DEVELOPMENT AND USE OF SSR MARKERS FOR ANALYSIS OF GENETIC DIVERSITY AND CORRELATION WITH LIGNIN CONTENT IN CARDAMOM (*Elettaria cardamomum* Maton) GERMPLASM

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DECLARATION

I hereby declare that the thesis entitled **"Development and use of SSR markers for analysis of genetic diversity and correlation with lignin content in cardamom (*Elettaria cardamomum* Maton) germplasm"** 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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Soumya S. Dharan

Dedicated to My Parents

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LIST OF ABBREVIATIONS

%	Percentage
µg	Microgram
µl	Microlitre
µ*M*	Micromolar
A_{260}	Absorbance at 260 nm wavelength
A_{280}	Absorbance at 280 nm wavelength
A_{440}	Absorbance at 440 nm wavelength
Ave_Het	Average heterozygosity
[Bmin][Cl]	1-n-butyl 3-methyl Immidazolium Chloride
bp	Base pair
C	Cytosine
CAP3	Contig Assembly Program 3
cDNA	Complementary DNA
cm	Centimetre
DNA	Deoxyribo nucleic acid
dNTPs	Deoxy nucleotide tri phosphates
EDTA	Ethylene diamine tetra acetic acid
EST	Expressed sequence tags
Exp_Het	Expected heterozygosity
Exp_Hom	Expected homozygosity
G	Guanine
g	gram
h	Nei's heterozygosity
I	Shannon's information index
M	Molar
mg	milligram
Mg^{2+}	Magnesium ion
min	Minute

ml	Millilitre
m*M*	Millimolar
mRNA	Messenger ribonucleic acid
NREL	National Renewable Energy Laboratory
ng	Nanogram
n*M*	Nanomolar
^{o}C	Degree Celsius
na	Number of alleles per locus
ne	Effective number of alleles
Obs_Het	Observed heterozygosity
Obs_Hom	Observed homozygosity
OD	Optical density
P	Percentage of polymorphic loci
PCR	Polymerase chain reaction
RAPD	Random amplified polymorphic DNA
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolution per minute
SD	Standard Deviation
sp.	Species
SNP	Single Nucleotide Polymorphism
SSR	Simple sequence repeat
TBE	Tris-borate EDTA buffer
TE	Tris-EDTA buffer
Tm	Melting temperature
U	Enzyme unit
V	Volt
var.	Variety
via	through

INTRODUCTION

1. INTRODUCTION

Ancient India, along with the richness of its culture was also recognised and glorified for the fragrance of its spices. Cardamom is one among the most ancient and economically important spice crop, which serves as a constituent in medical preparations and is also used for flavouring food, beverages and confectioneries. Cardamom which is indigenous to South India and Sri Lanka is also branded as the 'Queen of spices'.

The tropical evergreen rainforests of the Western Ghats of South India that lies at altitudes between 600 and 1500 masl is inferred to be the natural habitat of cardamom. Wild populations still reside in the remote squares of their natural habitats; however, much of today's commercial production and marketed products comes from cultivated sources. Cardamom is a sciophyte that prefer cool and humid climate for its growth. Therefore most of the cardamom plantations are established under the forest canopy.

Small cardamom, (*Elettaria cardamomum* Maton) belonging to the family Zingiberaceae, is a perennial herbaceous monocot. Dried fruits are the commercially important part of this plant. The fruits are tri-ocular, ovoid, oblong or greenish-brown capsules containing about 15-20 reddish brown seeds. Flowering initiates with the first fall of rain in April or May during the reproductive phase and the fruits take about six months to ripen. Bulk propagation of cardamom is generally performed *via* seeds but the descendants are genetically not uniform and display variation due to natural cross pollination.

Cardamom is a major export product of Kerala. In India, cultivation of cardamom spreads over 71,285 hectares with a production of 15,000 tonnes and Kerala contributes the maximum production of 11,440 tonnes. Depending upon the fruit size, small cardamom varieties are broadly classified into two, as *Elettaria cardamomum* var. major consisting of wild indigenous types and *Elettaria cardamomum* var. minor comprising the cultivated types *viz*., Malabar, Mysore and Vazhuka.

Small cardamom is affected by many diseases which have been identified as the major source of production constrain. 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 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.

Cardamom has tremendous diversity with respect to plant morphology, habitat specificity, inflorescence and fruit characteristics. The observed diversity might be the product of complex interactions between genetic and environmental factors. Understanding the genetic variability present in various morphotypes is important for developing sustainable and high-quality cardamom varieties.

The study aims to analyze the natural variation inherent in the cardamom genome using microsatellite markers and establishing a correlation with the lignin content which is an important quantitative trait. Lignin is a complex chemical compound which forms an integral part of the secondary cell walls of plants and some algae (Argyropoulos and Menachem, 1998). Abundance of lignin has been implicated as an important defence mechanism against pests and plant pathogens, so in general enhanced lignin level could result in improved pest resistance.

Microsatellite markers have frequently been used for genetic diversity studies, because of their 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 lignin content and microsatellite markers. Correlation analysis was conducted to determine if the genetic variability as estimated by SSR markers is a good indicator for that at lignin content among the various germplasm accessions.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Zingiberaceae the family to which *Elettaria cardamom* Maton belongs, is one of the largest families of monocotyledons, comprising of about 52 genera and 1500 species. The family generally encompasses aromatic perennial herbs with creeping horizontal or tuberous rhizomes. Mostly the members in the family possess distichous, simple leaves and the aerial stem is formed by encircling leaf sheaths. The species of zingiberaceae are found widely distributed over the tropical and subtropical areas, especially in South East Asia with an elevated concentration in Indonesia, Malaysia and Singapore (Sirirugsa, 1999).

Cardamom also known as the 'Queen of spices' is one of the most ancient and valuable spice crop. It is the third most expensive spice in the world after saffron and vanilla. Cardamom shows an optimal growth at higher altitudes, with heavy rainfall and perennial shade canopy (Agnihotrudu, 1987). India has a rich genetic diversity of cardamom as is believed to be originated in the moist evergreen forest of Western Ghats of Southern India and many wild populations are still confined to this region (Ravindran and Madhusoodanan, 2002). However Guatemala outstripped India in global trade and arouse as the primary producer of cardamom (Elizabeth *et al*., 2006).

The generic name *Eletteria* was derived from the 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).

Cardamom is extensively cultivated in the hilly regions of Kerala, Karnataka and Tamilnadu, of which Kerala contributes to 70% of the total production in the country. Suresh (1984) in his thesis 'Economics of cardamom plantation in Kerala', has prepared an exhaustive cost benefit analysis of cardamom cultivation, which throws much light into various cost components of cardamom production, processing and marketing Even though the flowers are hermaphrodite, cardamom species are usually cross pollinated and propagation is

via seedlings and suckers that create a considerable variation in the progenies (Padmini *et al*., 2000). Pollination is employed by honey bees, particularly by *Apis dorsata*.

2.1 COMMON MORPHOLOGY OF CARDAMOM

Elettaria cardamom Maton belongs to family Zingiberaceae, under the natural order Scitamine, is a herbaceous perennial monocot. Leafy shoots developed from the subterrenian rhizomatous root stock, clumps to form the pseudo stem. Lanceolate leaves that tapers to the lamina has a length of 25-90 cm and width 5-15 cm. Leaves that appears dark green and shiny on the upper surface can be either glabrous or hairy at the lower surface. Inflorescence arises from the base as long racemose panicle with attractive petalloid bisexual flowers. Fruits are trilocular capsules that have pleasant aroma and flavour, are the commercially important part of the plant.

2.1.1 Use of cardamom

From the late second century BC itself, the fruits of cardamom are being used for medicinal and culinary purposes. It has its own space among sweet spices and is used predominantly to flavour sweets, baked goods, and coffee, particularly in the Asian and Arab countries. Scientific studies have proved that cardamom is a good source of antioxidants, which guards the body from aging and stress, and combat common sicknesses and physical strife (Ravindran, 2002).

The phytochemical indole-3-carbinol present in cardamom is a well-known cancer fighter, helping to specifically ward off hormone-responding cancers like breast cancer, ovarian cancer, and prostate cancer. Previous studies suggest that consuming cardamom regularly will reduce the occurrence of these forms of cancer. In addition cardamom is used for curing problems associated with digestion, including heartburn, intestinal spasms, irritable bowel syndrome, intestinal gas, constipation, liver and gallbladder complaints, and loss of appetite. It is also used as a remedy for common cold, cough, bronchitis, sore mouth and

throat. Some people use cardamom as a stimulant and for urinary problems (Ravindran, 2002).

2.2 LIGNIN

Lignin is a complex polymer composed of a number of monomeric units of aromatic alcohols known as monolignols. Lignin forms an integral part of the secondary cell walls of plants (Lebo *et al*., 2001) and some algae (Martone *et al*., 2009). Lignin a major component of lignocellulose that contributes 15-25 %, is the second most abundant source of renewable organic biopolymer after cellulose (Argyropoulos and Menachem, 1998; Bozell, 2008). Being an enormous renewable resource lignin grabbed the attention of technologist and scientists across the globe.

The physical and chemical properties of lignin also serve as an obstacle against the invasion of pests and pathogens (Vance *et al*., 1980; Bhuiyan *et al*., 2009). Moussa *et al*. (1991) observed that lignin concentration was negatively correlated with duration of larval stage. It acts as a physical barrier to microbial attack by limiting cell wall digestion and as the plants mature the concentration of both fibre and lignin tends to increase (Soest, 1978; Chaves *et al*., 2002). Jung and Fahey (1983) reported that even the precursors of lignin have anti-microbial properties. However scientific studies have proved that lignin benefits plants by providing mechanical support, water conduction in the system and protection from insects (Waghorn *et al*., 2002).

Lignification reduces the mechanical pressure exerted during the penetration of fungal appressoria and hence enhances resistance; it also makes the cell wall less permeable to water and thus less accessible to cell wall-degrading enzymes (Nicholson *et al*., 1992; Zeyen *et al*., 2002). Yu *et al*. (2001) reported that the lignin content in woody plants is under genetic control. In numerous plant species including maize, sorghum, cotton, etc the Quantitative trait loci (QTL) affecting lignin properties have been mapped. A better understanding of the role

of cell wall components such as lignin as defence mechanism allows modification of crop to withstand pests and diseases (Santiago *et al*., 2013).

Lignin plays a major role in water conduction. It creates a hydrophobic surface available for the plants that allow transport of water to heights greater than 100 m (Koch *et al*., 2004). Lignin acts as free radical scavengers and this natural antioxidant property makes it extremely suitable in cosmetics and tropical formulations. Carbon nanotubes have been prepared from lignin and are found that lignin based rigid polyurethane shows excellent flame retardance.

Quantifying lignin is quiet complicated because of its extensive cross linkages with other structural components like cellulose and hemicellulose and also due to the insolubility of this polymer (Hatfield *et al*., 1994). Soest (1967) developed a protocol for acid detergent lignin estimation which is widely used with necessary modifications. Yamasaki *et al*. (1981) first developed a technique for isolating residual lignin from kraft pulp by enzymatic hydrolysis. Fort and co-workers performed C-NMR spectral analysis of ionic solvent [Bmim] [Cl] for the quantification of lignin (Fort *et al*., 2006).

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).

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 (2002) 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).

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, 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).

2.4.1 Primer design using Bioinformatics.

The modern tactics of Bioinformatics permit the prompt discovery of SSRs from the ever-increasing collection of widely available sequences databases comprising many genomes. Designing a primer requires to satisfy several factors viz., the length of the primer, melting temperature, GC contents, 3'-end sequence, dimer formation, false priming, specificity, and the occurrence of secondary hairpin structures (Dieffenbach *et al*., 1995; Singh and Kumar, 2001).

Adams *et al*. (1991) define Expressed sequence tags (ESTs) as partial sequences, representing the set of expressed genes, developed from mRNA via reverse transcription and cloning the cDNA fragments in suitable plasmids. Currently the most widely accepted nucleotide sequences for the studies based on molecular markers are the Expressed sequence tag, as it denotes the transcribed part of the genome.

The huge aggregate of EST dataset available on the unrestricted public domain such as NCBI can be utilised for the development of SSR markers and to perform software based in silico analysis for functional annotation. SSR marker development by this method is more preferred now a days (Scott *et al*., 2000; Temnykh *et al*.,2000) as they are cost effective, less time consuming and more informative in comparison to conventional method (Zane *et al*., 2002).

GenBank is an exclusively open source of nucleotide sequence database associated with bibliographic and biological annotation (Benson *et al*., 2010). Expressed sequence tags (ESTs) form the basic material for gene expression and annotation studies. EST data are available for download from ftp.ncbi.nih.gov/ repository/dbEST/ (Boguski *et al*., 1993).

Since ESTs symbolises the coding regions of the genome, it is particularly attractive for marker development and it also offers development of markers for

numerous genomes in a short time span (Gu *et al*., 1998; Picoult-Newberg *et al*., 1999; Kantety *et al*., 2002; Varshney *et al*., 2005).

Varshney *et al*. (2002) reported that the flanking sequences of a particular microsatellite locus in a genome are thought to be conserved within species, among different species in a genus, and perhaps even across related genera. Kumpatla and Mukhopadhyay (2005) described the use of a software TRIMEST program of EMBOSS, that has customised for removing the poly A – poly T ends of the EST sequences prior to SSR identification, for reducing the redundancy in further analysis.

For clustering and assembling the nucleotide sequences into contigs and singletons, sequence assembling process uses a program called CAP3 (Huang and Madan, 1999). A cluster of overlapping DNA segments that collectively represents a consensus region of DNA is known as a contig. Compared to the existing methodologies CAP3 gathers ESTs for identical genes, under more stringent criteria and is able to discriminate gene family members while abiding sequencing error.

There are several marker prediction tools in bioinformatics, which reduce human effort and time consumed for the development of molecular markers. WebSat is a simple to use web software, for microsatellite molecular marker prediction and development. WebSat permits the submission of sequences, visualization of microsatellites and the design of primers suitable for their amplification. The program is made user friendly, tolerating the complete regulation of parameters and the easy export of the resulting data, thus aiding the development of microsatellite markers (Martins *et al*., 2009).

NetPrimer is devised to identify and fix the hybridisation-related parameters for newly designed primers, it analyses a primer for fitness, melting temperature, molecular weight, %GC, nmol per A260, and the vigour of stability as a hairpin, as a dimer, and as a palindrome (http://www.genengnews.com/best-of-the-web/netprimer-calculate-and-check-pcr-primers/2330/).

EST-derived SSR markers are currently being used in marker assisted selection (MAS), generation of high yielding varieties, molecular mapping and quantitative trait loci (QTL) analysis (Varshney *et al*., 2005).

2.5 MOLECULAR ANALYSIS

2.5.1 DNA Isolation

Over the last few decades, numerous procedures for the isolation of DNA, from various tissue sources has been developed and published. In general, all methods encompass the disruption and lysis of the initial material, followed by the removal of proteins and other contaminants and finally the recovery of DNA (Doyle, 1996). But the protocols show a discrepancy in the quality and integrity of the isolated nucleic acid, which is the key component that directly touch the outcomes of all succeeding scientific researches (Cseke *et al*., 2004).

It's hard to isolate DNA from plants rich in metabolites, such as polyphenols and polysaccharides, which incorporates with nucleic acids during the course of extraction and interfere with the isolation procedure (Puchooa and Khoyratty, 2004; Mishra *et al*., 2008). Previous studies reported that, these metabolites also hinder with the action of some of the biological enzymes like polymerases, ligases and restriction endonucleases (Prittila *et al*., 2001; Diadema *et al*., 2003; Karaca *et al*., 2005; Varma *et al*., 2007; Moyo *et al*., 2008; Singh and Kumar, 2010; Sahu *et al*., 2012).

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 analyses 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 specific segments of DNA from minute quantities of even low quality source DNA material (Erlich, 1989). It's a quick and easy method for generating unlimited copies of DNA that revolutionized the research in molecular biology and is one of those scientific developments that actually deserve the norm 'breakthrough' (Joshi and Deshpande, 2010).

Polymerase Chain Reaction was developed by the American biochemist, Kary Mullis in the year 1983 and he was awarded with Nobel Prize in 1993 along with Michael Smith for his work on PCR (Bartlett and Stirling, 2003). The technique has got wide acceptance and is increasingly applied in molecular biology laboratories, medical diagnostics, forensic science, anthropology, archaeology and molecular systematics (Sambrook and Fritsch, 1989; Innis *et al*., 1990; Mcperson *et al*., 1991; Hoelzel, 1992; Mullis *et al*., 1994; Wink and Wehrle, 1994).

PCR is often carried out in a thermal cycler and process relies on thermal cycling, entailing cycles of repeated heating and cooling of the reaction mix, for the denaturation of the parent strand and the enzymatic replication of DNA (Pavlov *et al*., 2006). The temperatures used, duration, and the number of cycles

vary depending on different parameters and has to be standardised. The specified parameters comprise the polymerase enzyme used for DNA synthesis, the concentration of divalent ions and dNTPs in the reaction mix, and the melting temperature (Tm) of the primers (Terri, 2006).

Taq polymerase is one of the widely used enzymes for DNA synthesis, which has an optimum activity at temperature 75–80°C. The activity and fidelity of the polymerase enzyme is heavily depended on the concentration of Magnesium ions, so the magnesium concentration is a key component for successful PCR amplification. It is suggested that each PCR should contain 0.5 to 2.5 mM magnesium over the total dNTP concentrations (Innis and Gelfand, 1990).

Various additives such as DMSO (1-10%), glycerol (5-20%) non-ionic detergents, formamide (1.25-10%), bovine serum albumin (10-100 Lg/ml) and polyethyleneglycol (5- 15%) can be incorporated into the reaction to increase PCR specificity (Cheng *et al*., 1994; Newton and Graham, 1994).

The PCR technique mainly involves three steps, starting with denaturation, followed by annealing and ends with extension. Initial denaturation of DNA prefers high temperatures (from 90-97^0 C), as it involves the cleavage of hydrogen bonds. The annealing happens at a lower temperature, where the primer hybridise with the template strand and it usually ranges from 50-60°C for 1-2 minutes, is highly depended on the melting temperature of the primer. DNA Polymerase adds nucleotides to the annealed primers and extension occurs at 72°C for 2-5 min. The duration of the final step relies both on the DNA polymerase itself and on the length of the DNA fragment to be amplified. The three-stages are repeated 25–40 times in a typical PCR procedure. After the last cycle, samples are usually incubated at 72°C for 5 min to fill in the protruding ends of newly synthesized PCR products (Joshi and Deshpande, 2010).

The average yield of a PCR protocol can be described by the following equation:

$$N= n (1+E) c$$

Where 'N' is the final amount of target DNA, 'n' is the initial amount of DNA, 'E' denotes the efficiency of amplification and 'c' represents the number of PCR cycles (Keohavong *et al*., 1988). By standardising the annealing and extension temperature and by minimizing the quantity of dNTP and Mg^{2+} ion concentration the rate of misincorporation can be reduced considerably (Eckert *et al*., 1990).The DNA strands synthesized during each cycle provides a new template for the succeeding reaction (Fox and Parslow, 1988).

PCR is now a common and often indispensable technique used in medical and biological research labs for a variety of applications. 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 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

The markers are heritable characteristics associated with and are useful for the identification and characterization of specific genotypes. Barcaccia *et al.* (2000) defines molecular marker as a genomic locus, that can be identified *via* the usage of a specific probe or a primer which, in virtue of its presence, undeniably

differentiate the chromosomic trait which it signifies, as well as the flanking regions at the 3' and 5' extremity.

2.6.1 Biochemical markers

The oldest known molecular markers are the isoenzyme markers, which have been effectively used in numerous crop improvement programmes (Vallejos, 1983; Glaszmann *et al*., 1989; Baes and Custsem, 1993). Isozymes were defined as the enzymes having similar catalytic function but differ in their molecular structure. Whereas allozymes are allelic variants of an enzyme encoded by a single gene locus. Even though allozymes are co-dominant markers having high reproducibility the major drawbacks that limit their application is their relatively low abundance and low level of polymorphism. Moreover, proteins with identical electrophoretic mobility (co-migration) may not remain homologous for distantly related germplasm. In addition, their selective neutrality may also be in question (Berry and Kreitman, 1993; Hudson *et al*., 1994; Krieger and Ross, 2002).

2.6.2 DNA based markers

Molecular markers commonly called DNA markers are unique segments of DNA, found at specific locations in the genome, and are transmitted from one generation to the next as per the standard laws of inheritance (Semagn *et al*., 2006). The introduction of DNA marker technology has revolutionized the field of genetics by altering the pace and precision of genetic analysis (Cullis, 2002; Dodgson *et al*., 1997; Rafalski and Tingey, 1993).

It is not necessary that molecular markers always correlate a phenotypic expression with a genomic trait. But they offer several advantages over conventional, phenotype-based alternatives, as they are more consistent and detectable in all tissues irrespective of growth, differentiation, development, or defence status. Moreover, they are not confounded by environmental, pleiotropic and epistatic effects (Mondini *et al*., 2009). Scientific studies have proved that molecular markers are highly heritable and exhibit adequate polymorphism to discriminate genotypes of diverse crop plants (Kumar, 1999).

A wide array of techniques including DNA sequencing, minisatellite, RFLP and more recent by PCR based methods such as AFLP, ISSR, SNP, etc provide opportunity for more sophisticated analysis in the structure of population genetics and further measures of their evolutionary routes (Welsh and Mc Clelland, 1990; Williams *et al*., 1990; Vos *et al*., 1995; Wolfe and Liston, 1998; Degen *et al*., 2001; Hardy, 2003; Hill and Weir, 2004). Bhat *et al.* (2010) reported that to conserve diversity in any germplasm molecular markers could be an appropriate choice of study.

Compared to the traditional morphology based approaches, DNA based identification system is more prompt and informative and can serve a vital role in highly critical field of conservation biology (DeSalle and Amato, 2004).

2.6.2.1 Hybridisation Based Markers

Molecular markers that rely on restriction-hybridization technique were employed in the field of plant genetic studies at the initial periods. This approach combines the use of restriction endonucleases with the system of hybridisation (Southern, 1975).

2.6.2.1.1 Restriction Fragment Length Polymorphism (RFLP)

The use of RFLP marker was first reported in the year 1975, for the detection of DNA polymorphism for genetic mapping of a thermo subtle mutation in Adino virus serotypes (Grodzicker *et al*., 1975). Restriction Fragment Length Polymorphism (RFLP) is a technique in which individuals are differentiated by analysing the patterns derived from the cleavage of their DNA after restriction digestion and southern hybridisation. RFLP markers offer several advantages over the conventional approaches in genetic analysis including its high polymorphic nature, co-dominant inheritance, good transferability between laboratories, no sequence information requirement and is easily scorable due to large variation in the fragment size.

Limitations include the requirement of pure DNA in a relatively high quantity (Potter and Jones, 1991; Roy *et al*., 1992; Young *et al.,* 1992), laborious and expensive nature with large time consumption (Yu *et al*., 1993) and the requirement of hazardous radioactive nucleotides (Holtke *et al*., 1995). These limitations in turn led to the development of a novel set of fewer technically intricate procedures known as the PCR-based techniques.

2.6.2.2 PCR based Molecular markers

The development of Polymerase Chain Reaction marked the commencement of the era of PCR based molecular markers (Mullis *et al*., 1986). 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).

2.6.2.2.1 Random Amplified Polymorphic DNA (RAPD)

Unlike RFLP, RAPD is a PCR based procedure that detects nucleotide sequence polymorphisms by using arbitrary chosen primers usually 8-10 bp long. RAPD differs from rest of the molecular markers in its utility for the assessment of genetic diversity, because of their simplicity, speed and cost effectiveness related to other molecular markers (William *et al*., 1990; Rafalski and Tingey, 1993). With the oligonucleotide primers used under standardized conditions, this technique compares the unique fingerprint evidence derived from the distinct pattern obtained for each of the analysed sample, and discloses the relationship between the samples (Leal *et al*., 2004).

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).

2.6.2.2.2 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).

2.6.2.2.3 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, 2001; Kwok, 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, 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 the requirement of only a small amount of purified DNA.

2.6.2.2.4 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 have 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).

2.6.2.2.5 Inter Simple Sequence Repeats (ISSR)

This technique relies on the amplification of inter-SSR DNA sequences by using microsatellite core sequences as primers for PCR reaction and was first reported in 1994 by Zietkiewicz *et al*. About 10-60 fragments generated from multiple loci are separated by gel electrophoresis and analysed by scoring in the presence or absence of bands, arranged depending on the size of each fragment (Bracci *et al*., 2011).

MATERIALS AND METHODS

3. MATERIALS AND METHODS

This research work was carried out at Biotechnology and Bioinformatics division of Jawaharlal Nehru Tropical Botanic Garden and Research Institute (JNTBGRI), Palode during 2013-14.

3.1 SAMPLE COLLECTION

Plant materials used for the present study (small cardamom, *Elettaria cardamomum* Maton) were obtained from the cardamom germplasm conservatory of JNTBGRI, Palode. They were originally collected from different parts of Kerala with special emphasis on germplasm collection from Idukki. Fresh samples were used throughout the work. Samples were collected in clean polythene bags. For the present study, 18 accessions including true wild collections and cultivars were used (Table 1).

Table 1: List of Cardamom accessions used for the study

Sl No.	Accession	Germplasm	Location
1	C59	ICRI 6	Myladumpara, Idukki
2	C62	ICRI 5	Myladumpara, Idukki
3	C63	Valli Green Gold	Myladumpara,Idukki
4	C57	ICRI 1	Myladumpara, Idukki
5	C58	ICRI 7	Myladumpara, Idukki
6	C55	Palakudi	Idukki
7	C68	Pink tiller	ICRI, Idukki
8	C69	Panikulangara 2	Idukki
9	C51	Kanniyelam	Maniyaramkudi, Idukki
10	C70	Wild	Kakki, Periyar
11	C24	Wild	Pandimotta, Kulathuppuzha
12	C60	Panikulangara 1	Myladumpara,Idukki
13	C38	Wild	Cheenikala, Kulathupuzha
14	C53	Wild	Bonakkadu,Vithura
15	C65	PV 2	KAU,Pampadumpara
16	C61	ICRI 2	Myladumpara, Idukki
17	C56	Vander Cardamom	Myladumpara, Idukki
18	C52	Wild	Agasthyamala

3.2 LIGNIN ESTIMATION

3.2.1 Sample preparation

The leaves and pseudo stem of cardamom samples were taken for lignin extraction. The harvested samples were washed first under tap water and then with sterile distilled water. After measuring fresh weight, the samples were dried at 50˚C in a hot air oven (Universal Oven, New York) for 24 hours. From the dried samples, 10g each were then grounded into fine powder using a blender (Sumeet, Chennai).

Soxhlet extraction was performed prior to lignin analysis to remove the non structural extractives. This was done based on the National Renewable Energy Laboratory (NREL) analytical procedures for standard biomass analysis (NREL, 2008a). The powdered samples were filled in the cellulose thimple. This was then introduced into the soxhlet assembly attached to the round bottom flask having 250 ml distilled water and was refluxed until the solvent coming out from the siphon became colourless. The extract was discharged and the sample material was taken out and dried and was subjected to methanol extraction with the same protocol. The extracts were then air dried and covered with aluminium foil and paper, and stored at 25˚C under moisture free environment in sealed plastic bags.

3.2.2 Lignin extraction and estimation

Lignin estimation was done using UV Visible spectrophotometric analysis of lignin dissolved in an ionic solvent 1-n-butyl 3-methyl Immidazolium Chloride [Bmin][Cl] (Sigma Aldrich, New Delhi). For estimating the lignin content in the biomass, 1.5 mg pre-treated sample (from 3.2.1) was mixed with the [Bmin][Cl] by continues stirring at a temperature of 80 ˚C for about six hours. The lignin content was obtained by measuring absorbance at a wavelength of 440 nm using spectrophotometer (XP3001 Xplorer, Germany) in pure [Bmin][Cl] as blank.

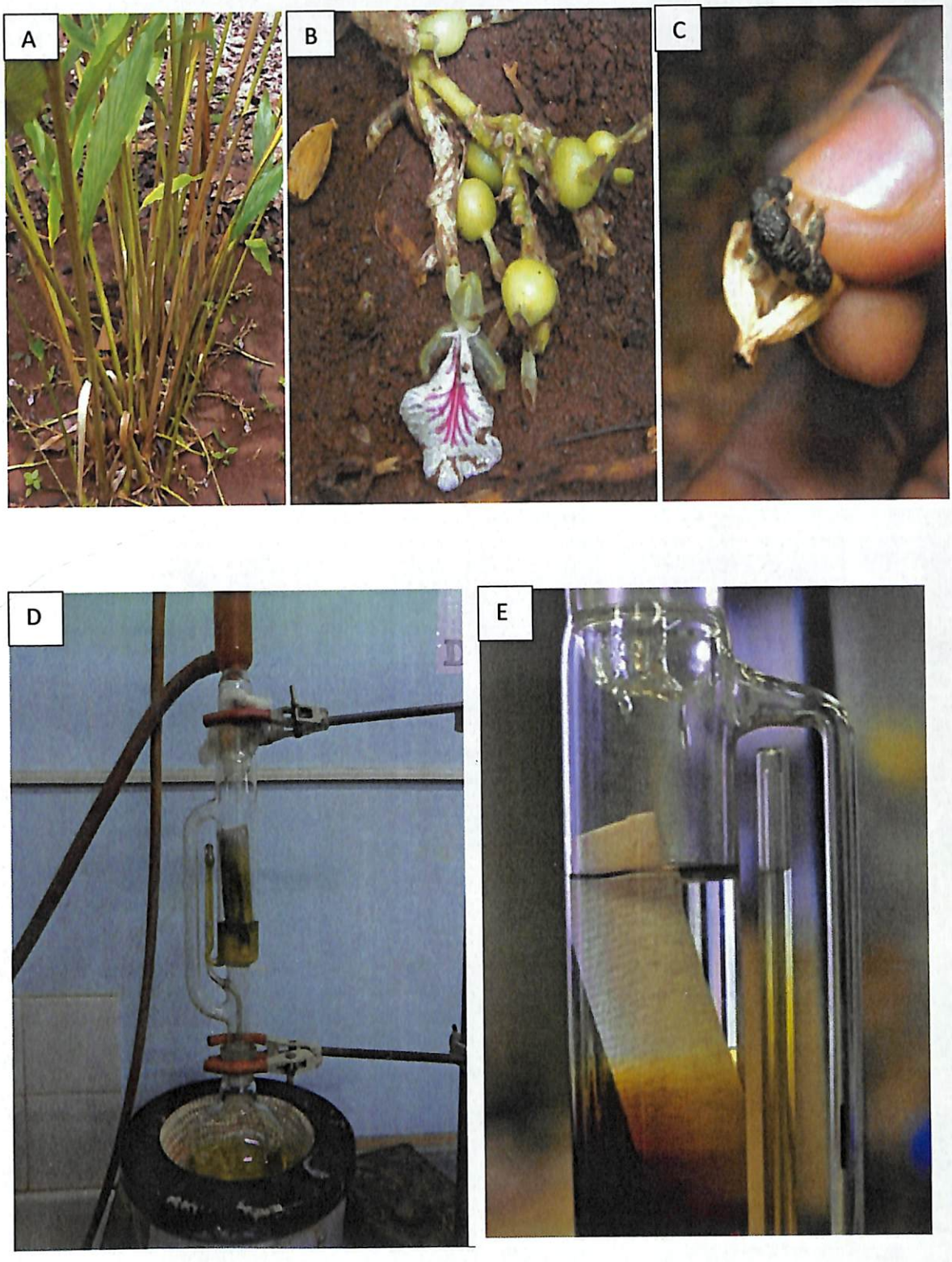

Plate 1: (A) Cardamom plant (B) cardamom flower (C) Cardamom capsule (D) and (E) soxhlet apparatus

3.2 MOLECULAR MARKER ANALYSES

3.2.1 Primer designing

With the keyword '*Curcuma longa'* a set of 12678 EST sequences were 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.

The retrieved sequences were then uploaded in TRIMEST program of EMBOSS suite (http://genome.csdb.cn/cgi-bin/emboss/trimest). This software was used to remove the poly A and poly T tail region of the EST, thus reduced the redundancy of the sequence for further analysis. The default settings used in the programme were, (i) the minimum length of poly-A tail as 4 (ii) the minimum number of contiguous mismatch allowed in a tail as 4 (iii) write the reverse complement when poly-T is removed (yes) and (iv) remove poly-T tails at the 5′ end of the sequence (yes).

The resulted trimmed sequences were then assembled using contig assembly program CAP3 in the mobyle portal platform (http://mobyle.pasteur.fr/cgi-bin/portal.py). The non-redundant dataset thus generated was uploaded in WEBSAT (wsmartins.net/websat/) for the detection of SSR region and designing suitable primers. A total of 206 primers were designed collectively for the contigs and singlets output from mobyle portal.

The efficiency of the newly designed primers was verified. The primer pairs with a score of exactly hundred percent were selected for synthesis. A score of hundred percent indicates the absence of self and cross dimerization of primers. This task was accomplished by a software NETPRIMER (http://www.premierbiosoft.com/netprimer/index.html), used for the quality validation of designed primers. A selected set of 20 primers were synthesized in IDT (Integrated DNA technologies, New Delhi).

3.2.2 DNA Isolation

DNA Isolation was performed from 18 cardamom accessions using DNeasy plant mini kit. Leaf samples collected from different plant varieties were wiped with sterile distilled water and subjected to total genomic DNA isolation with the following procedure.

100 mg fresh weight of the leaf samples were disrupted in mortar and pestle using liquid nitrogen. 400 µl Buffer AP1 and 4 µl RNase A were added to the samples, the mixture was then vortexed and incubated at 65 ˚C in waterbath (JEI0 TECH, Korea) for 10 minutes for accompanying cell lysis and removal of RNA. After incubation, 130 µl neutralization buffer P3 was added, mixed and incubated on ice for 5 minutes and the lysates were centrifuged at 14,000 rpm for 5 minutes. This step precipitated molecules other than nucleic acid, such as proteins and polysaccharides. The lysate was then pipetted into a QIAshredder spin column placed in a 2ml collection tube and centrifuged (Eppendorf, Chennai) for 2 min at 14,000 rpm. The flow-through was then transferred into a new tube and 1.5 volumes of wash Buffer AW1 was added and mixed by pipetting. From the mixture 650 µl was transferred into a DNeasy Mini spin column placed in the 2 ml collection tube and centrifuged for 1 min at 8000 rpm. After discarding the flow-through, the step was repeated with the remaining sample. For improving the quality of DNA obtained, the spin column was again placed in a 2 ml collection tube, and 500 µl wash buffer AW2 was added, and centrifuged for 1 min at 8000 rpm. After discarding the flow-through the spin column was transferred into a 2ml microfuge tube and the DNA was eluted by adding 100 µl AE buffer and centrifuging for 1 minute at 8000 rpm after 5 minutes incubation at room temperature. The isolated DNA was stored at -20 ˚C freezer (VESTFROST, Denmark).

3.2.2.1 Agarose Gel Electrophoresis

The DNA preparations were analysed using agarose gel electrophoresis. 3 µl aliquots from each DNA sample were loaded on 0.8 per cent agarose gel. The

gel was run at 110 V with 1x tank buffer in horizontal gel electrophoresis unit (Chromous Biotech, Banglore) for about 3 hours. The genomic DNA bands in the gel were visualized by ethidium bromide staining under the gel documentation system (UVP, UK) with software Vision Work LS.

3.2.2.2 Biophotometer analysis

The quantity and quality of the isolated DNA samples were determined using biophotometer (Eppendorf India Limited). The instrument was calibrated with 50µl Eluting buffer as blank. Biophotometer required a sample size of 1µl DNA diluted with 49µl eluting buffer 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)

3.2.3 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:

Reagents	Volume
Water	19.3 µl
10x buffer A	2.50 µl
dNTPs (10 mM each)	0.50 µl
Forward primer (25 nM)	0.60 µl
Reverse primer (25 nM)	0.60 µl
Template DNA (50 ng μl^{-1})	1.00 µl
Dynazyme (2 U μl^{-1})	0.50 µl
Total volume	25.0 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 100 bp DNA ladder was loaded along with the samples to compare the size of resultant bands.

3.2.3 SSR analysis

The bands were scored from the gels to identify polymorphism and other genetic diversity parameters. Genetic analysis was carried out using the population genetic software POPGENE version 1.32.

RESULTS

4. RESULTS

The results of the research work entitled "Development and use of SSR markers for analysis of genetic diversity and correlation with lignin content in cardamom (*Elettaria cardamomum* Maton) germplasm" conducted at the Biotechnology and Bioinformatics division of Jawaharlal Nehru Tropical Botanic Garden and Research Institute (JNTBGRI), Palode during the academic year 2013-14 are presented in this chapter.

4.1 LIGNIN ESTIMATION

4.1.1 Morphological Observations

Prior to the systematic analytical procedures, the morphological characteristics such as plant height, number of tillers and number of leaves of the plants were recorded (Table 2).

Table 2: Morphological observations of the accessions used for the study

Sl No.	Accession no.	Plant height (cm)	No. of tillers	No. of leaves / tiller
1	24	234	3	14
2	38	93	3	9
3	51	150	1	13
4	53	160	1	12
5	55	174	1	12
6	56	140	1	6
7	57	141	2	13
8	58	142	1	12
9	59	189	2	16
10	60	194	3	12
11	61	127	2	10
12	62	167	3	17
13	63	118	3	8
14	65	161	3	11
15	68	212	3	10
16	69	134	2	11
17	70	201	3	16

4.1.2 Spectrophotometric analysis

Lignin estimation of the water and methanol extracted samples were performed *via* spectrophotometric analysis. The samples were dissolved in ionic compound 1-n-butyl 3-methyl Immidazolium Chloride [Bmin][Cl] and the absorbance was measured at 440 nm. The details are described in Table 3.

Table 3: Details of samples used and absorbance at 440 nm obtained for estimating lignin content

Sl No.	Accession	Fresh Weight	Dry Weight	Weight after extraction	Absorbance A_{440}
1	24	10.5652	2.1843	1.1958	0.394
2	38	4.7431	0.6065	0.2679	0.419
3	51	9.3290	1.5552	0.8905	1.133
4	53	8.1224	1.4177	0.6323	1.733
5	55	7.7738	1.3893	0.7762	1.319
6	56	8.4116	1.11939	0.4793	1.543
7	57	9.9545	1.6800	0.7750	1.751
8	58	9.6901	1.0496	0.5643	1.721
9	59	6.8374	1.5665	0.6917	0.416
10	60	12.8050	1.5134	0.8320	0.687
11	61	5.6658	0.8683	0.4074	0.916
12	62	12.8633	3.3935	1.7844	1.257
13	63	9.9740	1.4231	0.6681	1.340
14	65	11.6840	1.5748	0.8595	0.433
15	68	20.0873	5.3268	2.5860	1.816
16	69	7.4709	1.3778	0.6864	0.837
17	70	7.9525	1.4382	0.6703	0.943

4.2 MOLECULAR MARKER ANALYSIS

4.2.1 Primer designing and analysis

Two hundred and six primer pairs were designed for SSR markers in cardamom using different bioinformatics tools with the EST sequences of *Curcuma longa* belonging to the same family. The efficiency of the primers were analysed with the software NETPRIMER and 20 primer pairs having a score of hundred percent were custom synthesized and used for genetic diversity studies. The newly synthesised primers were GC rich and devoid of self-dimerization. Details of the primers used for the analysis are described in Table 4.

Table 4: Details of primers designed for genetic analysis of cardamom

Sl No.	Primer	Sequence	No. of Bases	GC (%)	Tm (^{0}C)	Annealing temperaure
1	CaSSR 21F	TCACGCTAAATGGATGGTCTAC	22	45.4	54.4	49.5
	CaSSR 21R	TATCTACCCACAGCGGAAGTTT	22	45.4	55.8	
2	CaSSR 22F	TGAGAGGGGAAGATAAGACCAA	22	45.4	54.9	51.5
	CaSSR 22R	GGAACTGTGGCAGGAGATGTAT	22	50.0	56.6	
3	CaSSR 23F	GAGGGAAGAGAGGAGAAGGAGA	22	54.5	57.0	54.9
	CaSSR 23R	TCAAGATGTCTGGTATTGGTGG	22	45.4	54.5	
4	CaSSR 24F	GTTGAGGAACAGCGACGAG	19	57.8	55.9	52.8
	CaSSR 24R	AACACTTGCTTCCCTTACTCCA	22	45.4	56.3	
5	CaSSR 25F	AGGTTTCTTCTTTAGGCGTCGT	22	45.4	56.4	57.1
	CaSSR 25R	GAGATGGGGCGAAAATGG	18	55.5	53.7	
6	CaSSR 26F	CTGGGATGGGTATCTACAATGA	22	45.4	53.6	52.9
	CaSSR 26R	CAGTGAGTCCACAGAAAGCAAT	22	45.4	55.3	
7	CaSSR 27F	AGCAGTTGAGAGGGTTCTTGAG	22	50.0	56.7	49.5
	CaSSR 27R	ATTATTCCCTTCCCGCATTTAC	22	40.9	53.0	
8	CaSSR 28F	AAGTCCTCCAAGAACAACAACG	22	45.4	55.4	49.5

	CaSSR 28R	CTTTAGCCCAAGTGATAGACGG	22	50.0	55.1	
9	CaSSR 29F	TCCTCCTCCACCTCCTCC	18	66.6	58.0	58.2
	CaSSR 29R	GGTTTTCACCTTCCCAACTCTA	22	45.4	54.6	
10	CaSSR 30F	CAAGAACAAGAAACAACATCGC	22	40.9	53.0	51.9
	CaSSR 30R	GTTCCAGACGATAACAACGACA	22	45.4	54.9	
11	CaSSR 31F	ATCTCAACCTGCTGCCTCTG	20	55.0	57.2	50.5
	CaSSR 31R	TTCATCGTAACATCCACAATCG	22	40.9	53.0	
12	CaSSR 32F	CTCCTCCTCCTCCTCCTCAT	20	60.0	57.3	57.1
	CaSSR 32R	GCATACCTGTTTCAGAGTGGC	21	52.3	56.1	
13	CaSSR 33F	TGAAACATAACTTCTTGGAGCG	22	40.9	53.2	49.5
	CaSSR 33R	TCTCTCTCTCACACACACACACA	23	47.8	57.5	
14	CaSSR 34F	CAGAATCAGCAAAACAAGCAAC	22	40.9	53.3	50.5
	CaSSR 34R	TATGGGCAGTCTTAGGCAATCT	22	45.4	55.8	
15	CaSSR 35F	CTTGACAGGACAGCAACAGAAC	22	50.0	56.1	56.1
	CaSSR 35R	CGATGACAGAAGAGAGAGAGCA	22	50.0	55.9	
16	CaSSR 36F	ACCGCCCTCCTACTTCTTCTC	21	57.1	58.3	62.0
	CaSSR 36R	ACTCCGTGTGATACTTGTGCG	21	52.3	57.3	
17	CaSSR 37F	GGAGGAGGAGAAGAAGAAGGAG	22	54.5	56.1	50.5
	CaSSR 37R	CGCAACACACAGACATCTATCA	22	45.4	55.0	
18	CaSSR 38F	AAACAGCAACATCAGTCAAACG	22	40.9	54.3	53.9
	CaSSR 38R	CAGAGTCACCAGTGCCCTTC	20	60.0	57.8	
19	CaSSR 39F	ACCAGTCTTCTCTTTCCGCTC	21	52.3	56.7	58.0
	CaSSR 39R	CTCCACTCCAGGTAGAGCATTC	22	54.5	56.8	
20	CaSSR 40F	CAATGGGACTACAGTGGCG	19	57.8	55.8	49.2
	CaSSR 40R	ATAAACAAACTCAACAGCAGCG	22	40.9	54.1	

4.2.2 DNA Isolation

DNA samples of 18 cardamom accessions were isolated using DNeasy plant mini kit. Good quality DNA bands were observed in 0.8 % agarose gel electrophoresis. The quality and quantity of the extracted samples were confirmed with biophotometric analysis (Table 5).

Table 5: Quality and quantity of isolated DNA

Sl No.	Accessions	Absorbance (A 260 nm)	Absorbance (A 280 nm)	Purity A 260/ A 280	DNA concentration
1	24	0.063	0.047	1.34	0.1563
2	38	0.086	0.065	1.31	0.2140
3	51	0.078	0.054	1.45	0.1962
4	53	0.046	0.043	1.07	0.1157
5	55	0.068	0.043	1.58	0.1691
6	56	0.078	0.056	1.39	0.1938
7	57	0.042	0.028	1.53	0.1053
8	58	0.080	0.052	1.52	0.1992
9	59	0.058	0.037	1.58	0.1450
10	60	0.053	0.040	1.30	0.1313
11	61	0.063	0.050	1.26	0.1573
12	62	0.071	0.043	1.67	0.1775
13	63	0.023	0.016	1.45	0.0574
14	65	0.074	0.057	1.30	0.1845
15	68	0.083	0.062	1.33	0.2078
16	69	0.054	0.039	1.37	0.1345
17	70	0.048	0.034	1.42	0.1193

***Table 6: Description of Plate 2**

Lane	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Acc. no	59	62	63	57	58	55	68	69	51	70	24	60	38	53	65	61	56	52

* Subsequent plates follow the same order (plates 3-16), L represents Ladder

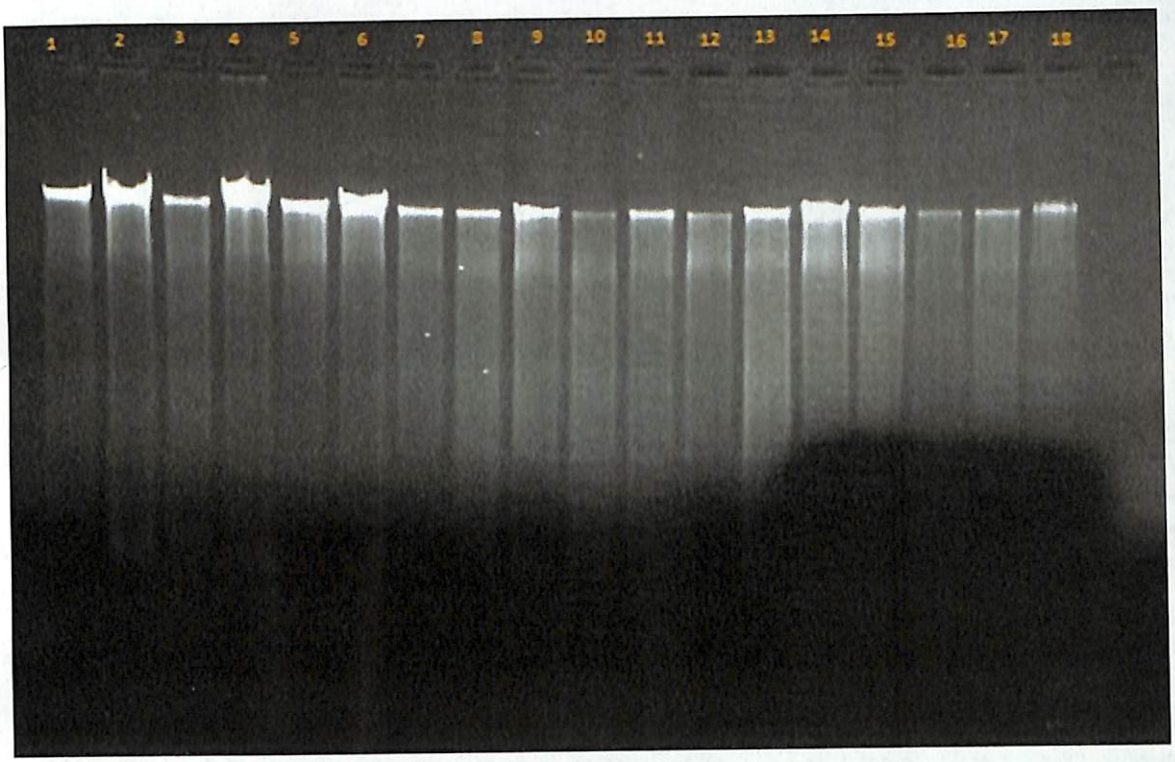

Plate 2: Genomic DNA isolated from 18 cardamom accessions. Description of each lane is represented with Table 6

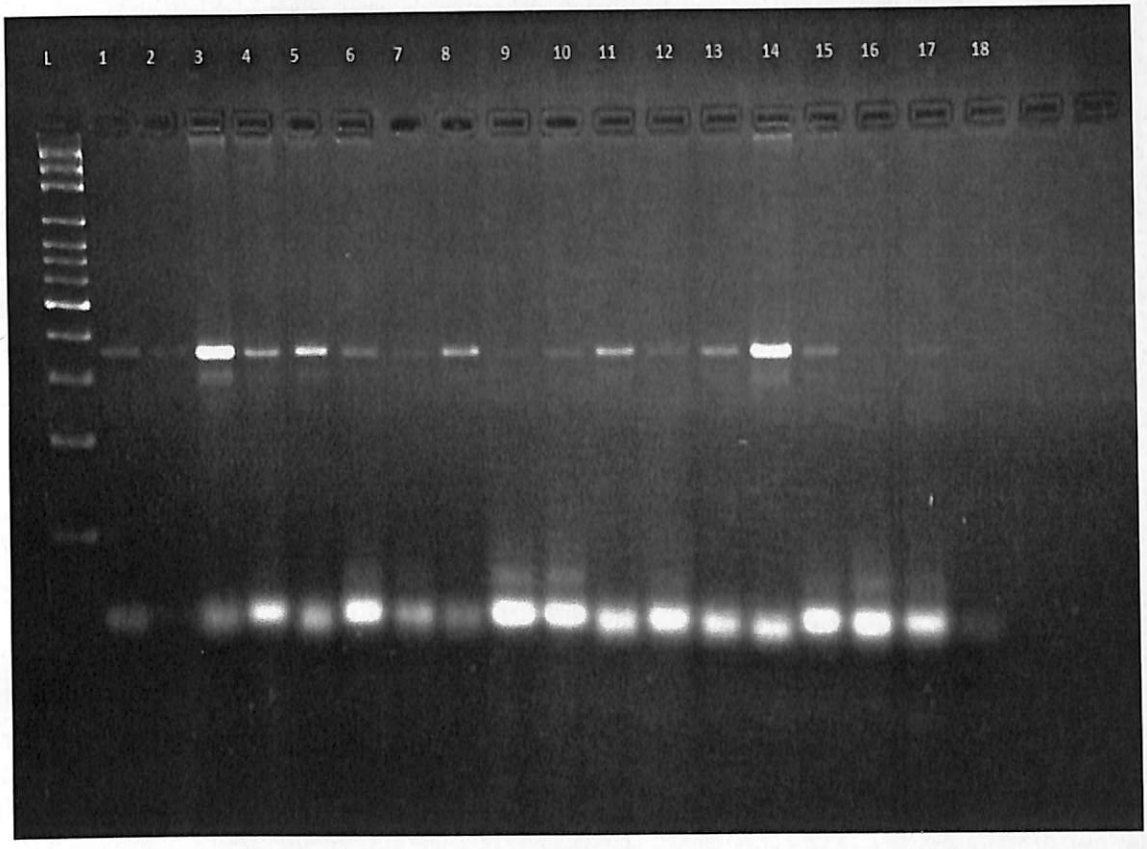

Plate 3: SSR Profile obtained for primer CaSSR 21

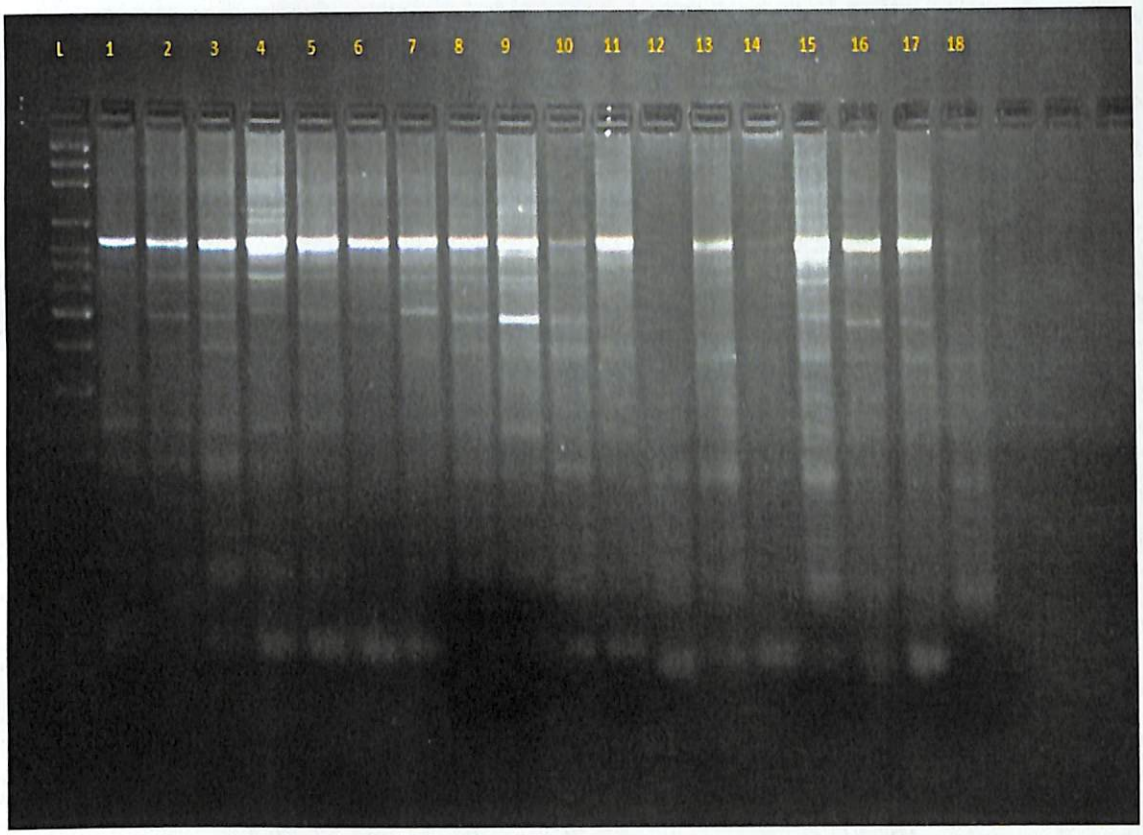

Plate 4: SSR profile obtained for primer CaSSR 23

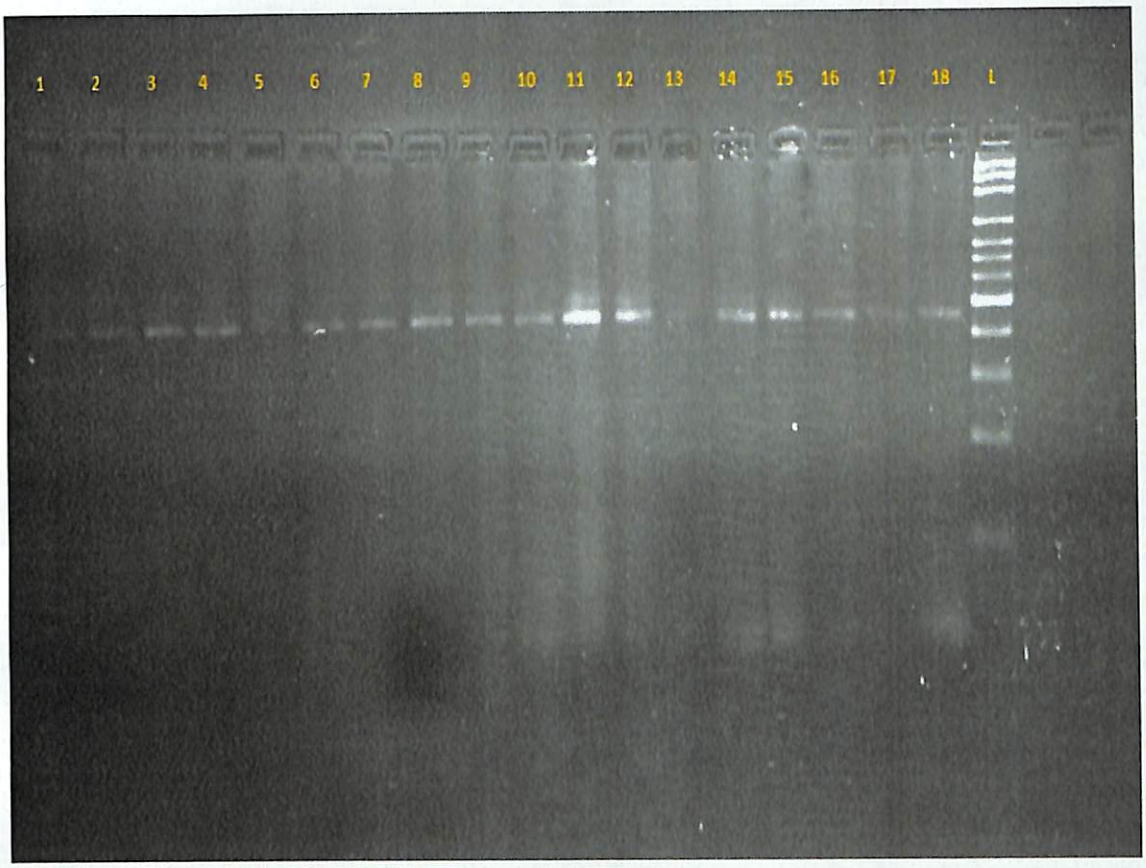

Plate 5: SSR Profile obtained for primer CaSSR 25

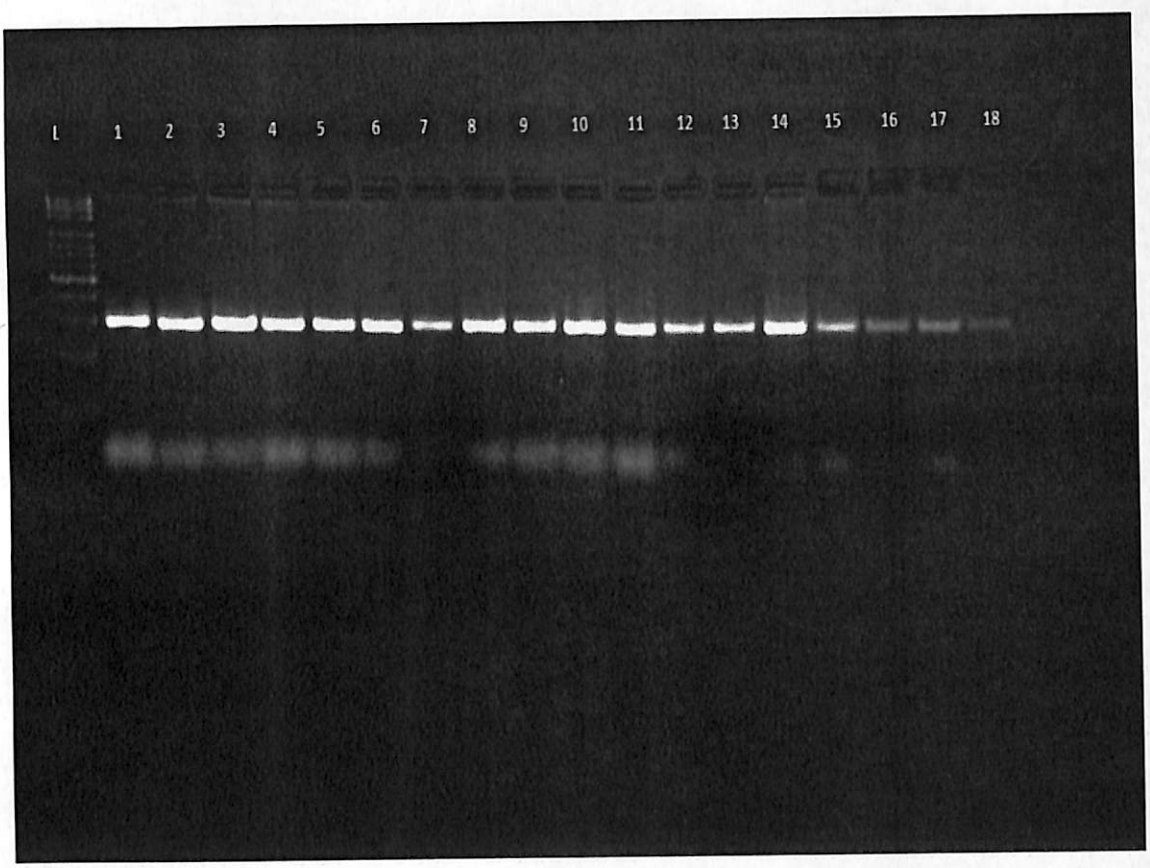

Plate 6: SSR Profile obtained for primer CaSSR26

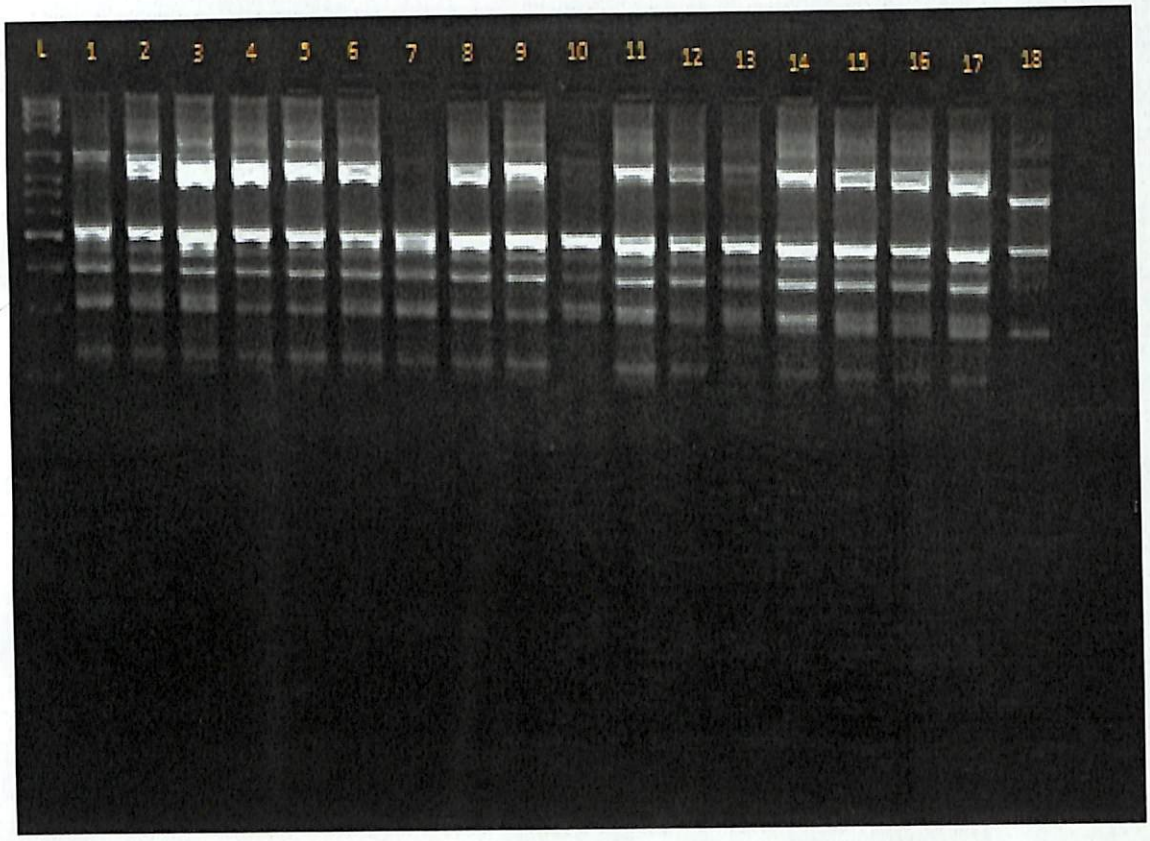

Plate 7: SSR profile obtained for CaSSR 29

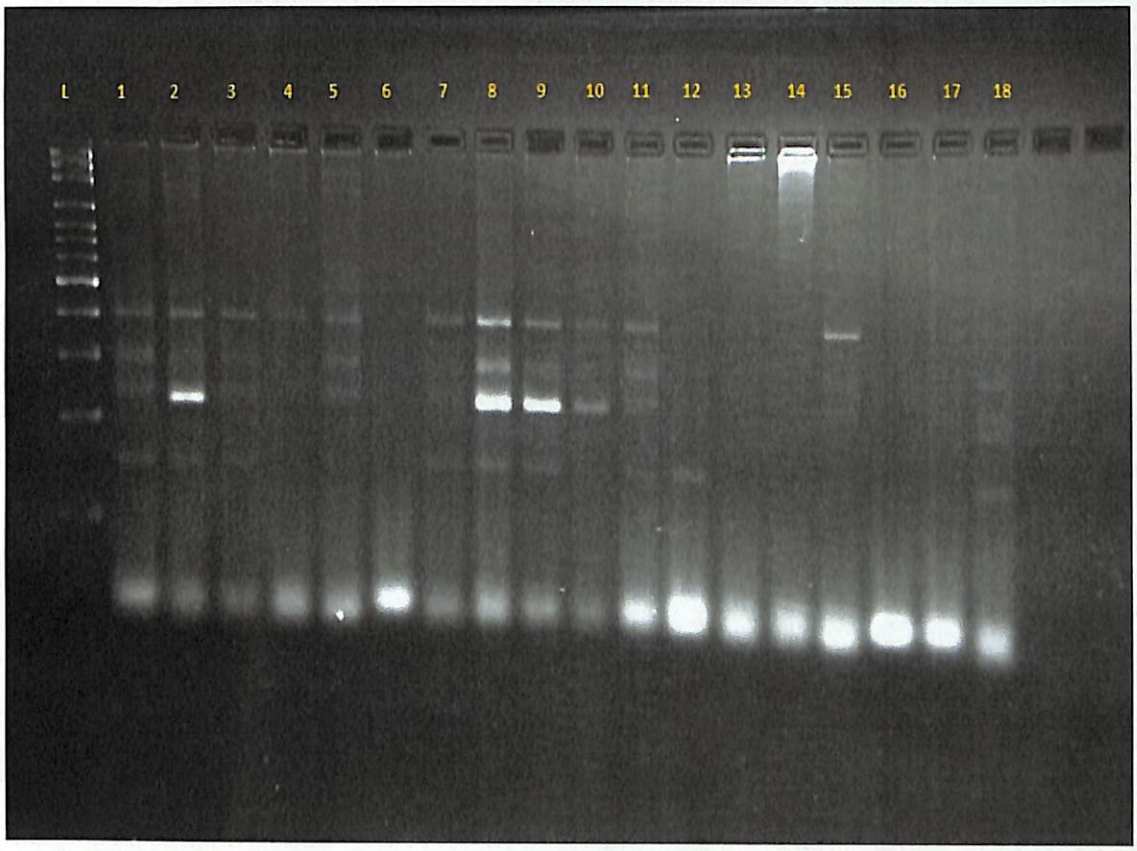

Plate 8: SSR profile obtained for primer CaSSR 30

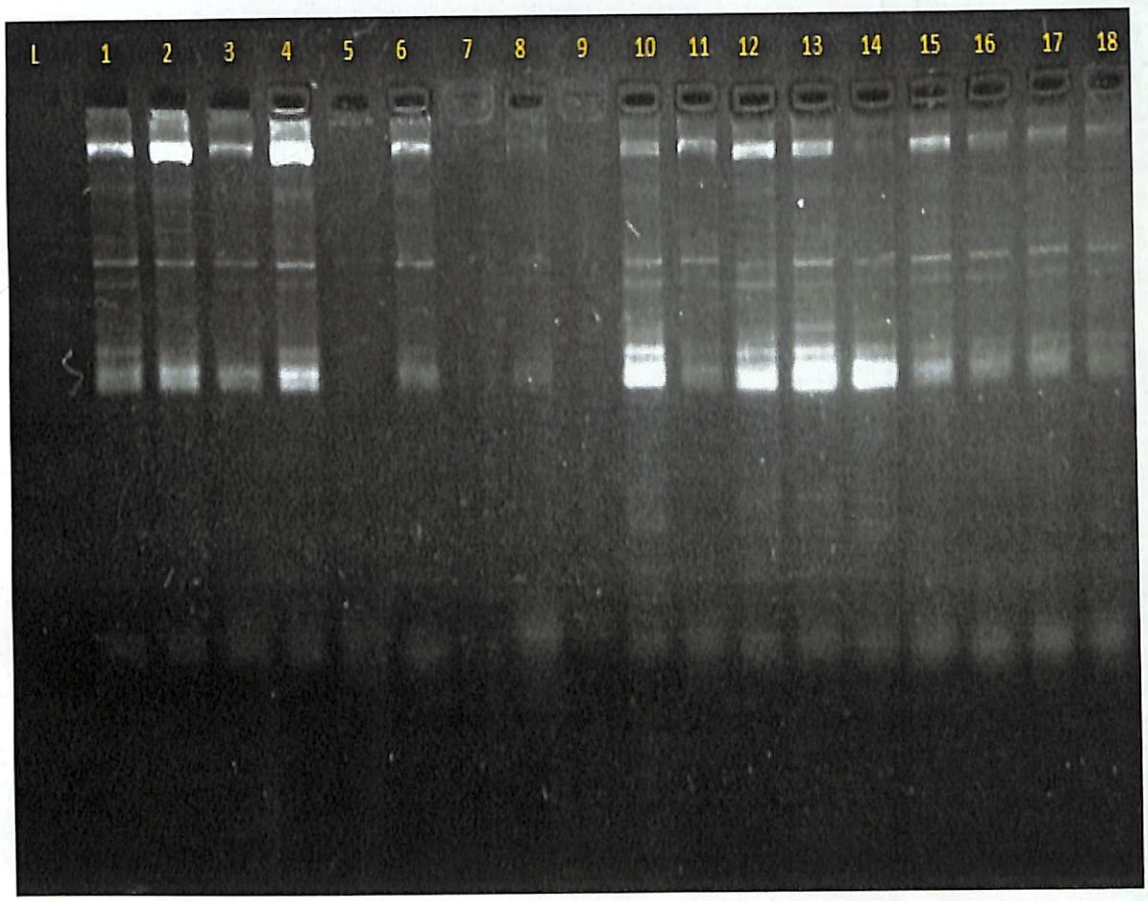

Plate 9: SSR profile obtained for primer CaSSR 32

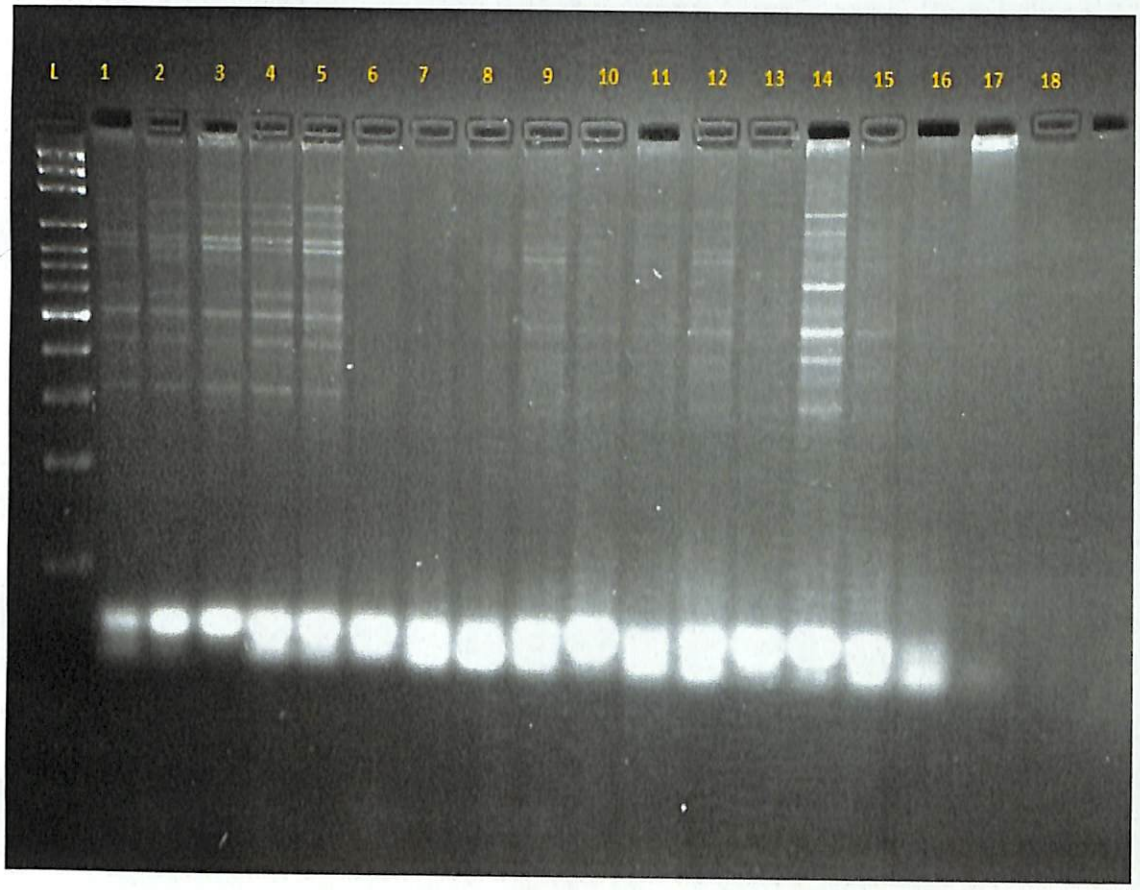

Plate 10: SSR profile obtained for primer CaSSR 33

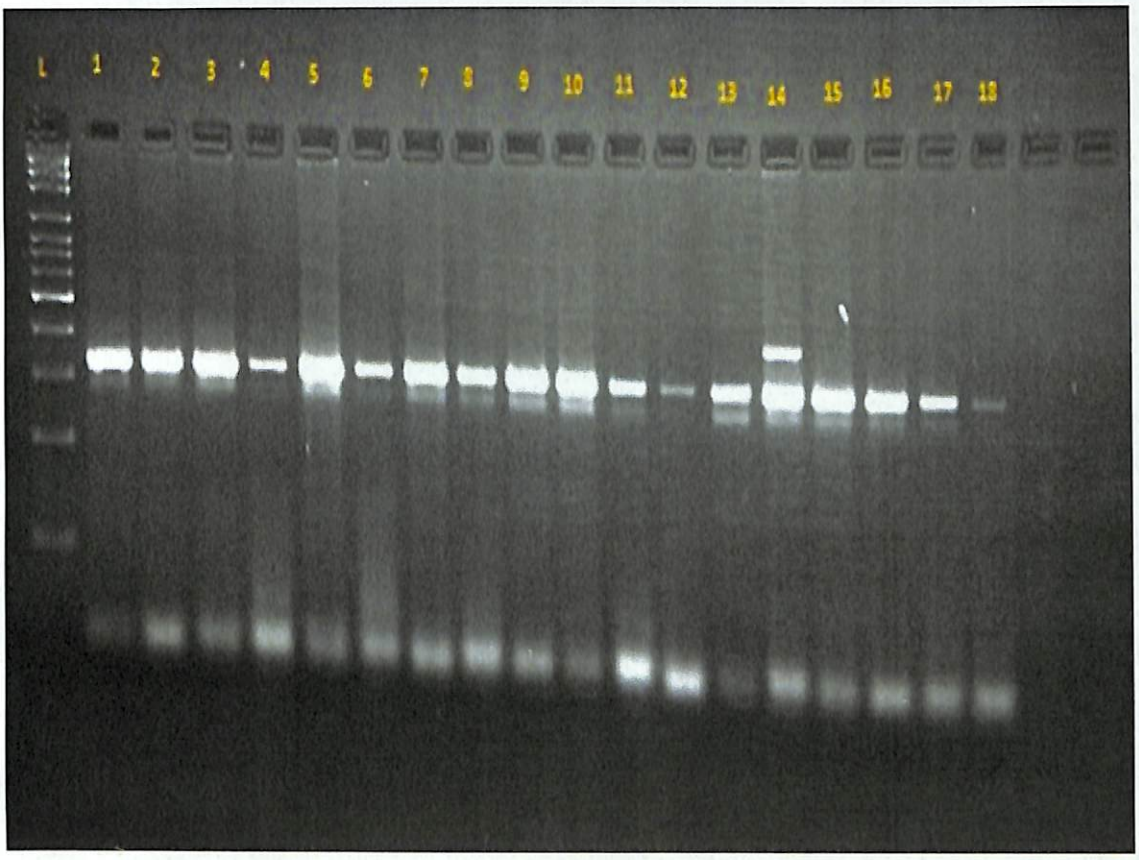

Plate 11: SSR profile obtained for primer CaSSR 34

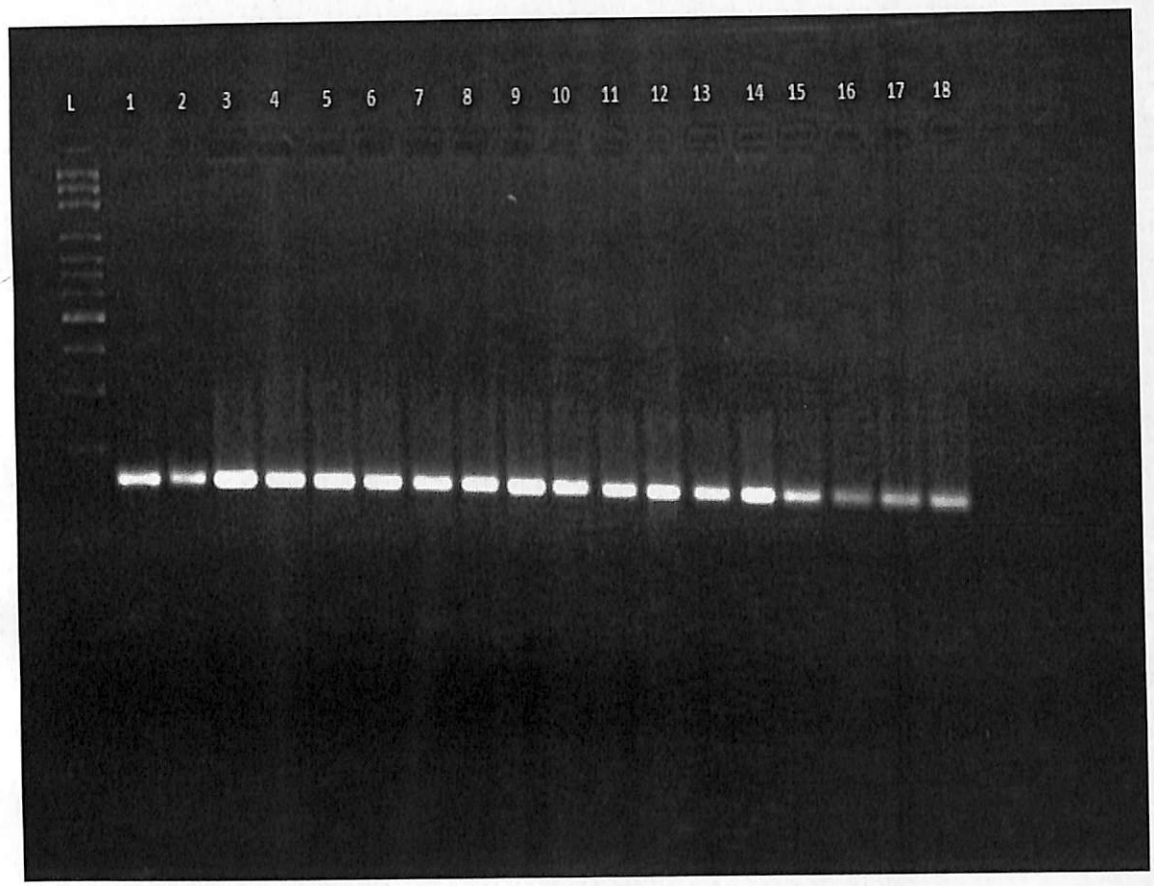

Plate 12: SSR profile obtained for primer CaSSR 35

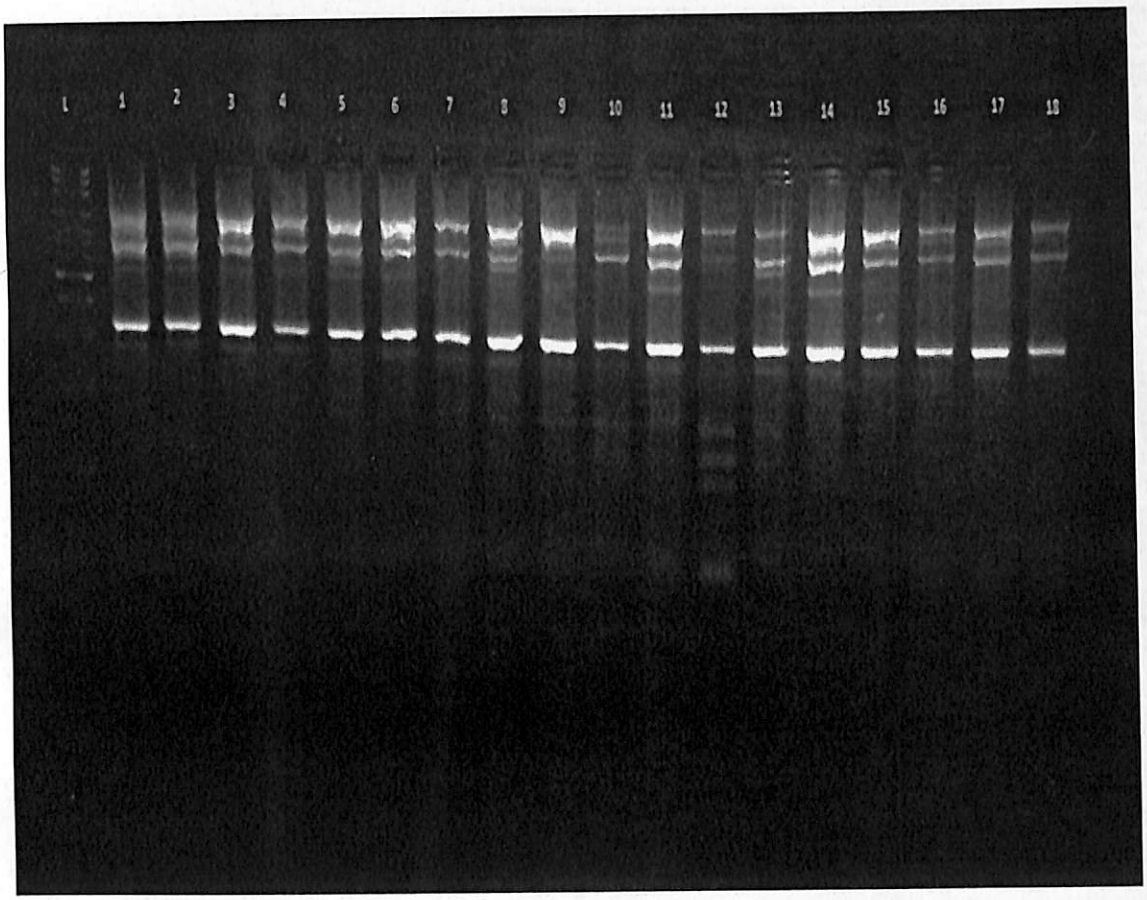

Plate 13: SSR profile obtained for primer CaSSR 37

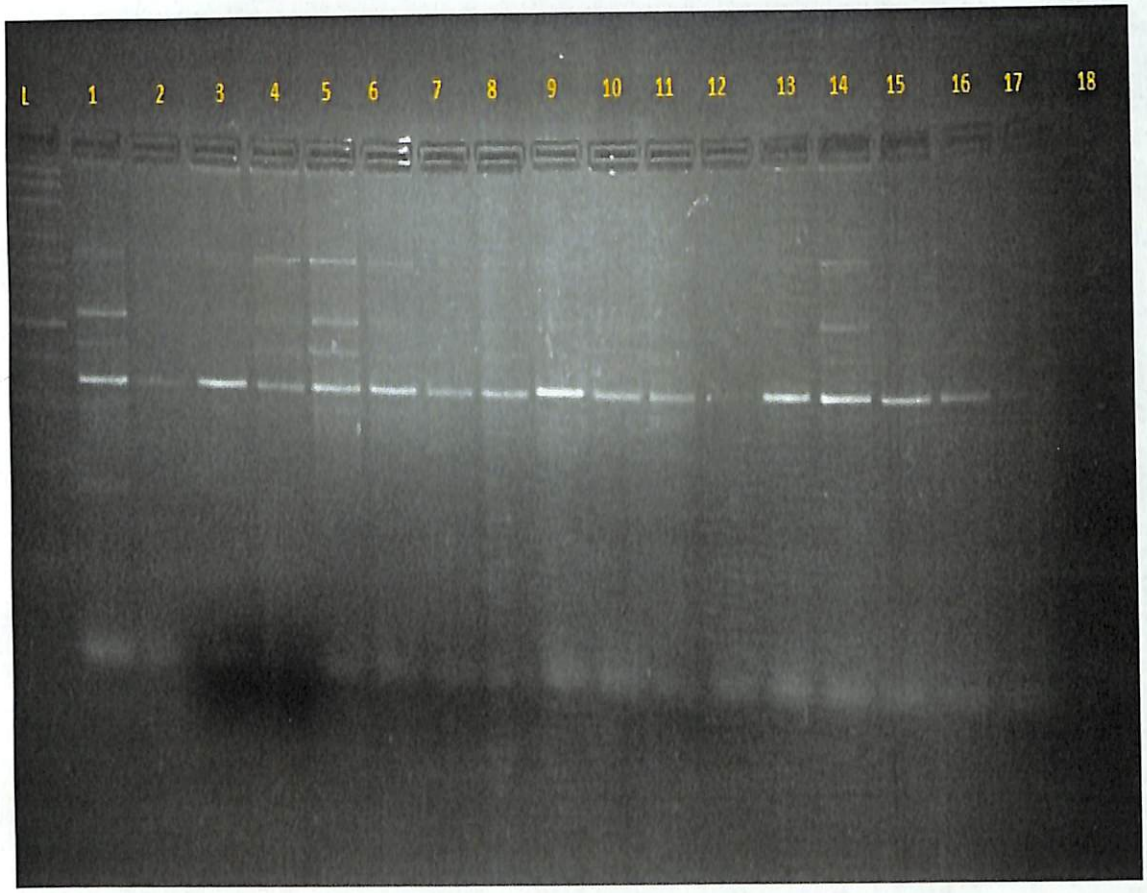

Plate 14: SSR profile obtained for primer CaSSR 38

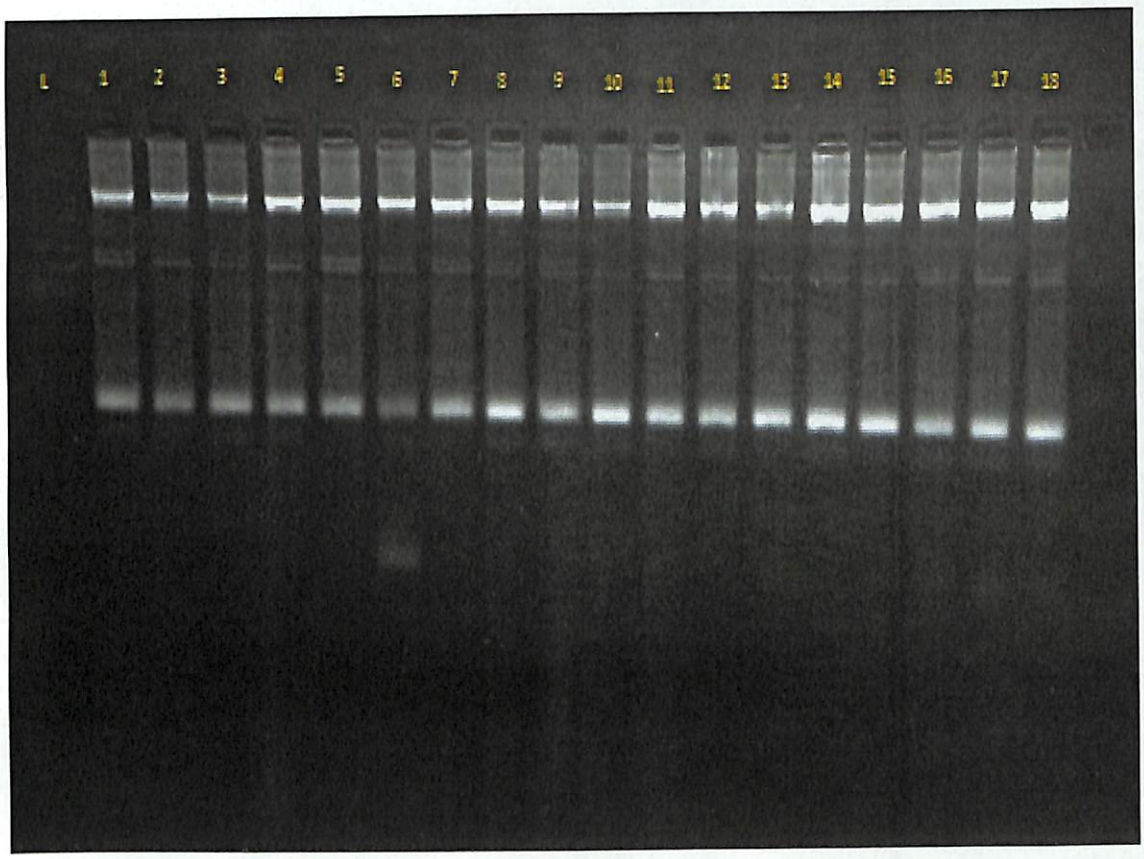

Plate 15: SSR profile obtained for primer CaSSR 39

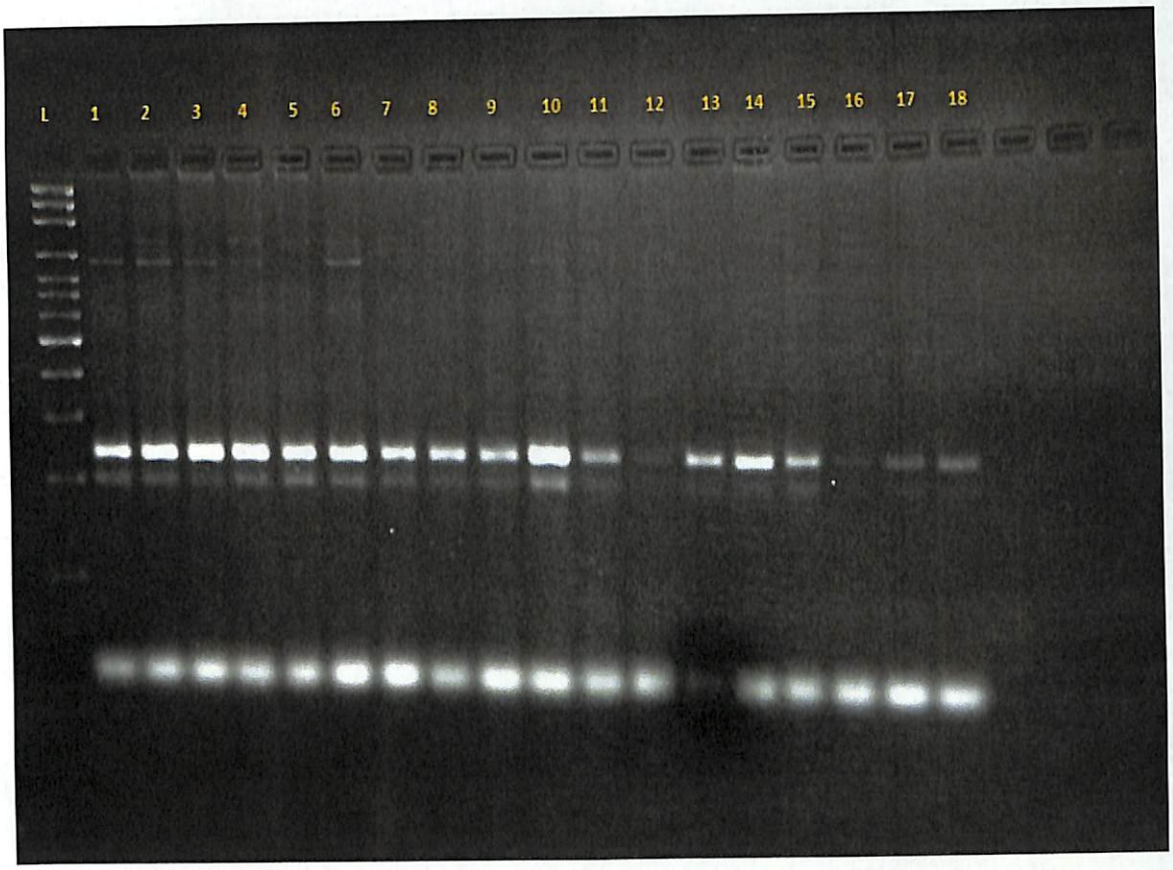

Plate 16: SSR profile obtained for primer CaSSR 40

4.2.3 SSR analysis

The number of bands obtained for each primer with respect to the accession number is represented with table 7.The maximum number of bands was observed for accession 58, variety ICRI 7 and the minimum was for accession 61 (ICRI 2) and 68 (Wild variety, pink tiller). Details of SSR analysis is summarized in table 8.

Table 7: Details of SSR analysis, number of bands obtained per primer for all accessions

Acc. No.	No. of bands for each Primer														Total no. of bands
	26	**34**	**35**	**21**	**25**	**32**	**23**	**40**	**37**	**33**	**38**	**29**	**30**	**39**	
Acc 24	1	2	1	2	2	4	3	4	7	3	3	10	5	4	51
Acc 38	1	2	1	1	1	7	3	3	7	1	4	8	0	4	43
Acc 51	1	2	1	1	1	0	6	3	6	5	2	9	4	4	45
Acc 53	1	3	1	2	1	4	2	4	7	7	6	10	0	4	52
Acc 55	1	1	1	2	1	5	3	5	6	0	5	10	0	4	44
Acc 56	1	2	1	1	1	5	5	2	6	0	1	10	2	4	41
Acc 57	1	1	1	2	1	6	8	4	6	8	4	9	1	4	56
Acc 58	1	2	1	2	1	1	6	4	6	9	5	9	6	5	58
Acc 59	1	2	1	1	1	6	3	4	7	7	6	7	4	4	54
Acc 60	1	1	1	1	1	6	1	1	5	7	1	10	1	4	41
Acc 61	1	2	1	1	1	5	4	1	6	0	1	10	0	4	37
Acc 62	1	2	1	1	1	4	6	5	7	8	1	10	5	3	55
Acc 63	1	2	1`	2	2	5	6	4	7	7	2	10	4	3	56
Acc 65	1	2	1	2	1	6	6	4	7	4	1	10	5	4	54
Acc 68	1	2	1	1	1	1	5	4	5	0	1	7	4	4	37
Acc 69	1	2	1	2	2	3	5	3	7	0	2	9	5	4	46
Acc 70	1	2	1	1	1	6	4	3	6	2	1	7	3	3	41

Table 8: Summary of SSR analysis

Primers	Number of bands	Minimum band size (bp)	Maximum band size (bp)
CaSSR 26	17	300	300
CaSSR 34	32	300	400
CaSSR 35	17	150	150
CaSSR 21	25	300	350
CaSSR 25	20	450	500
CaSSR 32	74	***	***
CaSSR 23	76	100	950
CaSSR 40	58	200	950
CaSSR 37	108	100	900
CaSSR 33	68	310	990
CaSSR 38	46	175	850
CaSSR 29	155	220	1000
CaSSR 30	49	150	400
CaSSR 39	66	150	1000

*** Marker was not clear

4.2.4 Genetic data analysis

Genetic data analysis of the 17 accessions was subjected to microsatellite analysis using 41 loci belong to 14 SSR primers using POPGENE (Yeh & Boyle, 1997). Results are presented in Table 9.

Table 9: Summary of genetic variation statistics for 17 cardamom accessions analyzed using 14 SSR primer pairs at 41 loci

Acc.No.	na	ne	l	Obs_ Hom	Obs_ Het	Exp_ Hom	Exp_ Het	h	Ave_ Het	P
Acc 24	1.50	1.50	0.35	0.50	0.50	0.50	0.50	0.25	0.20	36.59
Acc 59	1.47	1.47	0.33	0.53	0.47	0.53	0.47	0.24	0.18	41.46
Acc 38	1.34	1.34	0.24	0.66	0.34	0.66	0.34	0.17	0.21	24.39
Acc 65	1.45	1.45	0.31	0.55	0.45	0.55	0.45	0.23	0.19	34.15
Acc 60	1.32	1.32	0.22	0.68	0.32	0.68	0.32	0.16	0.20	21.95
Acc 69	1.43	1.43	0.30	0.57	0.43	0.57	0.43	0.22	0.21	31.71
Acc 61	1.48	1.48	0.33	0.52	0.48	0.52	0.48	0.24	0.23	26.83
Acc 70	1.34	1.34	0.24	0.66	0.34	0.66	0.34	0.17	0.19	24.39
Acc 51	1.48	1.48	0.34	0.52	0.48	0.52	0.48	0.24	0.19	36.59
Acc 62	1.40	1.40	0.28	0.60	0.40	0.60	0.40	0.20	0.18	34.15
Acc 55	1.52	1.52	0.36	0.48	0.52	0.48	0.52	0.26	0.22	34.15
Acc 63	1.46	1.46	0.32	0.54	0.46	0.54	0.46	0.23	0.18	39.02
Acc 56	1.46	1.46	0.32	0.54	0.46	0.54	0.46	0.23	0.21	29.27
Acc 58	1.43	1.43	0.30	0.57	0.43	0.57	0.43	0.21	0.17	36.59
Acc 53	1.52	1.52	0.36	0.48	0.52	0.48	0.52	0.26	0.19	39.02
Acc 57	1.46	1.46	0.32	0.54	0.46	0.54	0.46	0.23	0.17	41.46
Acc 68	1.35	1.35	0.24	0.65	0.35	0.65	0.35	0.17	0.21	19.51
Min	**1.32**	**1.32**	**0.22**	**0.48**	**0.32**	**0.48**	**0.32**	**0.16**	**0.17**	**19.51**
Max	**1.52**	**1.52**	**0.36**	**0.68**	**0.52**	**0.68**	**0.52**	**0.26**	**0.23**	**41.46**
Avg	**1.44**	**1.44**	**0.30**	**0.56**	**0.44**	**0.56**	**0.44**	**0.22**	**0.20**	**32.43**
SD	**0.06**	**0.06**	**0.04**	**0.06**	**0.06**	**0.06**	**0.06**	**0.03**	**0.02**	**6.89**

4.2.4.1 Estimation of SSR polymorphism

The observed number of alleles per locus (na) ranged from 1.32 to 1.52 effective number of alleles (ne) also ranged from 1.32 to 1.52, Shannon's information index (I) ranged from 0.22 to 0.36, observed homozygosity (Obs_Hom) ranged from 0.48 to 0.68, observed heterozygosity (Obs_Het) ranged from 0.32 to 0.52, expected homozygosity (Exp_Hom) ranged from 0.48 to 0.68, expected heterozygosity (Exp_Het) ranged from 0.32 to 0.52, Nei's heterozygosity (h) ranged from 0.16 to 0.26, average heterozygosity (Ave_Het) ranged from 0.17 to 0.23 and percentage of polymorphic loci (P) ranged from 19.51 to 41.46.

4.2.5 Genetic relationships and cluster analysis

The pair-wise genetic similarity and distance matrix were prepared on the basis of SSR data (Table 10). The genetic similarity values varied from 0.54 to 0.91 with a mean value of 0.7358

Cluster analysis was performed on the SSR data using POPGENE software following the UPGMA method and the dendrograms were constructed through TREEVIEW (Page, 1996) showing overall genetic relatedness among the individuals (Fig. 1).

Table 10: Nei's original measures of genetic identity and genetic distance (Nei, 1972) estimated from the SSR analysis

Acc. No	59	62	63	57	58	55	68	69	51	70	24	60	38	53	65	61	56
59	****	0.84	0.81	0.86	0.80	0.68	0.61	0.71	0.75	0.77	0.78	0.80	0.65	0.79	0.76	0.63	0.65
62	0.18	****	0.90	0.82	0.85	0.71	0.69	0.76	0.80	0.79	0.75	0.75	0.69	0.79	0.82	0.70	0.76
63	0.21	0.11	****	0.81	0.82	0.73	0.67	0.84	0.80	0.76	0.83	0.70	0.71	0.75	0.84	0.71	0.77
57	0.15	0.19	0.21	*** *	0.84	0.73	0.64	0.73	0.77	0.70	0.77	0.75	0.72	0.82	0.86	0.68	0.70
58	0.22	0.17	0.20	0.18	*** *	0.66	0.72	0.73	0.81	0.67	0.76	0.69	0.65	0.86	0.80	0.60	0.65
55	0.38	0.35	0.31	0.31	0.41	*** *	0.64	0.83	0.65	0.70	0.75	0.65	0.81	0.63	0.75	0.82	0.81
68	0.49	0.37	0.40	0.44	0.33	0.44	*** *	0.73	0.73	0.71	0.68	0.57	0.68	0.54	0.75	0.69	0.77
69	0.35	0.27	0.17	0.31	0.32	0.19	0.31	*** *	0.79	0.75	0.87	0.57	0.75	0.61	0.76	0.78	0.84
51	0.29	0.22	0.22	0.26	0.22	0.44	0.31	0.24	*** *	0.68	0.74	0.60	0.66	0.70	0.69	0.65	0.70
70	0.26	0.23	0.28	0.36	0.40	0.36	0.34	0.29	0.38	*** *	0.79	0.74	0.73	0.62	0.76	0.78	0.82
24	0.24	0.29	0.18	0.26	0.27	0.28	0.39	0.14	0.30	0.24	*** *	0.65	0.76	0.71	0.82	0.72	0.74
60	0.23	0.29	0.35	0.28	0.37	0.44	0.57	0.55	0.52	0.31	0.43	*** *	0.68	0.69	0.72	0.70	0.63
38	0.43	0.36	0.35	0.33	0.43	0.21	0.39	0.29	0.41	0.32	0.27	0.38	*** *	0.66	0.76	0.78	0.78
53	0.24	0.24	0.28	0.20	0.15	0.46	0.62	0.49	0.36	0.48	0.34	0.37	0.42	*** *	0.76	0.65	0.61
65	0.28	0.20	0.17	0.15	0.23	0.28	0.29	0.28	0.36	0.27	0.20	0.33	0.27	0.28	*** *	0.74	0.81
61	0.47	0.36	0.34	0.38	0.50	0.20	0.38	0.25	0.43	0.25	0.33	0.35	0.25	0.43	0.30	*** *	0.91
56	0.43	0.27	0.26	0.35	0.43	0.21	0.26	0.17	0.35	0.20	0.30	0.46	0.25	0.50	0.21	0.10	*** *

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

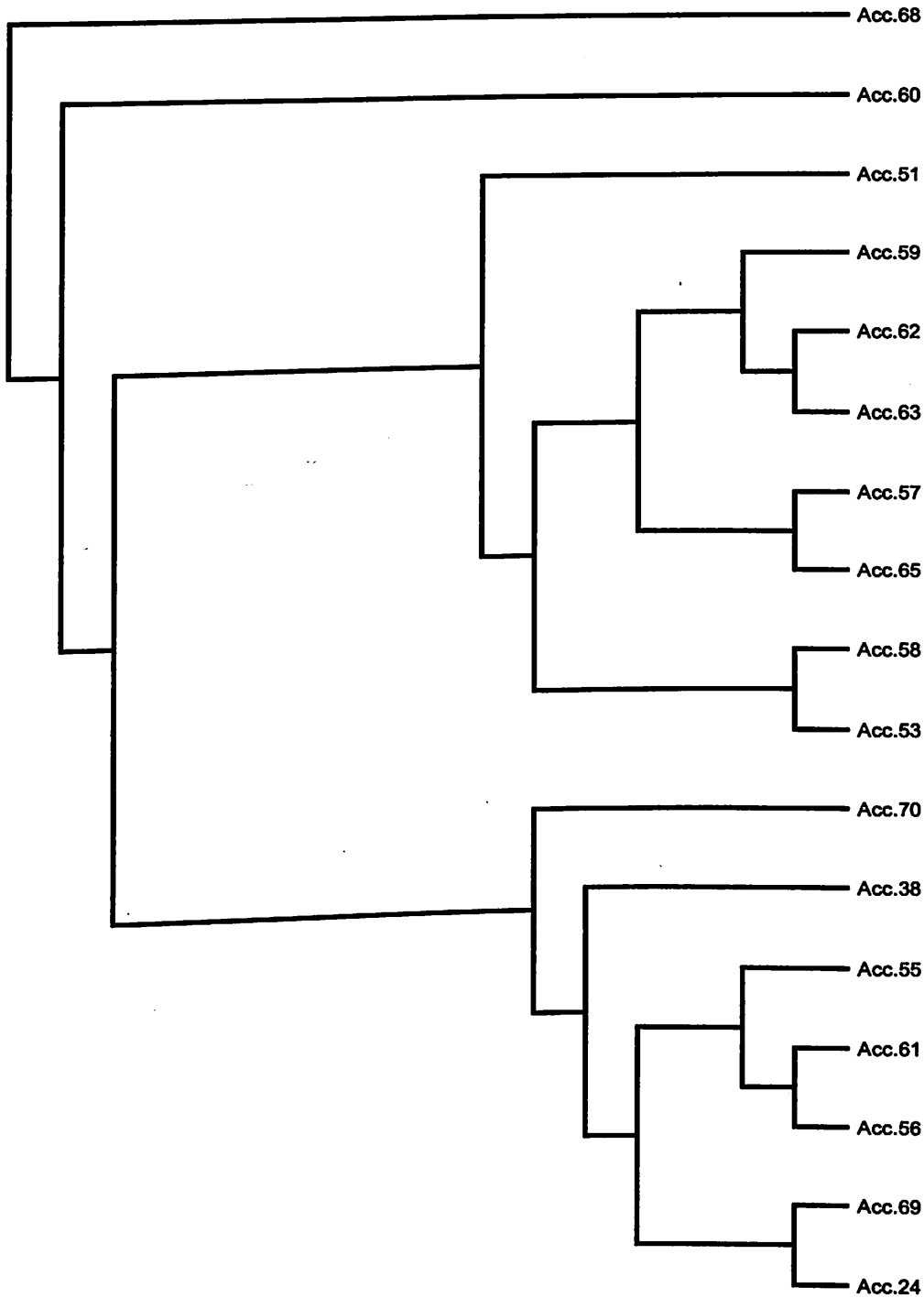

Fig. 1: Dendrogram based on genetic distance assessed using 14 SSR primers in 17 cardamom accessions

4.3 CORRELATION ANALYSIS

Analysis of correlation of microsatellite diversity with lignin concentration was carried out in the present study. For this, selected accessions were grouped into two having five accessions each: one with low lignin content and another group with high lignin content. When analyzed as individual groups, high correlation was observed for most of the genetic variability parameters with corresponding lignin content (Table 11). However, analysis of 10 accessions together yielded only marginal differences in coefficient of correlation estimates.

Table 11: Genetic variability and lignin content estimations for accessions grouped as low and high lignin content and their correlation coefficients

Acc. No.	na	ne	l	Obs_ Hom	Obs_ Het	Exp_ Hom	Exp_ Het	Nei	Ave_ Het	P (%)	Lignin
Acc 24*	1.50	1.50	0.35	0.50	0.50	0.50	0.50	0.25	0.20	36.59	0.39
Acc 59*	1.47	1.47	0.33	0.53	0.47	0.53	0.47	0.24	0.18	41.46	0.42
Acc 38*	1.34	1.34	0.24	0.66	0.34	0.66	0.34	0.17	0.21	24.39	0.42
Acc 65*	1.45	1.45	0.31	0.55	0.45	0.55	0.45	0.23	0.19	34.15	0.43
Acc 60*	1.32	1.32	0.22	0.68	0.32	0.68	0.32	0.16	0.20	21.95	0.69
Acc 55**	1.52	1.52	0.36	0.48	0.52	0.48	0.52	0.26	0.22	34.15	1.32
Acc 63**	1.46	1.46	0.32	0.54	0.46	0.54	0.46	0.23	0.18	39.02	1.34
Acc 56**	1.46	1.46	0.32	0.54	0.46	0.54	0.46	0.23	0.21	29.27	1.54
Acc 58**	1.43	1.43	0.30	0.57	0.43	0.57	0.43	0.21	0.17	36.59	1.72
Acc 57**	1.46	1.46	0.32	0.54	0.46	0.54	0.46	0.23	0.17	41.46	1.75
Both	0.24	0.24	0.24	-0.24	0.24	-0.24	0.24	0.24	-0.27	0.28	
Low	-0.71	-0.71	-0.71	0.71	-0.71	0.71	-0.71	-0.71	-0.04	-0.68	
High	-0.67	-0.67	-0.68	0.67	-0.67	0.67	-0.67	-0.68	-0.63	0.27	

Accessions with low and high lignin content are marked with * and ** respectively.

DISCUSSION

5. DISCUSSION

The biological diversity of our planet is rapidly diminishing as a direct and indirect consequence of human behaviour. A large number of species are already extinct, and the populations of many others have been reduced to levels where they risk extinction. The problem is severe for wild relatives of crop plants where due to selection, those less yielding forms are totally ignored and eventually eradicated. Even though wild or weedy forms are inferior in most of the agronomic traits, they have been demonstrated to contribute positively to yield enhancement in many crop species (Frey *et al*., 1983; Xiao *et al*., 1998; Moncada *et al*., 2001; Thomson *et al*., 2003; McCarty *et al*., 2004a; Sabu *et al*., 2006).

The mechanisms of control of lignin composition and quantity have wide implications regarding the adaptation and evolution of land plants and provide a basis for improved genetic manipulation of lignin for agroindustrial end uses besides its potential application for pest/microbial resistance characteristics. The transcription of genes that encode lignin biosynthetic enzymes may be important in the spatial and temporal control of lignifications and in regulating the quantity and composition of lignin. The expression of genes encoding lignin biosynthetic enzymes in response to different developmental and environmental cues may further influence the timing and localization of lignifications. Analysis of lignin in accessions of the same species growing under uniform environmental conditions offers an excellent system for studying the genetic variation of lignin biosynthesis and accumulation in these plants. In the present study, wide variation lignin content (absorbance at A440) was noted with minimum value of 0.394 and maximum of 1.816 (average was 1.098 ± 0.514). This is a clear indication on diversity in lignin content present in the cardamom accessions used in the study.

The advent of DNA marker technology has revolutionized the field of genetics by changing the pace and precision of genetic analysis (Cullins, 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 (Gupta *et al*., 1996; Thiel *et al*., 2003), characterized by the relative abundance, hyper variable, locus specific, codominant, and multi allelic nature (Thiel *et al*., 2003). SSRs are ubiquitous in the coding and non-coding regions of prokaryotes and eukaryotes. The standard method of microsatellite development involves the creation of a small insert genomic library, subsequent hybridization with tandemly repeated oligonucleotides and its capture followed by sequencing of candidate clones, thus making the entire process a time consuming and laborious one. With the rapid increase in the deposition of nucleotide sequences in the public databases and with the advent of bioinformatics tools, it has become a cost effective and fast approach to scan for microsatellite repeats and exploit the possibility of converting it into potential genetic markers. Another approach is to test transferability of SSRs developed for one species to another closely related species.

In the present study, 20 primers were developed through bioinformatics tools from the ESTs of *Curcuma longa*. Out of which, 6 primers having no amplification or only with monomorphic bands in some of the accessions were removed from further analysis. Remaining 14 SSR primers were used for the genotyping study. The polymorphic SSR markers generated in the present study revealed one to eight loci in the cardamom accessions analyzed. The polymorphism generated by the SSR primers which are developed from ESTs of curcuma through bioinformatics analysis in various cardamom accessions offers possibility for their future utilization in studying the genetic diversity of the cardamom or even for any related genera under Zingiberaceae. Development of primers from ESTs is a common approach for species where there is no sequence information available (Poncet *et al*., 2004; Lee *et al*., 2011; Demdoum *et al*., 2011).

Analysis revealed that the values obtained for all genetic variability parameters except polymorphic loci varied slightly as evidenced from the low standard deviation (SD) values (Table 9). Analysis of data from previous studies (Pineiro *et al*., 2009) found that higher values for SD in connection with percentage of polymorphic loci comparing to other variables such as observed heterozygocity, Shannon index, etc are not uncommon.

In cluster analysis (Fig 1), wild and cultivars of cardamom did not grouped separately except for Pink tiller, a wild variety from ICRI, Idukki which showed greater variation from rest of the cardamom germplasm since it remained as a stand-alone cluster. In general, distribution of various wild accessions along with cultivars may be due to limited number of loci analyzed and could be inferred that more number of loci needs to included in a study aimed to find out the varietal differences.

The high rate (60%) of cross species transferability of the SSR markers generated from *Curcuma longa* might be due to the sequence conservation in the ESTs of Curcuma and cardamom. Thus, the SSR markers generated in the present study offer potentiality for its future use in characterization and genetic diversity analysis of related cardamom species. Moreover, the transferability efficiency indicates that the approach of utilizing existing SSRs or EST sequences distributed in the public databases would help to generate reliable markers for comparative mapping in crops whose genetic/molecular information is little known.

Correlation analysis conducted in accessions with low and high lignin content showed positive correlation (0.70) for observed and expected heterozygocities (Table 11) where as other parameters exhibited mostly negative correlation. Even though low coefficient of correlation (0.24) was observed when all the ten accessions analyzed together, the former results obtained clearly indicates possibility for using those accessions with low and high lignin content having strong correlation with genetic parameters could be used as potential

candidates for quantitative trait loci (QTL) mapping of lignin content in cardamom. This could eventually be useful to develop lines with lignin content which would be having higher pest resistance.

SUMMARY

6. SUMMARY

The research work entitled “Development and use of SSR markers for analysis of genetic diversity and correlation with lignin content in cardamom (*Elettaria cardamomum* Maton) germplasm” was carried out at Biotechnology and Bioinformatics division of Jawaharlal Nehru Tropical Botanic Garden and Research Institute (JNTBGRI), Palode during 2013-14. The objective of the study was to estimate the natural variation inherent in the cardamom genome using microsatellite (simple sequence repeats, SSR) markers and test correlation with lignin content which is an important quantitative trait often attributed to pest tolerance characteristics.

Cardamom is an important spice crop, which ranks third position in the global market. It’s a native of tropical ever green forests in Western Ghats where presumably rich and untapped genetic diversity of the species still resides. Small cardamom is affected by many pests and diseases, and that have been identified as a major production constrain. More knowledge and better understanding on the genetic diversity and variation existing in the germplasm could result in the development of sustainable and high quality cardamom varieties.

Lignin forms an integral part of the secondary cell wall of plants. The function of lignin in the cell wall is to act as a matrix material that binds the plant polysaccharides thereby imparting strength and rigidity to the cell wall. Lignification imparts resistance to most of the insects and plant pathogens due to its insolubility in water and complex nature that prevents enzymatic degradation. Thus lignin plays a vital role in plants defence mechanism.

Eighteen accessions from different geographical locations that are conserved in the cardamom germplasm conservatory of JNTBGRI, were used for the present study and this included wild collections, landraces, and released varieties.

The work started from thousands of expressed sequence tags (ESTs) available for ginger and curcuma to develop SSR markers for cardamom. 20 SSR

primers were developed using accessible bioinformatics procedures and genetic variability parameters were analysed. The use of DNeasy plant minikit for extraction yield good quality DNA for molecular analysis.

Lignin quantified via spectrophotometric analysis at 440 nm was subjected to correlation study with the genetic parameters. The lignin content has shown considerable level of variation among different accessions and the study clearly demonstrated the existence of genetic diversity in various cardamom samples.

The observed number of alleles per locus (na) ranged from 1.32 to 1.52 effective number of alleles (ne) also ranged from 1.32 to 1.52, Shannon's information index (I) ranged from 0.22 to 0.36, observed homozygosity (Obs_Hom) ranged from 0.48 to 0.68, observed heterozygosity (Obs_Het) ranged from 0.32 to 0.52, expected homozygosity (Exp_Hom) ranged from 0.48 to 0.68, expected heterozygosity (Exp_Het) ranged from 0.32 to 0.52, Nei’s heterozygosity (h) ranged from 0.16 to 0.26, average heterozygosity (Ave_Het) ranged from 0.17 to 0.23 and percentage of polymorphic loci (P) ranged from 19.51 to 41.46.

Diversity analysis with dendrogram, revealed Pink tiller a wild variety from ICRI, Idukki showed greater variation from rest of the cardamom germplasm since it remained as a stand-alone cluster. A released variety Panikulangara 1, from Cardamom research station, Myladumpara, Idukki also showed considerable variation. Rest of the samples were aligned as two clusters.

On the basis of lignin estimation, the accessions were grouped into two, low and high lignin content groups. Statistical analysis revealed significant level of correlation between the lignin content and genetic diversity. Parameters such as observed homozygosity showed 70 and 67% correlation with low and high lignin groups. This correlation can be further elaborated to quantitative trait loci (QTL) studies for possible identification of alleles determining lignin content in cardamom and eventually help to breed elite cardamom lines with high lignin content.

Even though low coefficient of correlation was observed when all the ten accessions comprising of low and high lignin groups analyzed together, the results obtained when analyzed group-wise clearly indicate possibility for using those accessions as potential candidates for quantitative trait loci (QTL) mapping of lignin content in cardamom. This could eventually be useful to develop lines with lignin content which would be having higher pest resistance.

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APPENDICES

APPENDIX I

TBE Buffer (20 X) for 100 ml solution

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

APPENDIX II

Tracking dye

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

APPENDIX III

AE Buffer

Tris. HCl	10 mM
EDTA (pH 9.0)	0.5 mM

APPENDIX IV

Composition of Gel for AGE:

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

ABSTRACT

DEVELOPMENT AND USE OF SSR MARKERS FOR ANALYSIS OF GENETIC DIVERSITY AND CORRELATION WITH LIGNIN CONTENT IN CARDAMOM (*Elettaria cardamomum* Maton) GERMPLASM

By

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THESIS

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ABSTRACT

The research work entitled "Development and use of SSR markers for analysis of genetic diversity and correlation with lignin content in cardamom (*Elettaria cardamomum* Maton) germplasm" was carried out at Biotechnology and Bioinformatics division of Jawaharlal Nehru Tropical Botanic Garden and Research Institute (JNTBGRI), Palode during 2013-14.

India has rich genetic diversity of cardamom and this diversity might be the product of complex interactions between genetic and environmental factors. Understanding the footprints of divergence underlying the formation of various morphotypes/landraces is important for developing sustainable and high quality cardamom varieties.

The study aims to analyse the natural variation inherent in the cardamom genome using microsatellite (simple sequence repeats, SSR) markers and test correlation with lignin content which is an important quantitative trait often attributed to pest tolerance characteristics. Lignin is a phenolic polymer which is required for mechanical support, water transport and defence in vascular plants. The insolubility and complexity of lignin polymer makes it resistant to degradation by most microorganisms. Therefore, lignin has a very vital role in plant defence and it may be hypothesized that cardamom plants with high lignin content exhibit improved pest resistance.

Eighteen accessions including wild collections, landraces, and released varieties were selected for the present study. Twenty SSR primers were developed for cardamom and genetic diversity was analysed. Quantity of lignin was estimated and subjected to correlation analysis with genetic parameters.

The lignin content has shown considerable level of variation among different accessions and the study clearly demonstrated existence of genetic diversity in various cardamom samples. The accessions were grouped into two, based on low and high lignin content. Statistical analysis revealed significant level of correlation between the lignin content and genetic diversity. Parameters such as

observed homozygosity showed 70 and 67% correlation with low and high lignin groups. This correlation can be further elaborated to quantitative trait loci (QTL) studies for possible identification of alleles determining lignin content in cardamom and eventually help to breed elite cardamom lines with high lignin content.