- MS was done at NIIST, Pappanamcode. As per their report, the cardamom oil have the presence of around 45 components. The limonene percentage was identified from their fragmentation pattern by Mass Spectrometry using NIIST library. By comparing the retention time and area

percentage of D- Limonene of each 4 sample, the sample Pathumuriyan has got the lowest D- Limonene content of 0.1%. It indicated that has some sort of genetic variation related to the D- Limonene synthesis.

In order to validate the GC-MS results, gene specific primers were developed. Cardamom specific limonene sequence was not available in NCBI Genbank, so limonene specific gene sequences of various plant species were selected for the synthesis of functional SSR primers. By using Multiple Sequence Alignment (MSA) tool in Clustal Omega, sequences were aligned. The sequences were uploaded to the WEBSAT software for identifying the SSR regions residing within the sequences. However, no SSRs were detected within the target sequence. So for validating the results of GC-MS, gene specific primers for limonene were designed from the conserved regions obtained from Multiple Sequence Alignment (MSA).These newly synthesised primers were used for amplifying the thirty accessions in which sample Pathumuriyan has shown a distinct variation in its gel banding pattern indicating some genetic variation in the genes associated with D- Limonene synthesis. Study has also shown that D-Limonene, which is the tumour suppressor constituent in cardamom essential oil (Samir *et al.,* 2015) is found high in the current popular cultivars, Njellani and Green Bold compared to other cardamom varieties. Essential oils are more sensitive towards gram positive bacteria and also used as biopreservative agents. Limonene posses antimicrobial and antioxidant properties (Desislave *et al.*, 2016). The present study can be further elaborated to metabolic pathway studies as well as hybridisation techniques to develop elite cardamom varieties with increased D-Limonene content.

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8. APPENDIX I

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TBE Buffer (20X) for 100ml solution

Tris base	21.6g
Boric acid	11.0g
0.5M EDTA (Ph 8.0)	8.00ml

APPENDIX II

Tracking dye

Bromophenol blue	0.25%
Xylene cyanol	0.25%
Glycerol	30%
Water	69.5%

APPENDIX III

AE Buffer

Tris HCL	10Mm
EDTA (Ph 9.0)	0.5Mm

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APPENDIX IV

Composition of Gel for AGE

Agarose	0.75g
20X TBE buffer	2.5ml
EtBr	2.5µl

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DEVELOPMENT OF FUNCTIONAL SSR MARKERS FOR D-LIMONENE CONTENT AND ANALYSIS OF GENEIC POLYMORPHISM IN CARDAMOM (*Elettaria cardamomum* Maton)

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Abstract of Thesis

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9. ABSTRACT

The research work entitled "Development of functional SSR markers for D- Limonene content and analysis of genetic polymorphism in cardamom (*Elettaria cardamomum Maton*)" was carried out at the Biotechnology and Bioinformatics Division of Jawaharlal Nehru Tropical Botanical Garden and Research Institute (JNTBGRI), Palode during the academic year 2017-2018.

Elettaria cardamomum Maton, commonly known as green or true cardamom, is one of the most economically important spice crop owing to its very pleasant aroma and taste. It is highly valued from ancient time both for its medicinal as well as culinary uses. The study was carried out with an aim to analyse the genetic diversity inherent in the cardamom landraces and wild genotypes using cardamom specific SSR markers and the estimation and validation of its D-Limonene content.

The genetic diversity inherent in the cardamom germplasm were analysed with cardamom specific SSR markers. Thirty accessions including popular and non- popular cultivars, wild varieties, landraces and one large cardamom (as outgroup) were included in the present study. For the diversity analysis, six cardamom specific SSR markers were employed and POPGENE was used to estimate the genetic variation. The Limonene percentage in four samples was estimated through GC- MS. The present study has shown that D- Limonene, is the antimicrobial, antioxidant, and tumour suppressive constituent in cardamom essential oil, is found to be high in the current popular cultivars, Njellani and Green bold (2.76 and 1.73% respectively) compared to other cardamom varieties (Palakkudi, 0.31% and Pathumuriyan, 0.1%). For validating the results of GC-MS, gene specific primers for limonene were designed from the conserved regions of selected plant species. Thirty accessions were validated with these primers, in which the sample Pathumuriyan has shown distinct variation in the PCR products resolved using horizontal agarose gel electrophoresis which clearly demonstrates the variation in the genes involved in the biosynthesis of D-Limonene in this cultivar. The molecular marker and chemical analysis

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successfully validated the genetic variability within the species as well as established potential of the two popular cultivars in production of essential oil with high D-Limonene content. The present study can be further elaborated to metabolic pathway analysis with respect to essential oil content and would lead to the development of elite cardamom varieties with increased D- Limonene content through hybridisation techniques.