MOLECULAR CHARACTERIZATION OF EAST INDIAN LEMONGRASS (*Cymbopogon flexuosus* Stapf.) GERMPLASM ACCESSIONS

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THESIS

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CENTRE FOR PLANT BIOTECHNOLOGY AND MOLECULAR BIOLOGY
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2013

DECLARATION

I, hereby declare that this thesis entitled **"Molecular characterization of East Indian lemongrass (*Cymbopogon flexuosus* Stapf.) germplasm accessions"** is a bonafide record of research work done by me during the course of research and that it has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other university or society.

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CERTIFICATE

Certified that the thesis entitled **"Molecular characterization of East Indian lemongrass (*Cymbopogon flexuosus* Stapf.) germplasm accessions"** is a record of research work done independently by **Mr. Bansode Ravindra Dinkar** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, associateship, fellowship to him.

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We, the undersigned members of the advisory committee of **Mr. Bansode Ravindra Dinkar** a candidate for the degree of **Master of Science in Agriculture** with major field in **Plant Biotechnology,** agree that the thesis entitled **"Molecular characterization of East Indian lemongrass (*Cymbopogon flexuosus* Stapf.) germplasm accessions"** may be submitted by **Mr. Ravindra Dinkar Bansode** in partial fulfilment of the requirement for the degree.

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Bansode Ravindra Dinkar

Dedicated to my beloved parents

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ABBREVIATIONS

%	Percentage
>	Greater than
µg	Microgram
A	Ampere
AFLP	Amplified Fragment Length Polymorphism
AMPRRS	Aromatic and Medicinal Plant Research Station
AP-PCR	Arbitrarily Primed Polymerase Chain Reaction
bp	Base pair
cm	Centimetre
CPBMB	Centre for Plant Biotechnology and Molecular Biology
CTAB	Cetyl Trimethyl Ammonium Bromide
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
dNTPs	Deoxyribo Nucleoside Triphosphate
EDTA	Ethylene Diamine Tetra Acetic acid
g	Gram
ha	Hectare
ISSR	Inter Simple Sequence Repeat
Kb	Kilo basepairs
L	Litre
M	Molar
MAS	Marker Assisted Selection
mg	Milligram
ml	Millilitre
mM	Milli mole
ng	Nanogram
ºC	Degree Celsius
OD	Odakkali
OD	Optical Density

PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
P^H	Hydrogen ion concentration
PIC	Polymorphism Information Content
pM	Pico molar
PVP	Poly vinyl pyrolidine
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic acid
RNase	Ribonuclease
Rpm	Rotations per minute
SAHN	Sequential Agglomerative Hierarchical Non-overlapping
SCAR	Sequence Characterized Amplified Region
SSR	Simple Sequence Repeats
STS	Sequence Tagged Sites
TAE	Tris Acetate EDTA
TE	Tris EDTA
U	Unit
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
UV	Ultra violet
V	Volts
β	Beta
µl	Microlitre

Introduction

1. INTRODUCTION

Plant herbs are naturally gifted as they synthesize medicinal compounds. Hence it has economic value. The extraction and characterization of bioactive compounds from medicinal plants have resulted in the discovery of new drugs with high therapeutic value. Treatment using medicines of natural origin is gaining momentum nowadays on account of increasing concern about potentially harmful synthetic additives (Reische, 1998). East Indian lemongrass (*Cymbopogon flexuosus* Stapf.) has commercially valuable essential oils which are widely used in food technology, pharmaceutical and perfume industry.

Owing to the new attraction for natural products like essential oils, despite their wide use and being familiar to us as fragrances, it is important to develop a better understanding of their mode of biological action for new applications in human health, agriculture and environment. People nowadays are more aware on health issue due to the emergence of new diseases. Treatment using plant-based medicine appears to be an alternative approach due to the adverse effects associated with the use of synthetic drugs. The genetic diversity of plants has provided us not only survival, but a high degree of comfort and the most important thing of all is the potential treatment for various diseases (Payne *et al*., 1991).

East Indian lemongrass is an aromatic grass belonging to the family Poaceae grown in many parts of tropical and sub-tropical South East Asia and Africa. In India, it is cultivated along the Western Ghats (Maharashtra and Kerala), Karnataka and Tamil Nadu states besides the foot-hills of Arunachal Pradesh and Sikkim. It was introduced in India about a century back and is now commercially cultivated in these states. Most of the species of lemongrass are native to South Asia, South-East Asia and Australia. The so called East Indian lemongrass, also known as Malabar or Cochin grass is native to India, Sri Lanka, Burma and Thailand. Lemongrass oil is rich in active ingredients such as citral

(75 to 85%) and possesses a lemon like odour. Lemongrass oil is popularly known as “Cochin oil” in the world trade (Joy *et al.,* 2001).

During the early 1950s, India produced over 1800 t/annum of lemongrass oil and held monopoly both in production and world trade. At present, India grows this crop in 3,000 ha area, largely in states of Kerala, Karnataka, Uttar Pradesh and Assam. East Indian lemongrass oil is preferred due to its high citral content. The current world production of the oil is estimated at 1300 t/annum, with India contributing to the tune of 350 tones (Akhila, 2010).

Cymbopogons are highly heterozygous plants due to its cross pollination. Thus profound genetic variations are prevalent in the species (Sreenath and Jagadishchandra, 1991) that always demands better germplasm management and conservation practices. Knowledge of the genetic relationships among different accessions is essential for developing appropriate strategies for breeding, germplasm management and utilization of genetic resources (Paterson *et al*., 1991).

Many previous workers reported the genetic diversity among different taxa of *Cymbopogon* species based on oil constituents as well as molecular analysis (Sangwan *et al*., 2001b). The Aromatic and Medicinal plants Research Station (AMPRS), Odakkali has maintained around 400 accessions of lemongrass, collected from different parts of Kerala and other states. The accessions have been morphologically and biochemically characterized and no molecular data has been developed so far. Therefore 25 accessions of East Indian lemongrass diverse in oil and citral content were selected for the molecular characterization from the AMPRS, Odakkali.

Molecular markers show differences and similarities between individual and discern genomic diversity and are employed for identification and phylogenetic analysis. RAPD have been widely employed for the identification of cultivars (Sangwan *et al.,* 2001), ancestors (Shasany *et al.,* 2000), and discerning the genomic diversity (Sangwan *et al.,* 2001a; Khanuja *et al.,* 2005).

Another molecular system, Inter-simple sequence repeat (ISSR) markers, developed by Zietkiewicz *et al.* (1994) based on the amplification of a single primer containing a microsatellite 'core'sequence anchored at the 5′or 3′ end by a set of 2–4 purine or pyrimidine residues. This offers a high degree of reproducibility with the detection of rich level of polymorphism in a relatively simple procedure. Hence, it has been widely used in assessments of genetic diversity (Bornet and Branchard, 2001) and cultivar identification (Prevost and Wilkinson, 1999). Molecular analysis of different geographically scattered population of *Cymbopogon winterianus* through ISSR and RAPD fingerprinting provides a powerful tool for the generation of potential diagnostic markers for cultivar analysis (Bhattacharya *et al*., 2010a).

With this background, the present study on "Molecular characterization of East Indian lemongrass (*Cymbopogon flexuosus* Stapf.) germplasm accessions" was proposed with the following objective.

To characterize twenty five germplasm accessions of East Indian lemongrass (*Cymbopogon flexuosus* Stapf.) using molecular markers RAPD and ISSR.

Review of Literature

2. REVIEW OF LITERATURE

2.1 Lemongrass: an Aromatic and Medicinal Plant

East Indian lemongrass (*Cymbopogon flexuosus* Stapf.), also called Cochin grass or Malabar grass is native to Cambodia, Vietnam, India, Sri Lanka, Burma and Thailand. It is a perennial aromatic and medicinal grass producing an essential oil grass belongs to the family Poaceae. It is predominantly a highly cross pollinated crop. It is a diploid species with chromosome number 2n=20 (X=10) (Elangovan *et al.*, 2012).

Cymbopogons are highly heterozygous plants due to its cross pollination. that always demands better germplasm management and conservation practices. Knowledge of the genetic relationships among different accessions selected in present study is useful tool for developing appropriate strategies for breeding, germplasm management and utilization of genetic resources.

2.2 Brief description of East Indian lemongrass

2.2.1 Botanical description

East Indian lemongrass is a densely tufted robust perennial aromatic grass of about 2 m height. The leaves are linear and lanceolate, often very coarse, 50 to 120 cm long and 0.25 to 2.0 cm wide; glumes are 0.4 to 0.5 cm long. The inflorescences are very large. The panicles are often greyish or grayish green (rarely with a touch of purple), decompound, 60 x 30 cm in size, spreading and slightly hairy. The raceme pairs are in dense masses. The spikes are borne on tertiary branches with slender, long, flexuous and comparatively inconspicuous spathes. Spikes bear spikelets in pairs of which one is sessile and the other pedicellate. The sessile spikelet is an awned bisexual floret whereas the pedicellate is an awnless staminate floret. The lower glumes of the sessile spikelets are 4 to 5 mm long and 1 mm wide with 1 to 3 definite or obscure intra

carinal nerves. They are shallowly concave with one or two depressions (Joy *et al*., 2001).

2.2.2 Propagation

(a) Slips

For better quality and yield of oil, it is recommended to grow lemongrass by slips obtained by dividing well-grown clumps. Tops of clumps should be cut off within 20 to 25 cm of the root. The latter should be divided into slips and the lower brown sheath should be removed to expose young roots.

(b) Seeds

Lemongrass plants are left in the field without harvesting, for collection of seeds, as the yield of seeds from plants subjected to regular harvest is lower. On average, a healthy plant gives about 100 to 200 g of seeds. Seeds germinate in 5 to 6 days if temperature and moisture levels are correct and the seedlings are ready for transplanting when they are about 60 days old. It is advantageous to raise seedlings in a nursery whenever there is not an assured source of irrigation available. The transplanting of nursery-raised seedlings is better compared to direct sowing of seeds in the land.

3. Essential oil

Essential oils are volatile liquids, mostly insoluble in water, but freely soluble in alcohol, ether and vegetable and mineral oils. They are usually not oily to the touch. They were formed by varied and complex volatile mixtures of chemical compounds, with predominance of terpene associated to aldehyde, alcohols and ketone which were deposited in various structure of the plant (Linares *et al.,* 2005). It could be defined as a concentrated, hydrophobic liquid containing volatile aroma compounds from plant. Essential oils are also known as volatile, ethereal oils or aetherolea, or simply as the “oil of” the plant from which they were extracted. Oil is “essential” in the sense that it carries a distinctive

scent, or essence, of the plant. The origin of essential oil can be traced to an ancient concept essential quintessence.

Out of a total of about 1500 species of aromatic plants known, only a little over 300 species have been studied in some detail. Of the 50 species which find use as commercial source of essential oils and aroma-chemicals, the number of those having regular and large scale utilization hardly exceeds two dozens (Joy *et al*., 2001).

The essential oil is distributed in all parts of the grass, such as flower heads, leaves, and stems, with the flower heads containing the major portion (Akhila, 2010). Oil of lavender, for example, is derived from a flower, oil of patchouli from a leaf and oil of orange from a fruit. The oils are formed in the green (chlorophyll-bearing) parts of the plant and with plant maturity are transported to other tissues, particularly to the flowering shoots.

The oils are mainly extracted through distillation and have a long tradition of providing a variety of therapeutic benefits. Most of these benefits have been modified and confirmed through modern scientific research. Interest in essential oil has increased over the years with the improvement in aromatherapy, a branch of alternative medicine which uses the essential oils of aromatic plants and trees to promote health of body and serenity of mind. The use and application is gaining popularity in most industries and amongst individuals.

Nowadays, people worldwide are looking towards natural base products since there are no side effects when taken accordingly. Furthermore, there is also an interest in the production of functional, high value, natural products without chemical modification and residues of solvents or additives. This trend in consumer preference increases the demand tremendously with variety products range from essential oils (Nurul, 2005).

3.1 Lemongrass essential oil

Lemongrass is an aromatic grass belonging to genus *Cymbopogon*, which consist of about 80 species. Lemongrass is well known for its oil and it is one of the world's best known essential oils. There are two main types of lemongrass namely East Indian and West Indian. Although many sources use the two botanical names interchangeably, Charles Wells states that these two distinct varieties, citratus and flexuosus, (East Indian lemongrass). Lemongrass (*Cymbopogon flexuosus* Stapf.) is an aromatic crop cultivated for its commercial essential oil. The major component of this oil is citral. The East Indian lemongrass oil is obtained from *Cymbopogon flexuosus* Stapf. The species is considered to have originated in Kerala, the southernmost state of India. According to the colour of the stem, this is again divided into two types. The 'red grass' which is true *Cymbopogon flexuosus* Stapf is known as 'choomanna poolu' in Tamil; and 'white grass' known as 'wella poolu' has been identified as *Cymbopogon flexuosus* var. *albescens.* The oil obtained from this plant has very low aldehyde content and is poor in solubility (Jayasinha, 1999).

Lemongrass has been used in medicine in India for more than 2000 years. However, its first recorded distillation is in Philippines in the 17th century and in 1799 it was introduced to Jamaica. In 1917 it was grown for the first time in USA by Hood and during the World War I, grown in Guatamala by Julia Samayoa. Although the oil has been known, since very early times in India, the systematic cultivation and distillation of the grass were started in Kerala only about 100 years ago. Cultivation has assumed the status of a plantation crop after World War II (Jayasinha, 1999).

Traditional Indian medicine uses lemongrass for fever and infectious illness. Also used as insecticide and food flavouring. “Monoterpene citral as the major constituent (75%) finds uses as flavouring agents, and in perfumery and pharmaceutical industry and as a natural precursor of semisynthetic vitamin A”

(Sushil *et al.*, 2000). Flexuosus is often preferred by the perfume industry as it contains less myrcene and therefore, has a longer shelf life.

2.3.3 Production of Lemongrass oil

Lemongrass essential oil is extracted by steam distillation of the fresh or dried leaves of lemongrass. The oil of lemongrass is yellow in colour with a citrus/grass/lemon fragrance. The essential oils extracted from the West Indian and East Indian species of lemongrass are similar in fragrance and colour, although the West Indian lemongrass species is lighter and fresher.

3. Extraction Processes of Essential Oils

There are a few conventional and modern methods of extracting essential oils. It can be extracted by hydro-distillation (Steam Distillation and Water Distillation), cold pressing, enfleurage, hydro-diffusion, supercritical fluid extraction, vapo-cracking, turbo-extractor and microwave extraction.

Calderon *et al.* (2003) reported that the steam distillation is considered as the standard, and all other extraction techniques are compared to steam distillation in numerous research publications.

3.1 Steam Distillation

Many of the essential oils presently used in perfumery are obtained by steam distillation of flowers, leaves, bark, etc. Steam is widely used because of its high latent heat of evaporation, relatively cheaper and widely available. There are two types of steam distillation: water and steam distillation. The role of the distiller is to achieve oil as close as possible to the oil as it exists in the plant.

3.2. Uses of Lemongrass oil

Lemongrass is native to India widely used as an herb in Asian cuisine. It has a citrus flavour and can be dried and powdered, or used fresh. Lemongrass has been reported to have innumerable therapeutic and other health benefits.

(a) Stress: Lemongrass oil revitalizes the body and relieves the symptoms of jetlag, clears headaches and helps to combat nervous exhaustion and stress related conditions.

(b) Nervous system: It is a great overall tonic for the body and it boosts the parasympathetic nervous system, which is a boon when recovering from illness, as it also stimulates glandular secretions.

(c) Respiratory infections: It is useful with respiratory infections such as sore throats, laryngitis and fever and helps prevent spreading of infectious diseases. It is helpful with colitis, indigestion and gastro-enteritis.

(d) Muscle Aches: Lemongrass oil helps tone the muscles and tissue relieves muscle pains by making the muscle suppler.

(e) Insect repellent: It helps with correcting poor circulation and as an insect repellent. It helps to keep pets clean of fleas, ticks and lice.

(f) Pest Control: Lemongrass and citronella belong to the same family of plants and can repel pests.

(g) Athlete's Foot: It also is used for clearing up oily skin and acne, as well as athlete's foot. It alleviates excessive perspiration.

(h) Aromatherapy and massage oils: Lemongrass essential oils can be put in bath water or in oil burners for aromatherapy.

(i) Lotions: When making homemade lotions, lemongrass oil can be added to give lemony fragrance.

(j) Soaps: The antiseptic and astringent properties of lemongrass oil make it a natural choice for soaps. Soap with lemongrass and eucalyptus oil can work wonders for inflamed or irritated skin.

(k) Candles: A homemade lemongrass-scented candle can produce a nice and strong fragrance.

(l) Bath: This is one of the easiest ways of using essential oils especially when there is no one around to give a hand. Add 5 to 10 drops of the essential oil to warm bath once it has been run.

4. Genetic markers in plants

Genetic markers are measurable inherited genetic variations, used to understand genetic components. There are different types of genetic markers with different properties, each having its own advantages and disadvantages to assess the genetic variations among natural populations. Currently, the most commonly used genetic markers are molecular markers. Due to the rapid developments in the field of molecular genetics, a variety of different techniques have emerged to analyze genetic variation during the last few decades (Whitkus *et al.*, 1994, Karp *et al*., 1996, Parker *et al*., 1998; Schlötterer, 2004). These genetic markers may differ with respect to important features, such as genomic abundance, level of polymorphism detected, locus specificity, reproducibility, technical requirements and financial investment. No marker is superior to all others for a wide range of applications. The most appropriate and important genetic marker will depend on its specific application, the presumed level of polymorphism, the presence of sufficient technical facilities or know-how, time constraints and financial limitations. Generally markers are divided in to three broad classes: those based on visually assessable traits (morphological and agronomic traits), those based on gene product (biochemical markers), and those relying on a DNA assay (molecular markers). Genetic markers are widely used by breeders and conservationists to study genetic diversity and to assist in crop improvement.

4.1. Morphological markers.

Observation of phenotypes has been the classical approach to differentiate plant cultivars, morphological trait being the main target of such observations. The description of the plant morphology is an invaluable source of information of the genetic variability and has been the first criteria used to classify plant varieties.

Kuriakose (1990) compared observations of 15 collections of lemongrass on morphological characters like height per plant, length of culm, colour of leaf and leaf sheath and flowering nature. The data showed that there was significant

difference between the types for all the characters studied. Out of 15 collections, there were 3 red and 4 white types and remaining light red.

Kumar *et al*. (1993) observed variation for leaf and leaf sheath hairiness, leaf angle, midrib colour, nodal colour, nodal hairs, anther colour, ear shape and seed shape in pearl millet hybrids. Further they tested the key characters which are useful in identification.

Ahmed and Das (1994) evaluated for 19 quantitative characters of 85 rice indigenous glutinous rice varieties on the basis of pigment distribution. The entire germplasm were grouped into 47 categories.

The morphological characterization of 12 genotypes of lemongrass done by Singh and Singh (1999) showed considerable variability among the genotypes for all the characters studied, except plant height. The maximum range of variability was observed for fresh weight of herb followed by oil yield and plant height and minimum for oil content.

Sharma *et al*. (2002) analyzed morphological characters of lemongrass during different growth phases. The study revealed that plant height, number of leaves per plant, number of tillers per plant, biomass production and oil yield per plant were found optimal at flowering stage. The morphological characters plant height, number of leaves per plant and number of tillers per plant were found to be correlated to essential oil yield.

Sarma *et al.* (2005) studied morphological characters of lemongrass accessions and found to be different from each other. The leaf area of accession RLJ-TC-11 showed minimum value. The length of ligulae, auricle, awn and number of tillers per bush exhibited difference from each other.

Kumar *et al.* (2006) characterized 27 varieties of jute (*Corchorus olitorius* L. and C.*capsularis* L.) including 20 released or notified and seven varieties of common knowledge for three years using 16 qualitative morphological characteristics to establish distinctness among the varieties. No intra-varietal variation was observed for any of the characteristics. Among them, 10 characters

in C. *capsularis* were found mono-morphic and six characters were dimorphic where as in C. *olitorius* two characters were mono-morphic, eight characters were dimorphic and six characters were polymorphic between varieties indicating their potential for varietal characterization.

Wild wheat (*Aegilops* L. and *Triticum* L.) populations were characterized by Karagoz *et al.* (2006) for plant height, number of days to heading, growth habit, number of stems per plant and spike length. The highest variation was recorded for number of stems, while the lowest was for plant habit. They concluded that there was significant agro-morphological variation between the populations for the traits studied.

Gopal Reddy *et al*. (2007) evaluated 842 accessions of proso millet germplasm conserved at the International Crops Research Institute for the Semi-Arid Tropics. Data were recorded on various morpho-agronomic traits such as time to 50% flowering, plant height, growth habit, culm branching, sheath pubescence, ligule pubescence, leaf pubescence, and inflorescence traits like exsertion and inflorescence length. The characterization data reveals that dwarf plant height accessions were from Mexico and tall plant height accessions were from Sri Lanka. Good exsertion accessions were from Australia and China, and shorter panicle accessions occurred in former USSR, while the longest was found in Nepal.

Twenty six varieties of forage sorghum which included 20 released and notified and six indigenous local varieties were characterized using 40 morphological descriptors and subsequently examined for their Distinctiveness, Uniformity and Stability. No intra-varietal variation was observed for any of the visual characteristics and expression of characters in different varieties remained same for the two consecutive years confirming the uniformity and stability of the varieties (Joshi *et al*., 2009).

Lopes *et al*. (2009) characterized Italian rye grass landraces based on morphological traits of International Plant Genetic Resources Institute and

International Union for the Protection of New Varieties of Plants descriptors list. Each landrace showed inter-population variability mainly on “verdeal” landrace.

4.2. Biochemical markers

Biochemical markers also known as Isozymes, defined as structurally different molecular forms of an enzyme with, qualitatively, the same catalytic function. Isozymes originate through amino acid alterations, which cause changes in net charge, or the spatial structure (conformation) of the enzyme molecules and also, therefore, their electrophoretic mobility Isozymes analysis has been used for over 60 years for various research purposes in biology, *viz.* to delineate phylogenetic relationships, to estimate genetic variability and taxonomy, to study population genetics and developmental biology, to characterization in plant genetic resources management and plant breeding (Bretting and Widrlechner 1995, Staub *et al*., 1996). After specific staining the isozyme profile of individual samples can be observed (Hadacova and Ondrej 1972., Vallejos 1983., Soltis and Soltis, 1989). Nitrase reductase (NR) activity can be used as biochemical marker for screening lemongrass genotypes for growth and herbage yield (Sharma *et al.*, 2010).

4.3.1 Markers at DNA level

Plant molecular biology offers a great potential for plant breeding as it promises to provide several tools to reduce the time taken to produce crop varieties with desirable characters. With the use of molecular techniques it would now be possible to hasten the transfer of desirable genes among varieties and to introgress novel genes from related wild species. Polygenic characters which were previously very difficult to analyse using classical genetic analysis, would now be easily tagged using molecular markers.

The DNA markers assumed importance since they can be used as genetic markers that are associated with economically important traits (Darvasi and Soller, 1994). DNA markers because of their heritable nature were found to act as

versatile tools in the fields like taxonomy, physiology, embryology, genetic engineering *etc*., Major applications of these DNA markers in genetics and breeding are in i) Diversity analysis and phylogenetic studies, ii) Mapping genes iii) Marker Assisted Selection (MAS).

Techniques which are particularly promising in assisting selection for desirable characters involve the use of two types of molecular markers such as hybridization based molecular markers such as Restriction Fragment Length Polymorphisms (RFLP) (Botstein *et al*., 1980) and Polymerase Chain Reaction (PCR) based molecular markers such as Random Amplified Polymorphic DNA (RAPD) (Williams *et al*., 1990), Sequence Characterized Amplified Regions (SCAR) (Williams *et al*., 1991), Simple Sequence Repeats (SSR) (Hearne *et al*., 1992), Sequence-Tagged Sites (STS) (Fukuoka *et al*., 1994), Inter-Simple Sequence Repeat (ISSR) (Zietkiewicz *et al*., 1994) and Amplified Fragment Length Polymorphism (AFLP) (Vos *et al*., 1995). The utility of molecular markers in crop breeding is reviewed by Mohan *et al*. (1997) and Gupta and Roy (2002).

Among the various molecular marker techniques, RFLP was the first DNA marker used for the construction of genetic maps of agronomically important species and for mapping of heritable traits (Tanksley *et al.,* 1989). However, their utility has been hampered due to the involvement of radioactive isotopes, labour intensive and time consuming steps. Among the marker systems available SSRs have been proved to be more useful in marker assisted selection (Jena and Mackill, 2008).

Molecular markers have several advantages over the phenotype based markers that were previously available to plant breeders. They offer greater scope for improving the efficiency of plant breeding by carrying out selection indirectly on the trait of interest by using a set of tightly linked markers to the trait of interest. The markers at DNA level are used to construct the linkage maps, locate

the genetic loci on specific chromosomes, characterize the germplasm for its genetic diversity and finally to exercise marker assisted selection (MAS).

4.3.2. Polymerase Chain Reaction (PCR)

The Polymerase Chain Reaction (PCR) technique has been the basis of a growing range of new techniques for genome analysis based on the selective amplification of genomic DNA fragments (Saiki *et al*., 1988). Williams *et al.* (1990) reported the use of PCR with short oligonucleotide primers of arbitrary (random) sequence to generate markers, the basis of the Random Amplified Polymorphic DNA (RAPD). Welsh and McClelland (1990) also reported on Arbitrarily Primed Polymerase Chain Reaction (AP-PCR). DNA Amplification Fingerprinting (DAF) was also reported as another technique of PCR used in various experiments (Caetano-Anolles *et al*., 1991). The PCR reaction requires deoxynucleotides, DNA polymerase, primer, template and buffer containing magnesium (Taylor, 1991). Typical PCR amplification utilises oligonucleotide primers which hybridize to complementary strands. The product of DNA synthesis of one primer serves as template for another primer. The PCR process requires repeated cycles of DNA denaturation, annealing and extension with DNA polymerase enzyme, leading to amplification of the target sequence. This results in an exponential increase in the number of copies of the region amplified by the primer (Saiki *et al*., 1988). The technique can be applied to detect polymorphism in various plants, animals, bacterial species and fungi.

The introduction of the PCR technique has revolutionized standard molecular techniques and has allowed for the proliferation of new tools for detecting DNA polymorphism (Hu and Quiros, 1991). The electrophoresis pattern of fragments generated by each primer for one isolate can be used as DNA fingerprints for assaying diversity (Tommerup *et al*., 1998). Polymorphism between two individuals is generally scored as presence or absence (non-amplification) of a particular DNA fragment. The absence may result from deletion of a priming site or insertion rendering site too distant for successful

amplification. Insertion can change the size of a DNA fragment without preventing its amplification (Williams *et al*., 1990). PCR is simple, fast, specific, sensitive and the main advantage of this technique over others is its inherent simplistic analysis and the ability to amplify extremely, small quantities of DNA (Welsch *et al*., 1991).

4.4. Types of DNA markers

4.4.1 Restriction Fragment Length Polymorphism (RFLP) markers

Variations in DNA sequences have been extensively exploited as genetic markers for genome mapping in the last 10 years. One of the most important achievements is the advent of RFLP (Botstein *et al.,* 1980). Restriction Fragment Length Polymorphism (RFLP) analysis is a powerful tool for developing precise high-density molecular genetic maps because it reveals reliable and stable polymorphism. Markers based upon DNA probes have introduced a new dimension to the development of genetic maps and the mapping of agronomically and physiologically important characters. The major strength of DNA probes is that they have the potential to reveal an almost unlimited number of polymorphism (Wyman and White, 1980).

4.4.2 Randomly Amplified Polymorphic DNA (RAPD) markers

RAPD procedures were first developed (Williams *et al*., 1990) by using PCR to randomly amplify anonymous segments of nuclear DNA with an identical pair of primers 8 to 10 bp in length generated simultaneously. RAPD are commonly inherited as dominant markers, where the presence of a particular band is dominant and its absence is recessive (Tingley and Tufo, 1993). RAPD analysis has found applications in population studies (Welsh *et al*., 1991), biosystematics (Stiles *et al*., 1993), and gene tagging (Ranade *et al*., 2001) and fingerprinting (Virk *et al*., 1995; Mackill, 1996). RAPD has also been successfully applied in molecular ecology (Hadrys *et al*., 1992), genetic analysis (Williams *et al*., 1993), assessing variation in plants (Newbury and Ford-Lloyd,

1993) combining ability in rice (Radhidevi *et al*., 2002) and to study the extent of diversity within plant germplasm (Virk *et al*., 1995).

The principle is that, a single, short oligonucleotide primer, which binds to many different loci, is used to amplify random sequences from a complex DNA template. This means that the amplified fragment generated by PCR depends on the length and size of both the primer and the target genome. The assumption is made that a given DNA sequence (complementary to that of the primer) will occur in the genome, on opposite DNA strands, in opposite orientation within a distance that is readily amplifiable by PCR. These amplified products (of up to 3.0 kb) are usually separated on agarose gels (1.5 to 2.0%) and visualized by ethidium bromide staining.

The use of a single decamer oligonucleotide promotes the generation of several discrete DNA products and these are considered to originate from different genetic loci. Polymorphisms result from mutations or rearrangements either at or between the primer binding sites and are detected as the presence or absence of a particular RAPD band. This means that RAPDs are dominant markers and therefore, cannot be used to identify heterozygotes.

The standard RAPD utilized short synthetic oligonucleotides (10 bases long) of random sequences as primers to amplify nanogram amounts of total genomic DNA under low annealing temperatures by PCR. Primers are commercially available from various sources (e.g. Operon Technologies Inc., California; Biosciences, Bangalore; Eurofinns, Bangalore; GCC Biotech, Kolkata). Welsh and McClelland (1990) independently developed a similar methodology using primers about 15 nucleotides bp long and different amplification and electrophoresis conditions from RAPD and called it the arbitrarily primed polymerase chain reaction (AP-PCR) technique.

PCR amplification with primers shorter than 10 nucleotides has also been used to produce more complex DNA fingerprinting profiles. Although these approaches are different with respect to the length of the random primers,

amplification conditions and visualization methods, they all differ from the standard PCR condition2 in that only a single oligonucleotide of random sequence is employed and no prior knowledge of the genome subjected to analysis is required.

At an appropriate annealing temperature during the thermal cycle, oligonucleotide primers of random sequence bind several priming sites on the complementary sequences in the template genomic DNA and produce discrete DNA products if these priming sites are within an amplifiable distance of each other. The profile of amplified DNA primarily depends on nucleotide sequence homology between the template DNA and oligonucleotide primer at the end of each amplified product. Nucleotide variation between different sets of template DNAs will result in the presence or absence of bands because of changes in the priming sites. Recently, Sequence Characterized Amplified Regions (SCARs) analysis of RAPD polymorphisms showed that one cause of RAPD polymorphisms is chromosomal rearrangements such as insertions or deletions. Therefore, amplification products from the same alleles in a heterozygote differ in length and will be detected as presence and absence of bands in the RAPD profile, which is similar to that of low stringency minisatellite DNA fingerprinting patterns and is therefore also, termed RAPD fingerprinting. On average, each primer directs amplification of several discrete loci in the genome so that allelism is not distinguishable in RAPD patterns.

4.4.3. Advantages of RAPD markers

In the two decade, the RAPD technique based on PCR has been one of the most commonly used molecular techniques to develop DNA markers. RAPD markers are amplification products of anonymous DNA sequences using single, short and arbitrary oligonucleotide primers, and thus do not require prior knowledge of a DNA sequence. Low expense, efficiency in developing a large number of DNA markers in a short time and requirement for less sophisticated

equipment has made the RAPD technique valuable although reproducibility of the RAPD profile is still the centre of debate.

The discovery that PCR with random primers can be used to amplify a set of randomly distributed loci in any genome facilitated the development of genetic markers for a variety of purposes. The simplicity and applicability of the RAPD technique have captivated many scientists' interests. Perhaps the main reason for the success of RAPD analysis is the gain of a large number of genetic markers that require small amounts of DNA without the requirement for cloning, sequencing or any other form of the molecular characterization of the genome of the species in question (Bardakci, 2001).

4.4.4. RAPD for genetic diversity analysis

Shasany *et al.* (2000) reported on phenotypic and RAPD diversity among *Cymbopogon winterianus* Jowitt accessions in relation to *Cymbopogon nardus* Rendle. These accessions were analysed for similarity and genetic distances at the molecular level via RAPD profiling using 20 random primers. More than 50 per cent divergence was observed for all the *C. winterianus* accessions in relation to *C. nardus* accession CN2. The clustering based on the similarity matrices showed a major cluster of six accessions, consisting of two sub-clusters. CN2 got carved out along with two *C. winterianus* accessions, CW2 and CW6. On the other hand, the accessions CW2 and CW6 demonstrated distinct identities compared to CN2 at the DNA level.

Sangwan *et al.* (2001a) used RAPD marker for molecular characterization of *cymbopogon*. Eleven elite and popular Indian cultivars of *Cymbopogon* aromatic grasses of essential oil trade types *viz.,* citronella, palmarosa and lemongrass were characterized by means of RAPDs to discern the extent of diversity at the DNA level between and within the oil biotypes. Primary allelic variability and the genetic bases of the cultivated germplasm were computed through parameters of gene diversity, expected heterozygosity, allele number per locus. Lemongrasses displayed higher (1.89%) allelic variability per locus than

palmarosa (1.63%) and citronella (1.40%). Also, RAPDs of diagnostic and curatorial importance were discerned as ‘stand along’ molecular descriptors. The molecular distinctions were discussed in relation to oil-chemotypic variations.

Khanuja *et al.* (2002) performed RAPD analysis of interspecific relationships in *Cymbopogon* (Lemongrass) species. RAPD technique was used to analyse the intra- and interspecific relatedness among *Cymbopogon* spp. from different regions throughout India. Out of 25 primers screened, 11 responded positively, producing discrete bands in different species. The scorable bands ranged from 4 to 9, with an average of 6.1 bands per primer. Sixty eight bands were scored. Five of 11 primers amplified more than 5 polymorphic bands that could differentiate between the accessions. Of the 68 bands, 60 were polymorphic, 4 were monomorphic along with 4 unique bands representing 88.2 per cent polymorphism and 5.8 per cent monomorphism. All accessions were grouped into clusters.

Nayak *et al.* (2003) evaluated agronomically useful somaclonal variants in Jamrosa (a hybrid *Cymbopogon*) and detected genetic changes through RAPD. Among 100 somaclones of jamrosa screened, a wide range of variation was recorded for several traits, including plant height, tiller number, herb weight, oil content and total oil yield. Qualitative analysis of essential oil was carried out for 45 somaclones which performed better or equal to the donor parent. Eleven somaclones selected on the basis of total oil yield and quality was further evaluated in a replicated trial. Five superior somaclones showing a total oil yield twofold higher than the donor and possessing quality oil containing a high geraniol content >84 per cent were selected for further analysis in the field for six clonal propagations. Out of the five superior somaclones two improved somaclones which showed relative stability in oil yield and quality were subjected to RAPD analysis. Changes in RAPD banding pattern in the improved somaclone as compared to donor parent revealed occurrence of gross genetic changes.

Sangwan *et al.* (2003) reported genetic diversity among elite varieties of the aromatic grasses, *Cymbopogon martinii* enzyme and SDS-PAGE protein polymorphisms. The allelic score at each locus of the enzymes as well as presence and absence profiling in RAPDs, overall occurrence of band types etc. were subjected to computation of gene diversity, expected heterozygosity, allele number per locus, and similarity matrix. These, in turn, provide inputs to derive primary account of allelic variability, genetic bases of the cultivated germplasm, putative need for gene/trait introgression from the wild or geographically diverse habitat etc. in elite selections.

Khanuja *et al*., (2005) used RAPD to establish species relationship in *Cymbopogon* Spreng. Nineteen *Cymbopogon* taxa belonging to 11 species, two varieties, one hybrid taxon and four unidentified species were analysed for their essential oil constituents and RAPD profiles to determine the extent of genetic similarity and thereby the phylogenetic relationships among them. Remarkable variation was observed in the essential oil yield ranging from 0.3 per cent in *C. travancorensis* to 1.2 per cent in *C. martinii*. Citral, a major essential oil constituent, was employed as the base marker for chemotypic clustering. Based on genetic analysis, elevation of *C. flexuosus* to species status and separate species status for *C. travancorensis*, which has been merged under *C. flexuosus* were suggested towards resolving some of the taxonomic complexes in *Cymbopogon*. The separate species status for the earlier proposed varieties of C. martinii (motia and Sofia) is further substantiated by these analyses genotypes.

Kumar *et al.* (2007) assessed genetic diversity in *Cymbopogon* species using PCR-based functional markers. Genetic diversity among 25 accessions (involving 8 species, 2 interspecific hybrids and one hybrid mutant) of medicinally important genus *Cymbopogon* was assessed using 17 PCR-based functional markers that were designed from members of three different multigene families. We developed 16 primer pairs from two multigene families, 8 primer pairs each from cytochrome P450 and UDP-glucosyltransferase (UGT); one

primer pair was derived from 5S rRNA gene family. A total of 119 fragments were visualized, of which 108 (91%) were polymorphic.

Ganjewala. (2008) reported RAPD Characterization of 3 Selected cultivars OD-19, GRL-1 and Krishna of East Indian Lemongrass (*Cymbopogon flexuosus* Nees ex Steud) Wats. The genomic DNA isolated from fresh leaf tissues were amplified with 19 OPJ RAPD primers, which produces an overall 60 scorable bands in the cultivars studied, 29 of which were polymorphic and 31 were monomorphic. Dendrogram constructed by cluster analysis of RAPD markers showed that cultivar GRL-1 was very closely related with cultivar OD-19 while Krishna slightly distant from cv. OD-19 than GRL-1. However, these cultivars differ greatly by their essential oil content and compositions. The cultivar GRL-1 could be distinguished by the presence of high geraniol (92%) in essential oil from other two cultivars OD-19 and Krishna which are rich in citral 88 and 82 per cent respectively.

Bhattacharya *et al.* (2008) observed genetic polymorphism analysis of somatic embryo-derived plantlets of *Cymbopogon flexuosus* through RAPD assay. A total of 64 band positions were scored, out of which 19 RAPD bands were polymorphic. From genetic similarity coefficient based on RAPD band data sharing, it was found that the majority of the clones were almost identical or more than 92 per cent similar to the mother plant, except CL2 and CL9 (66%) which showed highest degree of genetic change with CL2 and CL9 showing presence of two non-parental bands each.

Kumar *et al.* (2009) analysed genetic diversity an in *Cymbopogon* species using DNA markers. Genetic diversity of 25 accessions of *Cymbopogon* aromatic grasses including eight species, two hybrids and one mutant strain were analyzed using DNA markers repeat (SSR) of rice genome. A total of 151 bands were produced ranging from 3 to 12 per primer pair. The polymorphic information content values varied from 0.143 to 0.916 with an average 0.715. Jaccard's similarity coefficient ranged from 64 to 87 per cent among the paired accessions.

generated by employing 20 primer pairs derived from cDNAs containing simple sequence

Bhattacharya *et al.* (2010a) carried out the work on molecular diversity in 11 elite germplasms of *Cymbopogon winterianus* across West Bengal, India using 10 RAPD and 9 ISSR markers. The RAPD primers developed 81 robust loci, which revealed 74.07 per cent polymorphism and 61 ISSR markers generated 47.50 per cent polymorphism. Genetic diversity parameters such as average and effective number of alleles, per cent polymorphism, average heterozygosity, intralocus gene diversity, polymorphic information content (PIC) for RAPD, ISSR, and RAPD+ISSR along with UPGMA clustering based on Jaccard's coefficient were estimated with a view to assess efficiency of the marker system in *Cymbopogon*.

Mumtaz *et al*. (2011) assessed genetic diversity in a set of twelve sugarcane genotypes using RAPD. A total of 32 primers were employed, 16 of them revealed amplification at 149 loci, out of which 136 were polymorphic. The genotype SPSG-26 showed the highest number of polymorphic loci, followed by CSSG-668 and HSF-242. Pairwise genetic similarity ranged from 67.2 per cent to 83.3 per cent. The UPGMA cluster analysis resolved most of the accessions in 2 groups. Ten primers revealed genotype specific bands among which 4 primers (K07, H02, K10 and F01) produced multiple genotype specific bands that aid genotype identification especially those with red rot resistance.

Pop *et al.* (2011) investigated the level of genetic diversity among maize inbred lines. Eighty-three maize inbred lines obtained from SCDA Turda were genotyped using 20 decamer primers. These primers generated, among the studied genotypes, a number of polymorphic bands comprised between 17 bands (OPA 03) and 7 bands (OPAB 11). The highest numbers of polymorphic bands were obtained with primer OPA 03, respectively 17 bands, followed by OPA 01, OPB 08 (16 polymorphic bands) and OPX 03 and OPAL 20 (13 polymorphic bands). UPGMA based dendrogram was constructed with Free Tree software.

Santos *et al.* (2010) verified the genetic diversity among 32 accessions of *Sorghum bicolor* (L. Moench) from Instituto Agronômico de Pernambuco and evaluate their use in future plant selection programs. With 20 primers used, a total of 737 bands were amplified with 34 being polymorphic and 10 monomorphic generating 77 per cent of polymorphic genotypes. A dendrogram based on UPGMA analysis grouped the 32 genotypes in nine distinct groups with a similarity coefficient ranging from 0.72 to 0.98 indicative of high level of genetic variation among the genotypes studied and the accession CSF-27 appeared as an external group. RAPD markers were efficient on grouping of the studied accessions.

Dey *et al.* (2012) analysed genetic variability in regenerated and germinated plantlets of ten *indica* rice genotypes (*Oryza sativa* L.) using RAPD markers. Three primers out of 10 produced a total of 35 bands of which 29 bands considered as polymorphic and the polymorphism per cent was 82.86. The primer G7 produced the highest per cent of polymorphic bands (84.61%), whereas OPF 15 produced the lowest (80%). The highest level of gene diversity value (0.5000) and Shannon's index (0.6931) were found in loci OPA 20-3, OPA 20-11 and G7-9, while, the lowest levels were found in OPA 20-8 and OPF15-10 (0.0950 and 0.1985) respectively. Dendrogram based on UPGMA indicated segregation of the 20 rice genotypes into two main clusters.

Adhikari *et al.* (2013) assessed genetic diversity in ten elite Indian cultivars of *Cymbopogon* species through RAPD analysis. Twelve decamer arbitrary primers were used. The amplification produced overall 64 scorable bands in the cultivars studied. Of which 52 were polymorphic and 12 were monomorphic. RAPD markers proved to be the efficient marker system with regard to detection of polymorphism, number of loci scored and PIC values. Polymorphism differed substantially within the discrete groups of cultivars and was approximately 71.88 per cent in palmarosa, 6.25 per cent in citronella and 46.88 per cent in lemongrasses.

Agarwal *et al*. (2013) studied genetic diversity of seventeen wheat cultivars with RAPD markers to provide important support for genetic improvement and parent selection in the breeding programme. Thirteen RAPD primers in the study yielded 86 polymorphic bands that discriminated 17 wheat genotypes in 2 clusters showing nearly 60 per cent polymorphism. Primer 4 SS gave highest polymorphism (80%) followed by 8 SS with 71.4 per cent polymorphism among 17 genotypes showing the great promise of RAPD in genetic diversity analysis.

Prasad and Shekhar. (2013) used RAPD marker to characterize genetic diversity in 3 species of *Cymbopogon* (*C. martinii, C. citratus* and *C. nardus*). Dendrogram constructed by cluster analysis of RAPD markers showed that *C. martinii* and *C. citratus* are closely related. Since morphological differences among these species are indistinctive, RAPD characterization can be helpful in their Discrimination. Also knowledge of genetic diversity can be used to identify the specific antimicrobial agent in these *Cymbopogon* species.

4.5. Inter Simple Sequence Repeats (ISSR)

Inter Simple Sequence Repeat (ISSR) technique is a PCR based method, which involves amplification of DNA segment present at an amplifiable distance in between two identical microsatellite repeat regions oriented in opposite direction. The technique uses microsatellites, usually 16 to 25 bp long, as primers in a single primer PCR reaction targeting multiple genomic loci to amplify mainly the inter simple sequence repeats sequences of different sizes. The microsatellite repeats used as primers can be di-nucleotide, tri-nucleotide, tetra-nucleotide or penta-nucleotide. The primers used can be either unanchored (Gupta *et al*., 1994; Meyer *et al*., 1993; Wu *et al.*, 1994) or more usually anchored at 3'or 5' end with 1 to 4 degenerate bases extended into the flanking sequences (Zietkiewicz *et al*., 1994).

The technique combines most of the benefits of AFLP and microsatellite analysis with the universality of RAPD. ISSRs have high reproducibility possibly due to the use of longer primers (16 to 25bp) as compared to RAPD primers (10bp) which permits the subsequent use of high annealing temperature leading to higher stringency. The studies on reproducibility show that it is only the faintest bands that are not reproducible.

ISSRs segregate mostly as dominant markers following simple Mendelian inheritance (Gupta *et al.,* 1994; Tsumura *et al.,* 1996; Ratnaparkhe *et al.,* 1998 and Wang *et al.,* 1998). However, they have also been shown to segregate as co-dominant markers in some cases thus enabling distinction between homozygotes and heterozygotes (Wu *et al*., 1994; Akagi *et al*., 1996; Wang *et al*., 1998 and Sankar and Moore, 2001).

4.5.1. Advantage of ISSR marker

Recently, ISSR markers have emerged as an alternative system with reliability and advantages of microsatellites (SSR). The technique involves amplification of genomic segments flanked by inversely oriented and closely spaced microsatellite sequences by a single primer or a pair of primers based on SSRs anchored 5′ or 3′ with 1 to 4 purine or pyramidine residues. The sequences of repeats and anchor nucleates are arbitrarily selected. Coupled with the separation of amplification products on a polyacrylamide or agarose gels, ISSR amplification can reveal a much larger number of fragments per primer than RAPD. It is concluded that ISSR technique provides a quick, reliable and highly informative system for DNA fingerprinting. ISSR markers are inherited in Mendelian mode and segregated as dominant markers. This technique has been widely used in the studies of cultivar identification, genetic mapping, gene tagging, genetic diversity, evolution and molecular ecology (Wang, 2002).

4.5.2. ISSR marker for genetic diversity analysis

Ang and Song (2001) observed genetic variation and clonal diversity of 7 populations of the rhizomatous grass *P. villosa* from northwest China using inter ISSR markers. Out of the 84 primers screened, 12 primers that produced clear and reproducible fragments were analysed. These primers generated 173 DNA fragments (220-1400 bp), from which, 122 fragments (70.5%) were polymorphic, indicating considerable genetic variation at the species level. However, the percentage of polymorphic bands among the populations ranged from 6.1 to 26.87 per cent, indicating relatively low levels of polymorphism at the population level.

Yu *et al.* (2002) observed polymorphisms in sugarcane using ISSR marker. One hundred anchored ISSR primers from UBC 9 were used for PCR amplification from a sample set of 11 genotypes. Amplification products were resolved in 1.5 agarose gels and banding patterns were compared among the 11 genotypes. Of the 100 primers tested, 32 produced clear banding patterns that were easily scored. Nineteen of these 32 primers were designed to dinucleotide repeats, three to trinucleotide repeats, two to tetranucleotide repeats, and three to pentanucleotide repeats. Four primers designed to 5′ degenerate motifs also yielded discrete repeats. A similarity matrix and a dendrogram of 11 genotypes were generated, which were consistent with previous results.

Srivastava and Gupta (2008) evaluated potential of ISSR markers for molecular profiling in 42 sugarcane cultivars from subtropical India. ISSR amplification was achieved using di-, tri- and tetra-oligonucleotide repeats. All the nucleotide motifs were amenable to amplification. ISSR results suggested that the frequency and clustering of specific simple sequence repeats were variable and motif-specific. However, only a slight variation in di-, tri- or tetra-nucleotide ISSR profiles was observed. The ISSR amplification pattern was used to assess the genetic similarity among the sugarcane genotypes by Dice similarity

coefficients, which ranged from 0.68 to 0.97, resulting in a mean similarity value of 0.878.

Bhattacharya *et al*. (2010b) established an efficient plant propagation system through somatic embryogenesis in *Cymbopogon* pendulus, an aromatic grass followed by analysis of genetic status of regenerates using ISSR markers. Thirteen randomly selected regenerated clones were screened using six ISSR primers. Nine clones produced similar monomorphic amplification profiles while remaining clones showed minor variation with absence of certain parental bands and appearance of unique band. Majority of the regenerants maintained genetic fidelity with the generation of few variants as evidenced from similarity matrix estimates using Nei Li's coefficient of similarity data.

Zhou *et al.* (2009) performed ISSR analysis of hybrids of loose spike sorghum and sudangrass. The results showed that 451 bands of ISSR markers were amplified with 14 suitable primers and the length of DNA fragment ranged from 200 to 2000bp. A total of 416 bands were polymorphic and the percentage of polymorphic bands reached 92.2 per cent.

Sozen *et al.* (2010) assessed genetic variability and relationship among winter triticale (× Triticosecale Wittmack) cultivars using ISSR markers. Twenty ISSR primers were tested and twelve of them amplified clear and reproducible bands. The number of ISSR fragments generated per primer set ranged from 5 to 31 with fragment sizes varying from 320 to 2700 bp. A total of 209 ISSR fragments were detected, of which 159 were polymorphic (76.07%). All cultivars were clearly differentiated by their ISSR fingerprints. Based on UPGMA analysis a dendrogram was constructed and 11 triticale cultivars were grouped in two clusters.

Turki *et al.* (2011) assessed the genetic variation and relationship among different sorghum varieties. ISSR markers were used to assess the genetic diversity of 10 sorghum varieties, nine of Indians and one of Syrian origins. Out

of 20 ISSR primers screened, 9 primers were selected for their polymorphic and repeatable fragments. 110 fragments were polymorphic out of total 130 while the percentage of polymorphic bands value ranged from 63.6 per cent of $(AC)_8$ T to 100 per cent of $(AG)_8$ A with a mean of 84.61 per cent. The UPGMA clustering associated the varieties into two major clusters, separating Indian varieties from the Syrian one variety.

Suazo *et al.* (2012) established and optimized ISSR molecular markers as a tool for breeding programs of *Pinus radiate*. Two ISSR markers and twelve selective amplifications of microsatellite polymorphic loci (SAMPL) primer combinations were tested, using a first-generation full-sib family of 86 individuals. PCR products were visualized by capillary electrophoresis and polymorphism was detected by presence or absence of a particular fragment in one P. radiate parental. A total of 18 polymorphic fragments were found for two ISSR primers tested, with an average segregation distortion of 33 per cent.

Farsani *et al.* (2012) assessed genetic diversity of Bermuda grass (*Cynodon dactylon*) using ISSR Markers. Twenty seven Bermuda grass accessions and introductions, mostly from different parts of Iran, were assayed by ISSR markers to differentiate and explore their genetic relationships. Fourteen ISSR primers amplified 389 fragments of which 313 (80.5%) were polymorphic. The average polymorphism information content (PIC) was 0.328, which shows that the majority of primers are informative. Cluster analysis using UPGMA method and Jaccard's similarity coefficient (r = 0.828) grouped the accessions into six main clusters according to some degree to geographical origin, their chromosome number and some morphological characteristics.

Yang *et al.* (2012) assessed the genetic diversity of 12 natural populations in Yunnan, using ISSR markers. From 10 ISSR primers, 155 bands were generated, of which 153 were polymorphic (98.71%). A large proportion of the genetic variation (78.95%) resides among the individuals within populations, whereas only 21.05 per cent are found among populations. Mantel tests indicated

no significant correlation between genetic and geographic distances among the populations.

Rizkalla *et al.* (2012) carried out work on genetic Diversity based on ISSR and Protein markers associated with earliness Trait in wheat. Ten ISSR primers detected a total of 75 fragments, ranging in size from 128 to1531bp, 41 of them showed 54.66 per cent polymorphism. Number of bands varied from 3 to 12 with an average of 7.50 bands per primer. UPGMA analysis based on genetic similarities ranged between 0.933 and 0.080 indicating the genetic diversity among wheat genotypes.

Agarwal *et al.* (2012) reported ISSR markers, a useful tool for genetic diversity analysis in bread wheat (*Triticum aestivum* Thell.) genotypes. Thirteen ISSR primers used in the study amplified 164 bands out of which 123 bands were polymorphic and thus showed 75 per cent polymorphism. Dendrogram generated by the ISSR markers data showed that two genotypes NWL-6-4 and NIAW 1342 out of all the 17 genotypes were closely related to each other as they were having a highest similarity value of 0.91.

Bai *et al.* (2012) analysed the genetic diversity of turf bamboo species by using ISSR molecular marker technology. A total of 201 clear bands with good repeatability and high polymorphism were amplified with 21 ISSR primers, with a polymorphism rate of 93.1 per cent; similarity coefficients between different turf bamboo species ranged from 0.275 to 0.571, with an average similarity coefficient of 0.357; according to the results of ISSR markers, 10 different ornamental turf bamboo species were divided into three categories by using UPGMA cluster analysis method.

Huang *et al.,* (2012) explored the genetic diversity of *Cynodon radiatus* (Poaceae) accessions using ISSR markers. ISSR markers were applied to determine the genetic relationship among 29 *Cynodon radiatus* accessions from different regions of China. Fifty ISSR primers were screened, and 14 primers

were selected for their capability to produce clear and reproducible patterns of multiple bands to amplify specific *C. radiatus* genomic sequences. A total of 189 ISSR fragments from 300 bp to 3000 bp were amplified, of which 98.94 per cent were polymorphic. Genetic similarity coefficients among the 29 accessions ranged from 0.45 to 0.90, with an average of 0.68. Cluster analysis by 2 methods, unweighted pair-group method with arithmetic averages and principle coordinate analysis, showed that the *C. radiatus* accessions clustered into 4 distinct groups

Liet *et al.* (2011) used ISSR technique and 10 primers were used to study genetic diversity among 21 maize accessions consisting of 12 normal and 9 waxy maize accessions, collected from 3 provinces in the North mountain region of Vietnam and Laos. A total of 108 ISSR fragments were detected and all of them were polymorphic (100%). Polymorphism information content (PIC) values of ISSR primers ranged from 0.10-0.39. The average PIC value for each primer was 0.24. The resolving power (Rp) value ranged from 14.29-0.48 with an average of 4.48 per primer. Based on UPGMA analysis, using 70 per cent genetic similarity as the cutoff, a dendrogram was constructed and 21 maize accessions were grouped into three clusters. The similarity coefficients among accessions ranged from 0.52-0.90.

Idris *et al.* (2012) observed genotypes diversity study in Maize (*Zea mays* L.) by ISSR markers. In this study, Inter-simple sequence repeat (ISSR) markers were used to assess genetic diversity in a selected group of maize inbred (*Zea mays* L.) genotypes. A high level of polymorphism of 69 per cent was detected among these genotypes. The ISSR primers showed 10 fingerprints for six genotypes out of 9 studied which are Frantic, Huediba-1, Balady, Huediba-2, Giza 2 and Mogtamaa 45-2. The maximum genetic distance of 0.48 per cent was detected between Huediba-2 and Mogtama-45-2. While, the minimum genetic distance of 0.16 per cent was observed between Giza-2 and Var.113. The results indicated that variation can be attributed to use of ISSR.

Patil *et al.* (2012) reported analysis of genetic diversity by using ISSR markers in *Sorghum bicolor* (L.) Moench. Twenty five ISSR primers were used to study the genetic diversity among 16 genotypes of *Sorghum bicolor* (L.) Moench. A total of 288 scorable ISSR markers were generated from 16 genotypes, of which 262 were polymorphic. The polymorphic data generated from these markers were used to estimate the similarity index value and a dendrogram was constructed. The dendrogram classified the 16 genotypes into 13 distinct clusters.

Inter Simple Sequence Repeat is considered to be the most accurate and efficient molecular marker technology currently on variety identification. (Liu *et al.*, 2012) ISSR marker was used on 27 Bermuda grass individuals to identify genetic diversity and construct Bermuda grass fingerprints of 9 accessions. Thirteen primers produced 54 polymorphic bands, of which 34 bands were found to be polymorphic, 4.1 bands per primer on average. The percentage of polymorphic locus was 64.15 per cent on average. The Nei's genetic similarity coefficient changed from 0.547 to 0.962, the range was up to 0.415.

Lin *et al.* (2012) used ISSR technique for genetic diversity of *Pennisetum purpureum* germplasms in Taiwan. According to ISSR analysis, 14 UBC primers were polymorphic. Analysis molecular of variance analysis (AMOVA) was used to determine for the variance and genetic matrix of samples. Results showed that the variance was 96.95 per cent ($P<0.1391$) within population.

Dashchi *et al.* (2012) used ISSR markers to characterize and assess genetic diversity of Iranian bread wheat (*Triticum aestivum* L.) using 101 cultivars and breeding lines. Twenty-three ISSR primers amplified a total of 267 loci, of which 224 (83.9%) were polymorphic among the genotypes. The Dice similarity coefficient for the germplasm ranged from 0.76 (between two breeding lines) to 0.91 (between two breeding lines).

Devarumath *et al.* (2012) performed study to characterize 81 sugarcane genotypes. A total of 13 ISSR primers used and produced 65 amplified

fragments, of which 63 (96.5%) were polymorphic. The Polymorphic Information Content (PIC) value ranged from 0.11 (UBC824) to 0.45 (UBC825) primers with an average value of 0.28. The primer UBC 817 and UBC 825 exhibited highest resolving power (Rp) value 3.8 among thirteen primers. Genetic similarity (GS) by Jaccard's similarity coefficient ranged from 0.23 to 0.95 with a mean of 0.59. Dendrograms constructed using the UPGMA cluster analysis revealed low level of correlation between genetic similarities based on pedigree and DNA profiles.

Study of genetic diversity is an important factor in sugarcane breeding programs to identify genetically diverse parents for developing hybrid cultivars with improved cane and sugar yield. (Kalwade *et al.,* 2012). Genetic profiling studies of 17 sugarcane genotypes were carried out by using 27 ISSR markers having di, tri and tetra nucleotide repeat motifs. Out of the 252 amplicons amplified by 27 ISSR primers, 212 were polymorphic (84.13%) with an average of 9.3 alleles per locus. Cluster analysis by UPGMA method revealed similarity coefficient of 0.49 which was mainly attributed to inter specific diversity. Thus, ISSR markers can be used as a potential marker system for genetic profiling of sugarcane genotypes.

Xie *et al.* (2012) investigated the genetic diversity and genetic relationships of 81 Chinese wild zoysiagrass accessions and three commercial cultivars using inter-simple sequence repeat (ISSR) markers. ISSR primers produced 388 clear bands, among which 375 were polymorphic. The genetic similarity coefficients (GSCs) among 84 zoysiagrass accessions or cultivars ranged from 0.644 to 0.866 with an average of 0.751. The GSCs within species were significantly higher than that among species. Cluster analysis using an unweighted pair group method with arithmetic mean (UPGMA) method showed that the 84 zoysiagrass accessions could be classified into 10 major groups.

Kumbhar *et al.* (2013) reported molecular diversity in rice (*Oryza sativa* L.) using ISSR markers. Inter Simple Sequence Repeat (ISSR) fingerprinting was

used to assess the genetic diversity among fifty rice accessions. Out of 25 ISSR primers screened 13 primers produced polymorphic amplicons and were selected for genetic diversity analysis. It produced a total of 103 reproducible amplification products with an average of 7.92 amplicons per primer. All the markers displayed polymorphic amplicons. Of the total amplicons, 100 (97.08%) were polymorphic for more than one variety and three amplicons were monomorphic. The UPGMA based clustering analysis using Dice similarity coefficient grouped these genotypes into three major and eleven sub-clusters.

Zhang *et al.* (2013) used ISSR markers to evaluate the genetic variation within and among grass populations that were sampled from the Zhejiang and Guangdong Provinces of China. Based on nine ISSR primers, 206 clear and reproducible DNA fragments were generated. Relatively low levels of genetic diversity were determined among the populations. The coefficient of genetic differentiation among the populations was 0.52, which indicates that 52.3 per cent of the total molecular variance exists among the populations.

5. Lemongrass oil marketing strategy

Lemongrass oil is one of the 10 major essential oils. The main exporters of the oil are India and Guatemala (Jayasinha, 1999). Presently the market for lemongrass is at a good level. There are a lot of traders based in Mumbai, Delhi, Ahmadabad etc which are into selling and marketing the lemongrass oil in great amounts. There is also great scope and demand for lemongrass in other countries. Due to widespread use of essential oil of lemongrass in different industries, there is a great possibility of increase in its demand both nationally and internationally by well known and well established countries worldwide. Farmers can apply buy back guarantee as a marketing tool strategy to increase it demand in the market.

As we have seen above, lemongrass has a lot of utilities, one of them being low maintenance plant, i.e. once it is cultivated; there is no need to check back on it for the next 5 years. The lemongrass plant is very sturdy and can take harsh weather conditions. It also doesn’t require too watering and is not eaten by wild

animals or cattle. Selling lemongrass product i.e. oil and leaves is also fairly easy because of it great and enormous amount of uses. Because it is beneficial a lot of progressive farmers have chosen lemongrass farming over other agriculture products and this rate is only expected to increase in near future.

Materials and Methods

3. MATERIALS AND METHODS

The study on "Molecular characterization of East Indian lemongrass (*Cymbopogon flexuosus* Stapf.) germplasm accessions" was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara during the period 2011-2013. The materials used and methodologies adopted are discussed in this chapter.

3.1 Materials

3.1.1 Plant Materials

Twenty five accessions of East Indian lemongrass (*Cymbopogon flexuosus* Stapf.) *viz.*, OD-1, OD-2, OD-4, OD-7, OD-8, OD-9, OD-10, OD-11, OD-12, OD-13, OD-14, OD-15,OD-16, OD-17, OD-18, OD-20, OD-21, OD-23, OD-24, OD-25, OD-28, OD-29, OD-39, OD-40 and OD-320, diverse in oil and citral content were selected. The slips of the accessions were collected from Aromatic and Medicinal Plant Research Station, Odakkali, Kerala.

The morphological observations like length of culm, length of lamina, breadth of lamina and colour of leaf sheath (Table1) and biochemicals parameters like oil recovery (%FWB), oil recovery (%DWB) and citral content (Table 2) were provided by AMPRS. The geographical location of the selected accessions is shown in Plate1.

Collected slips were grown in polythene bags with potting mixture under polyhouse condition for a period of one month and maintained at CPBMB, College of Horticulture, Vellanikkara. After one month the raised plants were transplanted in earthen pots under open condition (Plate 2).

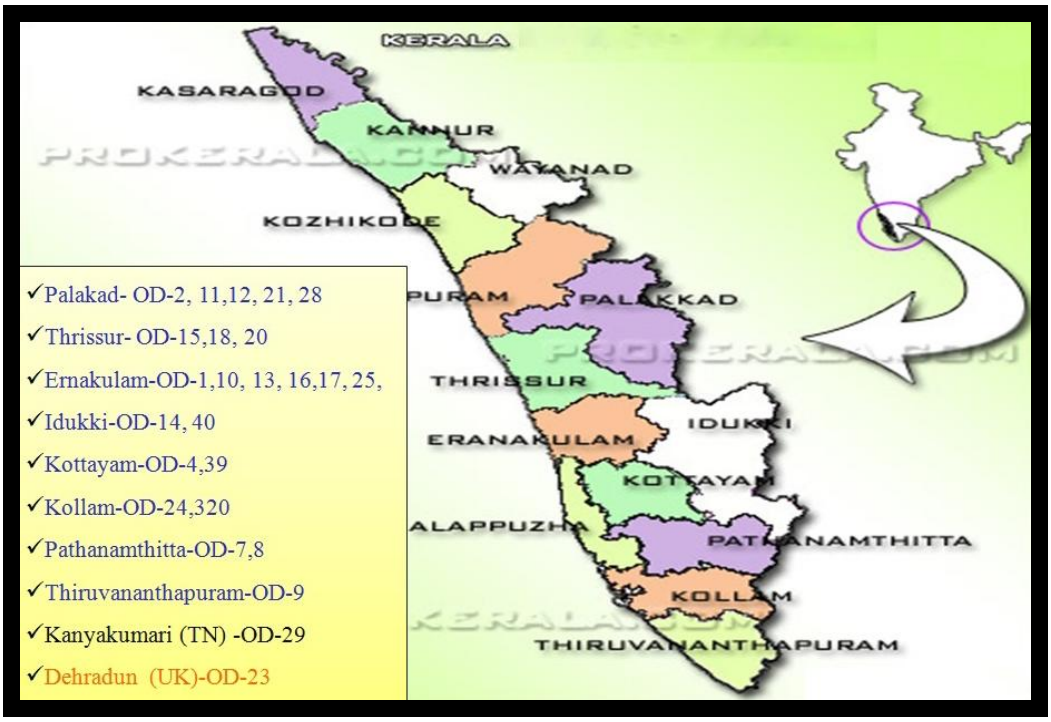


Plate 1. Geographical location of selected lemongrass accessions

Plate 2. Lemongrass accessions maintained for molecular characterization at CPBMB

Table 1. Morphological observations of selected lemongrass accessions collected from AMPRS, Odakkali

SI. No	Type	Place of collection	Length of culm (cm)	Distance between two ligules (cm)	Length of lamina (cm)	Breadth of lamina (cm)	Colour of leaf sheath
1	OD-1	Odakkali- Local	72.60	7.30	121.80	2.20	Light red
2	OD-2	Nelliampathy	56.30	2.30	99.10	1.60	Light green
3	OD-4	Shengalam	80.30	5.70	151.50	1.90	Red
4	OD-7	Pathanamthitta	63.30	7.50	148.60	1.70	Deep red
5	OD-8	Konni	64.60	6.80	102.70	1.60	Deep red
6	OD-9	Kallayi	57.60	5.60	109.30	1.50	Light red
7	OD-10	Kolencherry	64.00	6.80	119.80	1.80	Red
8	OD-11	Vadakkancherry-B	69.30	9.30	115.20	1.20	Light red
9	OD-12	Vadakkancherry-A	73.30	12.30	97.50	1.40	Light red
10	OD-13	Kunnamkulam	52.00	6.00	132.20	1.70	Light red
11	OD-14	Mannamkandam	71.30	5.70	141.00	2.10	Red
12	OD-15	Kunnamkulam	48.60	7.00	85.70	1.10	Light red
13	OD-16	Valayanchirangara	48.60	6.20	92.30	1.60	Light red
14	OD-17	Muvattupuzha	68.00	6.60	119.80	1.50	Light red
15	OD-18	Mulamkunnathukavu	76.30	11.10	141.10	1.70	Light red
16	OD-20	Pattikkad	65.60	4.70	147.80	1.50	Light red
17	OD-21	Nelliampathy	55.60	3.90	110.10	1.20	Light red
18	OD-23	Dehradun	88.00	8.80	137.70	1.50	Light red
19	OD-24	Kulathupuzha	77.30	7.80	114.80	1.70	Deep red
20	OD-25	Mamala	41.00	2.50	86.20	1.10	Deep red
21	OD-28	Nenmara	54.00	4.10	118.80	1.30	Deep red
22	OD-29	Trakkalai	72.70	5.40	114.50	1.80	Deep red
23	OD-39	Kulathupuzha	68.30	5.90	137.80	2.10	Red
24	OD-40	Thodupuzha	66.30	6.70	115.10	1.60	Light red
25	OD-320	Kootikal	54.70	3.60	128.90	1.60	Deep red

Table 2. Biochemical data of selected lemongrass accessions collected from AMPRS, Odakkali

SI.No	Type	Oil yield (ml/plant)	Oil recovery (%, FWB)	Oil recovery (%, DWB)	Citral (%)
1	OD-1	0.53	0.33	1.27	85.50
2	OD-2	0.17	0.50	1.56	71.50
3	OD-4	0.59	0.60	2.35	86.40
4	OD-7	0.53	0.48	1.64	84.40
5	OD-8	0.18	0.55	2.03	86.20
6	OD-9	0.78	0.45	1.73	84.00
7	OD-10	0.45	0.45	1.61	78.60
8	OD-11	0.70	0.55	2.04	78.80
9	OD-12	0.40	0.40	1.48	78.10
10	OD-13	0.34	0.30	1.15	81.70
11	OD-14	0.45	0.30	1.36	83.10
12	OD-15	0.15	0.30	1.16	78.60
13	OD-16	0.29	0.33	1.27	83.20
14	OD-17	0.66	0.40	1.23	82.40
15	OD-18	0.44	0.30	0.97	75.40
16	OD-20	0.76	0.38	1.15	86.80
17	OD-21	0.57	0.33	1.02	83.30
18	OD-23	1.11	0.58	1.74	88.50
19	OD-24	0.41	0.63	2.03	70.20
20	OD-25	0.57	0.50	1.67	82.00
21	OD-28	0.83	0.40	1.36	85.60
22	OD-29	0.73	0.30	1.07	82.10
23	OD-39	0.68	0.48	1.40	83.70
24	OD-40	0.78	0.50	1.47	84.30
25	OD-320	0.61	0.42	1.40	81.10

3.1.2 Laboratory chemicals, glasswares and plasticware

The chemicals used in the present study were of good quality (AR grade) procured from Merck India Ltd., HIMEDIA and SISCO Research Laboratories. The *Taq* DNA polymerase, dNTPs, *Taq* buffer and molecular marker (λDNA/*HindIII+EcoRI* double digest/100 bp ladder) were supplied by Bangalore Genei Ltd. All the plasticwares used were obtained from Axygen and Tarson India Ltd. The decamer primers were obtained from Operon Technologies Inc. (Alameda, Calif.) and ISSR primers were obtained from Sigma Aldrich Chemical Pvt. Ltd.

3.1.3 Equipment and Machinery

The present research work was carried out using molecular biology facilities and equipments available at CPBMB, College of Horticulture. Centrifugation was done in High speed refrigerated centrifuge (KUBOTA 6500). NanoDrop^{R} ND-1000 spectrophotometer was used for the estimation of quality and quantity of DNA. The DNA amplification was carried out in Eppendorf Master Cycler (Eppendorf, USA). Horizontal gel electrophoresis system (BIO-RAD, USA) was used for agarose gel electrophoresis. Gel Doc- BIO-RAD was used for imaging and documenting the agarose gel. The details of laboratory equipments used for the study are provided in Appendix I.

3.3 Molecular analysis

Molecular analysis of the 25 accessions of East Indian lemongrass were carried out with two different marker systems- RAPD and ISSR.

3.3.1 Genomic DNA extraction

The young tender, pale green leaves (first to third from the tip) from individual plants in each accession grown in open conditions were collected early in the morning. The surface of leaves was cleaned by washing with sterile water. The plant genomic DNA was isolated from pale green tender leaves (1st or 2nd

leaf from tip) of 25 accessions of East Indian lemongrass accessions using CTAB extraction method suggested by Rogers and Bendich (1994) with slight modifications.

3.3.1.1 Reagents:

The procedure for preparation of reagents used for DNA isolation is given in Annexure II.

1. CTAB extraction buffer (2x)
2. CTAB (10%)
3. β- mercaptoethanol
4. Chloroform : isoamyl alcohol (24:1)
5. Chilled isopropanol
6. Ethanol (70 %)
7. RNase A (DNase free)
8. TE buffer

3.3.1.2 Procedure for DNA isolation

Young and tender leaf tissue (1g) was weighed and ground in liquid nitrogen using mortar and pestle along with 50µl of β-mercaptoethanol and a pinch of Poly Vinyl Pyrrolidone (PVP). The sample was ground into fine powder using liquid nitrogen, 4 ml of extraction buffer (2x) and the powder was transferred to a sterile 50 ml Oakridge tube containing 3 ml of pre-warmed extraction buffer (total 7 ml). The homogenate was incubated for 30 minutes at 65ºC with occasional mixing by gentle inversion. Equal volume of chloroform: isoamyl alcohol (24:1) was added and centrifuged at 10,000 rpm for 15 minutes at 4ºC. The contents got separated into three distinct phases. The aqueous phase containing DNA was pipetted out into a fresh 50 ml Oakridge tube and incubated in water bath at 37ºC for 30 minutes. After incubation, one tenth volume of 10 per cent CTAB solution was added followed by purification with equal volume of chloroform: isoamyl alcohol (24:1) mixture and centrifuged at 10,000 rpm for

15 minutes at 4ºC. The supernatant was collected, 1/3 volume of chilled isopropanol was added and incubated at -20ºC for 30 minutes. The precipitated DNA was pelleted by centrifugation at 10,000 rpm for 15 minutes at 4ºC. The pellet was collected and washed first with 70 per cent alcohol. Pellet was air dried for 10 minutes at room temperature and dissolved in 100µl distilled water.

3.3.2 Purification of DNA

The DNA which had RNA as contaminant was purified by RNase treatment and further precipitation. The RNase treatment was carried out during the DNA isolation steps after pelleting the DNA.

3.3.3 Assessing the quality of DNA by Electrophoresis

The quality of isolated DNA was evaluated through agarose gel electrophoresis (Sambrook *et al*., 1989).

3.3.3.1 Reagents and Equipments

1. Agarose
2. 50X TAE buffer (P^H 8.0)
3. 6X Loading /Tracking dye
4. Ethidium bromide (0.5 µg/ml)
5. Electrophoresis unit, power pack (BIO-RAD), gel casting tray, comb
6. UV transilluminator (Wealtec)
7. Gel documentation and analysis system (BIO-RAD)

Chemical composition of buffers and dye are given in Annexure III. The procedure followed for agarose gel electrophoresis was as follows:

Agarose (0.8%) was weighed and dissolved in TAE buffer (1X) by boiling, added ethidium bromide (0.5µg/ml) and mixed well. The open end of the gel casting tray was sealed with cello tape and kept on a horizontal surface. The comb was placed desirably and the dissolved agarose was poured on to the tray. The gel was allowed to set for 30 minutes after which the comb was removed

carefully. The tray was kept in the electrophoresis unit with well side directed towards the cathode. 1X TAE buffer was added to the tank. Then DNA sample (5 µl) along with tracking dye (1 µl) was loaded into the wells using a micropipette carefully. λDNA/*Eco*RI+*Hind*III double digest was used as a molecular marker. After closing the tank, the anode and cathode ends were connected to the power pack and the gel was run at a constant voltage (100V) and current (50 A). The power was turned off when the tracking dye reached 2/3rd length of the gel.

Then the gel was taken from the electrophoresis unit and viewed under UV transilluminator for presence of DNA. The DNA fluoresces under UV light due to ethidium bromide dye. The image was documented in gel documentation system (Gel DOC-XR Imaging system BIO-RAD (USA). The gel profile was examined for intactness, clarity of DNA band, presence of RNA and protein.

3.3.4 Assessing the quality and quantity of DNA by Nanodrop Spectrophotometer

The quality and quantity of genomic DNA was estimated using NanoDrop^{R} ND-1000 spectrophotometer (NanoDrop Technologies Inc., USA). Before taking sample readings, the instrument was set to zero by taking 1µl autoclaved distilled water as blank. One micro litre of nucleic acid sample was measured at a wavelength of 260 nm and 280 nm and OD_{260}/OD_{280} ratios were recorded to assess the purity of DNA. A ratio of 1.8 to 2.0 for OD_{260}/OD_{280} indicated good quality of DNA. The quantity of DNA in the pure sample was calculated using the formula OD_{260}= 1 is equivalent to 50 µg double stranded DNA/µl sample.

1OD at 260 nm = 50 µg DNA/ml

Therefore $OD_{260} \times 50$ gives the quantity of DNA in µg/ml.

3.4 Molecular Markers used for the study

Two different types of markers *viz*., Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeats (ISSR) were used for the study in order to confirm the result at molecular level.

3.4.1 DNA amplification conditions

The PCR condition required for effective amplifications in RAPD and ISSR analysis include appropriate proportions of the component of the reaction mixture. The reaction mixture includes template DNA, assay buffer A or B, $MgCl_2$, *Taq* DNA polymerase, dNTPs and primers. The aliquot of this master mix was dispensed into 0.5 ml or 0.2 ml PCR tubes. The PCR was carried out in an Eppendorf Master Cycler (Eppendorf, USA)/Veriti Thermal Cycler (Applied Biosystem, USA).

3.4.2 RAPD (Random Amplified Polymorphic DNA) analysis

The good quality genomic DNA (40 to 50ng/µl) isolated from lemongrass leaf samples were subjected to RAPD as per the procedure. Random decamer primers supplied by 'Operon Technologies' USA with good resolving power was used for amplification of DNA. The decamer primers for RAPD assay were selected after an initial screening study of primers.

The amplification was carried out in an Eppendorf Master Cycler (Eppendorf, USA). PCR amplification was performed in a 20 µl reaction mixture as constituted below:

Composition of the reaction mixture for PCR

a) Genomic DNA (40ng)	- 2.0 µl
b) 10X *Taq* assay buffer B	- 2.0 µl
c) $MgCl_2$	- 1.5 µl
d) dNTP mix (10mM each)	- 1.5 µl
e) *Taq* DNA polymerase (1U)	- 0.4 µl
f) Decamer primer (10 pM)	- 2.0 µl
g) Sterile distilled water	- 10.6 µl
Total volume	- 20.0 µl

The thermo cycler was programmed as follows:

94°C for 4 minutes - Initial denaturation

92°C for 1 minute - Denaturation

32 to 34°C for 1 minute - Primer annealing

72°C for 2 minutes - Primer extension

} 39 cycles

72°C for 8 minutes - Final extension

4°C for infinity to hold the sample

3.4.2.1 Screening of random primers for RAPD analysis

Thirty five decamer primers in the series of OPA, OPC, OPD, OPK, OPG RN, and S were screened for RAPD analysis (Table3).

3.4.2.2 Random primers selected for RAPD Assay

Total 35 decamer primers were screened for RAPD analysis out of which 10 primers yielded the best amplification pattern were selected.

The amplified products were run on 2 per cent agarose gel using 1X TAE buffer stained with ethidium bromide along with marker (λDNA/*Eco*RI+*Hind*III double digest). The profile was visualized under UV (312 nm) transilluminator and documented using gel documentation system BIO-RAD Imaging system, (USA). The documented RAPD profiles were carefully examined for amplification of DNA as bands. The number of monomorphic and polymorphic bands was recorded for further analysis.

Table 3. List of RAPD primers used for screening of East Indian lemongrass accessions

Sl. No.	Name of Primer	Nucleotide Sequence
1.	OPA 02	5’TGCCGAGCTG3’
2	OPA 04	5’AATCGGGCTG3’
3	OPA 06	5’GGTCCCTGAC3’
4	OPA 08	5’GTGACGTAGG3’
5	OPA 10	5’GTGATCGCAG3’
6	OPA 12	5’TGGGCGATAG3’
7	OPA 27	5’GAAACGGGTG3’
8	OPA 28	5’GTGACGTAGG3’
9	OPC 01	5’TTCGAGCCAG3’
10	OPC 02	5’GTGAGGCGTC3’
11	OPC 04	5’GGTACGATGC3’
12	OPC 08	5’TGGACCGGTA3’
13	OPC 14	5’TGCGTGCTTG3’
14	OPD 10	5’GGTCTACCAC3’
15	OPD 15	5’CATCCGTGCT3’
16	OPD 20	5’ACCCGGTAAC3’
17	OPE 05	5’CTGAGAATCC3’
18	OPE 07	5’AGATGCAGCC3’
19	OPG 08	5’TCACGTCCAC3’
20	OPK 01	5’TGGCGACCTG3’
21	OPP 16	5’CCAAGCTGCC3’
22	OPP 17	5’TGACCCGCCT3’
23	OPU 03	5’CTATGCCGAC3’
24	OPU 07	5’CTACAGTGAG3’
25	OPU 13	5’GGCTGGTTCC3’
26	OPAH 1	5’TCCGCAACCA3’
27	OPAH 3	5’GGTTACTGCC3’
28	OPAH 5	5’TTGCAGGCAG3’
29	OPAH 6	5’GTAAGCCCCT3’
30	OPAH 9	5’AGAACCGAGG3’
31	RN 07	5’CAGCCCAGAG3’
32	RN 08	5’ACCTCAGCTC3’
33	RY 08	5’AGGCAGAGCA3’
34	S11	5’GTAGACCCGT3’
35	S 12	5’CCTTGACGCA3’

3.4.3 ISSR (Inter Simple Sequence Repeats) analysis

The good quality genomic DNA (30 to 40ng/µl) isolated from lemongrass leaf samples were subjected to ISSR as per the procedure reported by Menezes *et al.* (2009). ISSR primers supplied by Sigma Aldrich, USA with good resolving power were used for amplification of DNA. ISSR primers for ISSR assay were selected after an initial screening of primers.

The amplification was carried out in an Eppendorf Master Cycler (Eppendorf, USA). USA). PCR amplification was performed in a 20 µl reaction mixture as constituted below:

Composition of the reaction mixture for PCR

a) Genomic DNA (25ng)	- 2.0µl
b) 10X Taq assay buffer B	- 2.0 µl
c) $MgCl_2$	- 1.5µl
c) dNTP mix (10mM each)	- 1.5 µl
d) Taq DNA polymerase (1U)	- 0.3 µl
e) Primer (10 pM)	- 1.5 µl
f) Sterile distilled water	- 11.2 µl
Total volume	- 20.0 µl

The thermal cycler was carried out with the following programme

94°C for 1 minute	-	Initial denaturation	
94°C for 1 minute	-	Denaturation	35 cycles
52-58 °C for 1 minute	-	Primer annealing	
72°C for 1 minute	-	Primer extension	
72°C for 5 minutes	-	Final extension	

4°C for infinity to hold the sample

3.4.3.1 Screening of ISSR Primers for ISSR Analysis

Thirty one ISSR primers belonging to series UBC, ISSR and SPP were screened by PCR for ISSR analysis (Table 4)

3.4.3.2 ISSR primers selected for ISSR assay

Out of 31 primers screened for ISSR analysis, 10 primers yielded the best amplification products were selected.

The amplified products were run on 2 per cent agarose gel using 1X TAE buffer stained with ethidium bromide along with marker 100bp ladder. The profile was visualized under UV (312 nm) transilluminator and documented using gel documentation system BIO-RAD Imaging system, (USA). The documented ISSR profiles were carefully examined for amplification of DNA as bands. The numbers of monomorphic and polymorphic bands were recorded for further analysis.

Table 4. List of ISSR primers used for screening of lemongrass accessions

Sl. No.	Name of Primer	Nucleotide Sequence
1.	UBC 840	5'GAGAGAGAGAGAGAGAYT3'
2	UBC 844	5'CTCTCTCTCTCTCTCTRC3'
3	UBC 890	5'VHVGTGTGTGTGTGTGT3'
4	UBC 811	5'GAGAGAGAGAGAGAGAC3'
5	UBC813	5'CTCTCTCTCTCTCTCTT3'
6	UBC 815	5'CTCTCTCTCTCTCTCTG3'
7	UBC354	5'CTAGAGGCCG3'
8	UBC S2	5'CTCTCTCTCGTGTGTGTG3'
9	UBC 866	5'CTCCTCCTCCTCCTCCTC3'
10	UBC 826	5'ACACACACACACACACC3'
11	UBC 848	5'CACACACACACACACARG3'
12	UBC 845	5'CTCTCTCTCTCTCTCTRG3'
13	UBC 868	5'GAAGAAGAAGAAGAAGA3'
14	UBC 834	5'AGAGAGAGAGAGAGAGYT3'
15	UBC 835	5'AGAGAGAGAGAGAGAGY3'
16	UBC 836	5'AGAGAGAGAGAGAGAGY3'
17	UBC 807	5'AGAGAGAGAGAGAGAGT3'
18	UBC 817	5'CACACACACACACACAA3'
19	UBC 818	5'CACACACACACACACAG3'
20	UBC 820	5'GTGTGTGTGTGTGTGTC3'
21	ISSR 04	5'ACACACACACACACACC3'
22	ISSR 05	5'CTCTCTCTCTCTCTTG3'
23	ISSR 06	5'GAGAGAGAGAGAGAGAC3'
24	ISSR 07	5'CTCTCTCTCTCTCTTG3'
25	ISSR 08	5'GAGAGAGAGAGAGAGAT3'
26	ISSR 09	5'CTCTCTCTCTCTCTCTCG3'
27	ISSR 10	5'ACACACACACACACACG3'
28	ISSR 15	5'TCCTCCTCCTCCTCC3'
29	SPS 03	5'GACAGACAGACAGACA3'
30	SPS 08	5'GGAGGAGGAGGA3'
31	ISSR 2	5'GACAGACAGACAGACA3'

3.4.4 Scoring of bands and data analysis

Scoring of bands on agarose was done with the Quantity one software (Bio-Rad) loaded in Gel Doc. 100bp ladder was used as molecular weight size marker for each gel along with DNA samples. The bands were scored as 1 and 0 for the presence and absence respectively and their size recorded in relation to the molecular weight marker used. The result obtained from RAPD and ISSR were transformed into data matrix as discrete variables. Jaccard's coefficient of similarity was used to derive the dissimilarity matrix and dendrogram was generated by using Unweighted Pair Group Method with Arithmetic Mean (UPGMA). Only distinct and well resolved fragments were scored. The resulting data were analysed using the software package NTSYS pc version 2.02i (Rohlf, 2005).

Resolving power (Prevost and Wilkinson, 1999) was used to identify the primers that would distinguish the accessions most efficiently. Resolving power (Rp) of a primer was calculated as the sum of 'band informativeness' of all the bands produced by the primer. Band information (Ib) is = 1(2(0.5 – p)), where p is the proportion of the accessions containing the bands. Resolving power of the primer is represented as: Rp= Σ Ib.

The PIC value (Hollman *et al*., 2005) of a marker detects polymorphism within a population depending on the number of detectable alleles and their frequency. Value of a primer is represented as PIC = 1- Σ pi^2, where *pi* is the frequency of the i^{th} allele.

Results

4. RESULTS

The study on "Molecular characterization of East Indian lemongrass (*Cymbopogon flexuosus* Stapf.) germplasm accessions" was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara during the period 2011-2013. The result obtained in the present study is described below.

4.1 Molecular characterization

The results of the Molecular characterization of East Indian lemongrass (*Cymbopogon flexuosus* Stapf.) germplasm accessions were carried out using two different marker systems viz., RAPD and ISSR. The results of the experiment are described below.

4.1.1 Isolation, Purification and quantification of DNA

As reported in many other crops young leaves were selected as the ideal part for extraction of genomic DNA. The leaves were collected in the morning (8 to 9 am) from potted plants. Young tender, pale green leaves (1g) yielded good quality DNA in sufficient quantity.

Genomic DNA was isolated through the CTAB method (Roger and Benedich, 1999) with modification. The DNA had RNA contamination. (Plate3a). RNase treatment and further precipitation gave sufficient quantity of good quality DNA from leaf sample. The agarose gel electrophoresis indicated clear discrete band without RNA contamination and spectrophotometric analysis gave ratio of UV absorbance ($A_{260}/_{280}$) between 1.8 and 2.0. Quality and quantity of DNA isolated through the CTAB method for lemongrass accessions are indicated in Plate 3b and in Table 5.

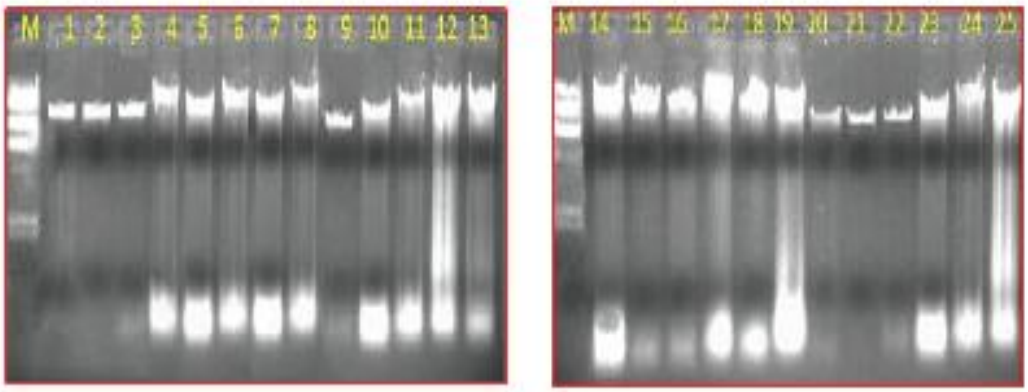


Plate 3a. DNA sample with RNA contamination

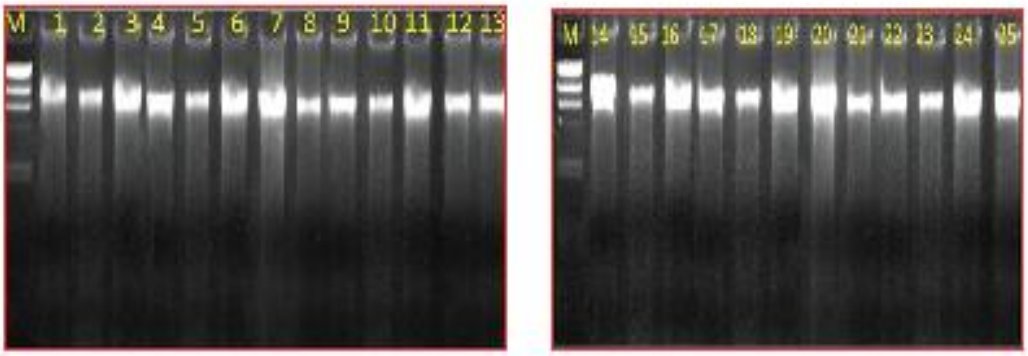


Plate 3b. DNA sample after RNase treatment

1-OD-1	6-OD-9	11-OD-14	16-OD-20	21-OD-28
2-0D-2	7-OD-10	12-OD-15	17-OD-21	22-OD- 29
3-OD-4	8-OD-11	13-OD-16	18-OD-23	23-OD-39
4-OD-7	9-OD-12	14-OD-17	19-OD-24	24-OD-40
5-OD-8	10-OD-13	15-OD-18	20-OD-25	25-OD-320

Plate 3. DNA isolation and its purification

Table 5. Quality and quantity of DNA isolated from lemongrass accessions assessed by Nano Drop spectrophotometer

Sl. No	Accessions	UV absorbance at 260 nm (A_{260})	UV absorbance at 280 nm (A_{280})	$A_{260}/_{280}$	Quantity (ng/µl)
1	OD-1	49.714	26.034	1.91	2485.70
2	OD-2	52.884	27.717	1.91	2644.45
3	OD-4	49.770	25.551	1.95	2488.48
4	OD-7	49.618	25.528	1.94	2480.92
5	OD-8	50.224	25.366	1.93	2811.20
6	OD-9	55.509	28.399	1.95	2775.45
7	OD-10	34.075	17.040	2.00	1703.75
8	OD-11	38.227	19.543	1.96	1911.36
9	OD-12	57.444	29.054	1.98	2872.19
10	OD-13	61.118	32.089	1.90	3055.91
11	OD-14	55.509	28.399	1.95	2623.70
12	OD-15	34.075	17.040	2.00	2177.03
13	OD-16	38.227	19.543	1.96	2306.90
14	OD-17	57.446	29.054	1.98	2593.03
15	OD-18	61.118	32.089	1.90	2948.60
16	OD-20	69.341	36.256	1.91	3467.06
17	OD-21	51.675	26.479	1.95	2584.25
18	OD-23	27.641	14.247	1.94	1382.03
19	OD-24	45.671	23.276	1.96	2283.55
20	OD-25	53.692	27.808	1.93	2684.62
21	OD-28	43.535	22.433	1.94	2176.75
22	OD-29	36.231	18.529	1.96	1811.56
23	OD-39	16.373	8.617	1.90	2818.66
24	OD-40	37.879	19.04	1.94	1893.93
25	OD-320	22.314	12.061	1.85	1615.68

The protocol for different marker assays- RAPD and ISSR were validated with bulked DNA of lemongrass accessions. Different primers were screened with the genomic DNA of ten selected accessions using the validated protocols.

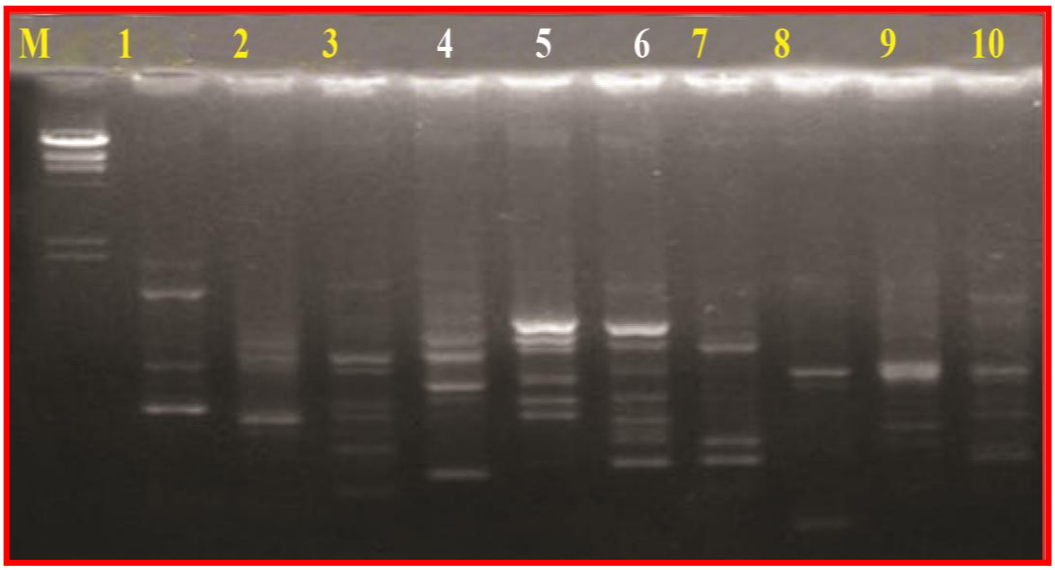
4.2.1 Random Amplified Polymorphic DNA (RAPD) analysis

Good quality genomic DNA from the lemongrass accessions were subjected to RAPD assay. The experiments carried out under this included screening of random primers and RAPD with selected primers.

4.2.1.1 Screening of primers for RAPD analysis

Thirty five primers belonging to series OPA, OPAH, OPC, OPD, OPE, OPP, OPU, RN, RY, S were tested for amplification of genomic DNA of lemongrass accessions. The amplification patterns of these primers are shown in Plate 4 and Table 6. The selected 10 RAPD primers are depicted in Table 7.

Out of seven OPA series primer screened, five primers *viz.*, OPA 2, OPA 4, OPA10, OPA 12, OPA 28 were selected for the final amplification with the 25 accessions of the lemongrass. Out of five OPC series primer screened three primers *viz.*, OPC1, OPC 2, OPC14 were selected for the final amplification with the 25 accessions of the lemongrass. Out of three OPD series primer screened only one *viz.;* OPD 12 was selected for the final amplification. Out of five OPAH series primer screened only one *viz*., OPAH 3 was selected for final amplification.



Lane: M: Molecular weight marker λ DNA/*Hind* III digest

1-OPA 28, 2-OPA 6, 3-OPA 8, 4-OPA 4, 5-OPA 12, 6-OPA 27, 7-OPA 06, 8-OPC 4, 9-OPC8, 10-OPD-10

Plate 4. Screening of RAPD primers for amplification of lemongrass DNA

Table 6. Screening of 35 random primers for RAPD assay in lemongrass accessions

Sl. No	Primers	Amplification pattern			
		No. of Bands	Type of bands		Remarks
			Distinct	Faint	
1	OPA 02	9	7	2	Selected
2	OPA 04	6	4	2	Selected
3	OPA 06	6	2	4	----
4	OPA 08	5	2	3	----
5	OPA 10	9	6	3	Selected
6	OPA 12	7	6	1	Selected
7	OPA 27	9	6	3	Selected
8	OPA 28	6	3	3	----
9	OPC 01	10	7	3	Selected
10	OPC 02	13	9	5	Selected
11	OPC 04	2	1	1	----
12	OPC 08	1	1	0	----
13	OPC 14	2	2	0	Selected
14	OPD 10	4	1	3	----
15	OPD 15	3	1	2	Selected
16	OPD 20	6	1	5	----
17	OPE 05	4	1	3	----
18	OPE 07	1	0	1	----
19	OPG 08	3	1	2	----
20	OPK 01	4	1	3	----
21	OPP 16	6	2	4	----
22	OPP 17	7	2	5	----
23	OPU 03	8	5	3	----
24	OPU 07	9	6	3	----
25	OPU 13	1	0	1	----
26	OPAH 1	7	2	5	----
27	OPAH 3	10	7	3	Selected
28	OPAH 5	8	6	2	----
29	OPAH 6	1	0	1	----
30	OPAH 9	7	1	6	----
31	RN 07	5	3	2	----
32	RN 08	5	2	3	----
33	RY 08	6	2	4	----
34	S11	3	2	1	----
35	S 12	1	1	0	----

Table 7. List of RAPD primers selected for molecular characterization of East Indian lemongrass accessions

Sl. No.	Name of Primer	Nucleotide Sequence
1.	OPA 02	5’TGCCGAGCTG3’
2	OPA 04	5’AATCGGGCTG3’
3	OPA 10	5’GTGATCGCAG3’
4	OPA 12	5’TGGGCGATAG3’
5	OPAH 3	5’GGTTACTGCC3’
6	OPA 27	5’GAAACGGGTG3’
7	OPC 01	5’TTCGAGCCAG3’
8	OPC 02	5’GTGAGGCGTC3’
9	OPC 14	5’TGCGTGCTTG3’
10	OPD 15	5’CATCCGTGCT3’

.3.1.2 Amplification with selected primers

The RAPD primer sets OPA, OPAH, OPC, OPD were selected for amplifying DNA of 25 accessions of lemongrass.

(i) OPA 2

The agarose gel profile for the amplification pattern was observed in the 25 accessions of lemongrass with primer OPA 2 (Plate 5a). A total of 12clear distinct loci were observed and out of which 5 were polymorphic and 7 were monomorphic. The percentage of polymorphism was 58 and the amplicons ranged from 400bp to 1400bp.

(ii) OPA 4

The agarose gel profile for the amplification pattern was observed in the 25 accessions of lemongrass with primer UBC 815 (Plate 5b). A total of 11 clear distinct loci were observed out of which 5 were polymorphic and 6 were monomorphic. The percentage of polymorphism was 45 and the amplicons ranged from 200bp to 1400bp.

(iii) OPA 10

The agarose gel profile for the amplification pattern was observed in the 25 accessions of lemongrass with primer OPA 10 (Plate 6a). A total of 7 clear distinct loci were observed out of which 4 were polymorphic and 3 were monomorphic. The percentage of polymorphism was 57 and the amplicons ranged from 400bp to 1100bp.

(iv) OPA 12

The agarose gel profile for the amplification pattern was observed in the 25 accessions of lemongrass with primer OPA 12 (Plate 6b). A total of 8 clear distinct loci were observed out of which 5 were polymorphic and 3 were

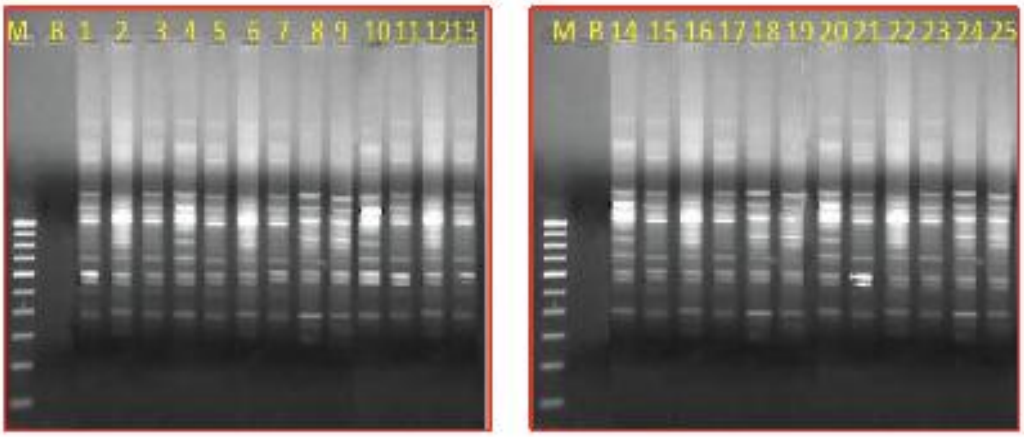


Plate 5a. Amplification with primer OPA 2

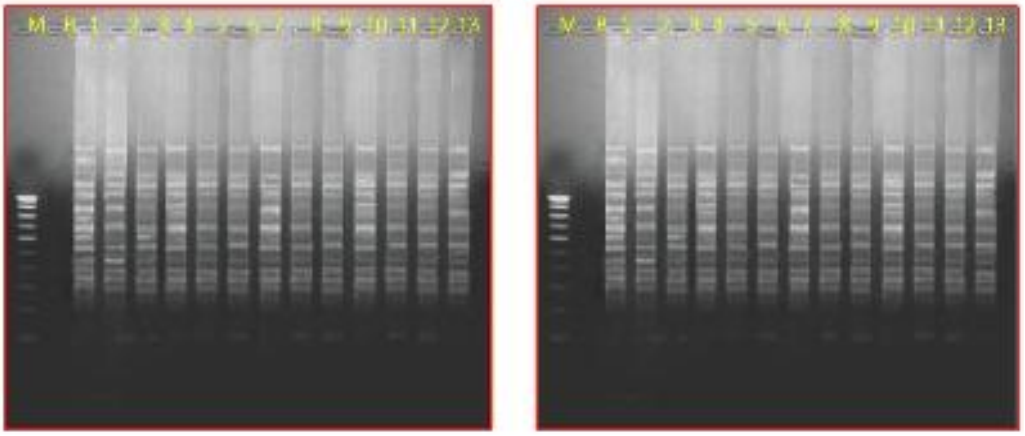


Plate 5b. Amplification with primer OPA 4

Lane-Marker 100 bp. B-blank

1-OD-1	6-OD-9	11-OD-14	16-OD-20	21-OD-28
2-0D-2	7-OD-10	12-OD-15	17-OD-21	22-OD- 29
3-OD-4	8-OD-11	13-OD-16	18-OD-23	23-OD-39
4-OD-7	9-OD-12	14-OD-17	19-OD-24	24-OD-40
5-OD-8	10-OD-13	15-OD-18	20-OD-25	25-OD-320

Plate 5. Amplification pattern of 25 accessions of East Indian Lemongrass with RAPD primers OPA 2 and OPA 4

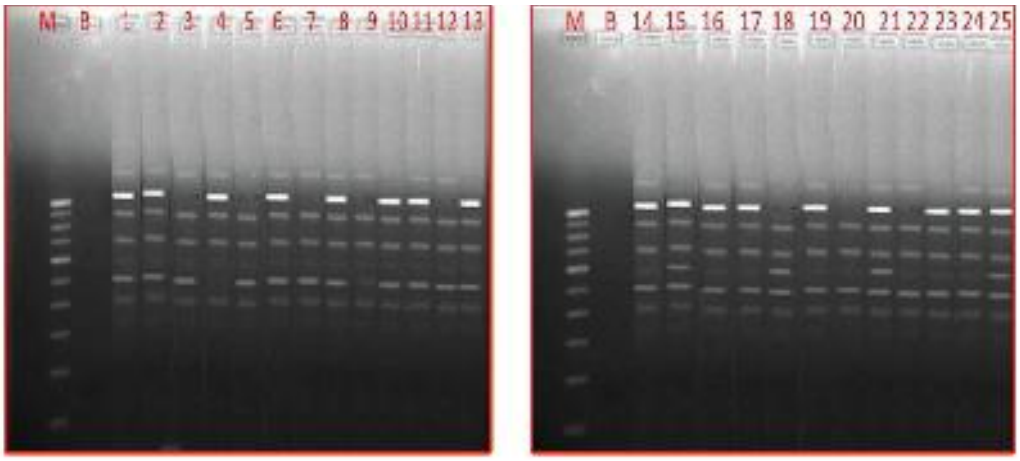


Plate 6a. Amplification with primer OPA 10

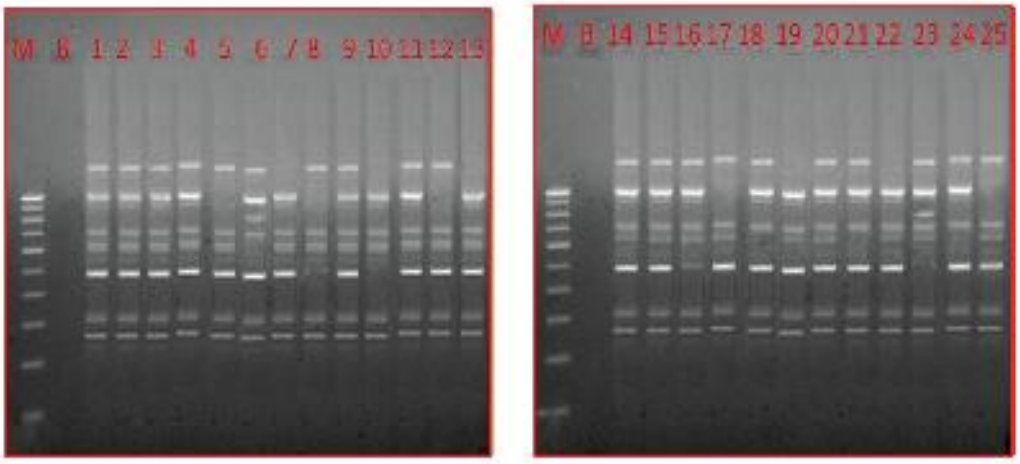


Plate 6b. Amplification with primer OPA 12

Lane-Marker 100 bp. B-blank

1-OD-1	6-OD-9	11-OD-14	16-OD-20	21-OD-28
2-0D-2	7-OD-10	12-OD-15	17-OD-21	22-OD- 29
3-OD-4	8-OD-11	13-OD-16	18-OD-23	23-OD-39
4-OD-7	9-OD-12	14-OD-17	19-OD-24	24-OD-40
5-OD-8	10-OD-13	15-OD-18	20-OD-25	25-OD-320

Plate 6: Amplification pattern of 25 accessions of East Indian Lemongrass with RAPD primers OPA 10 and OPA 12

monomorphic and the amplicons ranged from. The percentage of polymorphism was 52 and the amplicons ranged from 270bp to 1200bp.

(v) OPA 27

The agarose gel profile for the amplification pattern was observed in the 25 accessions of lemon grass with primer OPA 27 (Plate 7a). A total of 12 clear distinct loci were observed out of which 7 were polymorphic and 5 were monomorphic. The percentage of polymorphism was 58 and the amplicons ranged from 300bp to 1500bp.

(vi) OPAH 3

The agarose gel profile for the amplification pattern was observed in the 25 accessions of lemon grass with primer OPAH 3 (Plate 7b). A total of 10 clear distinct loci were observed out of which 5 were polymorphic and 5 were monomorphic. The percentage of polymorphism was 50 and the amplicons ranged from 350bp to 1150bp.

(vii) OPC 1

The agarose gel profile for the amplification pattern was observed in the 25 accessions of lemon grass with primer OPC 1 (Plate 8a). A total of 12 clear distinct loci were observed out of which 6 were polymorphic and 6 were monomorphic. The percentage of polymorphism was 50 and the amplicons ranged from 400bp to 1500bp.

(viii) OPC 2

The agarose gel profile for the amplification pattern was observed in the 25 accessions of lemon grass with primer OPC 2 (Plate 8b). A total of 9 clear distinct loci were observed out of which 7 were polymorphic and 2 were monomorphic. The percentage of polymorphism was 77 and the amplicons ranged from 300bp to 1100bp.

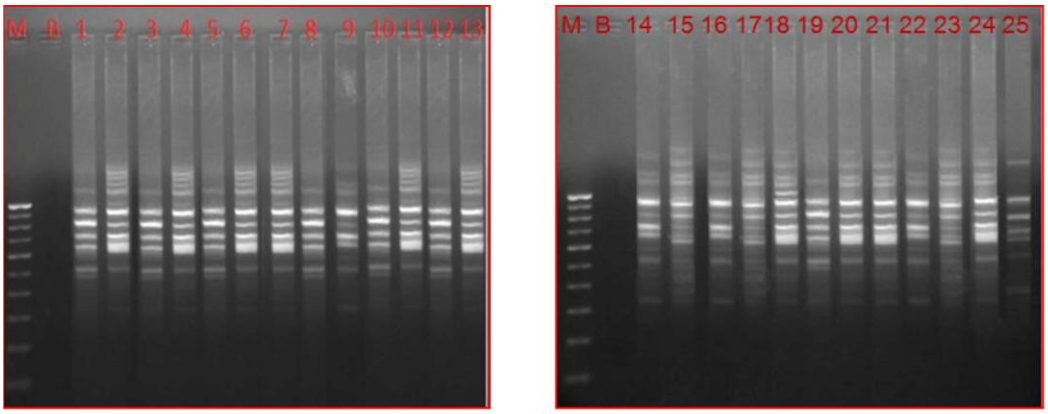


Plate 7a. Amplification with primer OPA 27

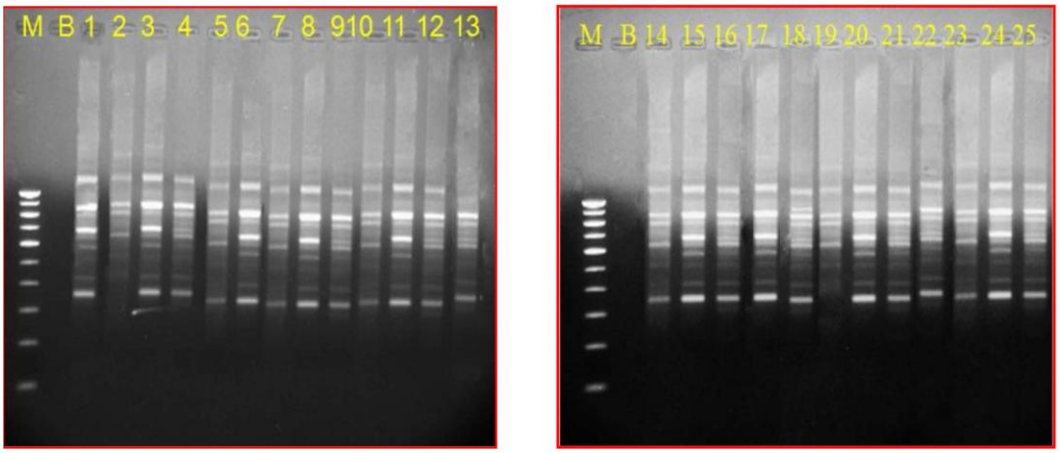


Plate 7b. Amplification with primer OPAH 3

Lane-Marker 100 bp. B-blank

1-OD-1	6-OD-9	11-OD-14	16-OD-20	21-OD-28
2-0D-2	7-OD-10	12-OD-15	17-OD-21	22-OD- 29
3-OD-4	8-OD-11	13-OD-16	18-OD-23	23-OD-39
4-OD-7	9-OD-12	14-OD-17	19-OD-24	24-OD-40
5-OD-8	10-OD-13	15-OD-18	20-OD-25	25-OD-320

Plate 7. Amplification pattern of 25 accession of East Indian lemongrass with RAPD primers OPA 27 and OPAH 3

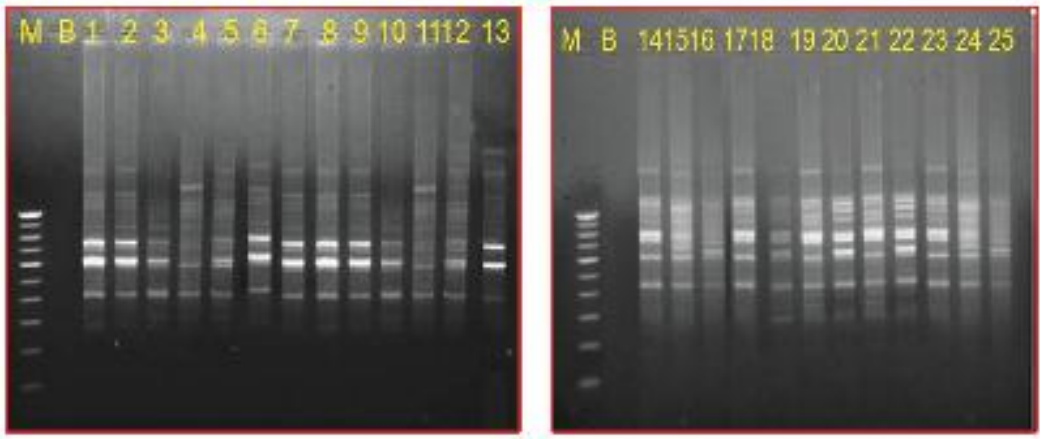


Plate 8a. Amplification with primer primer OPC 1

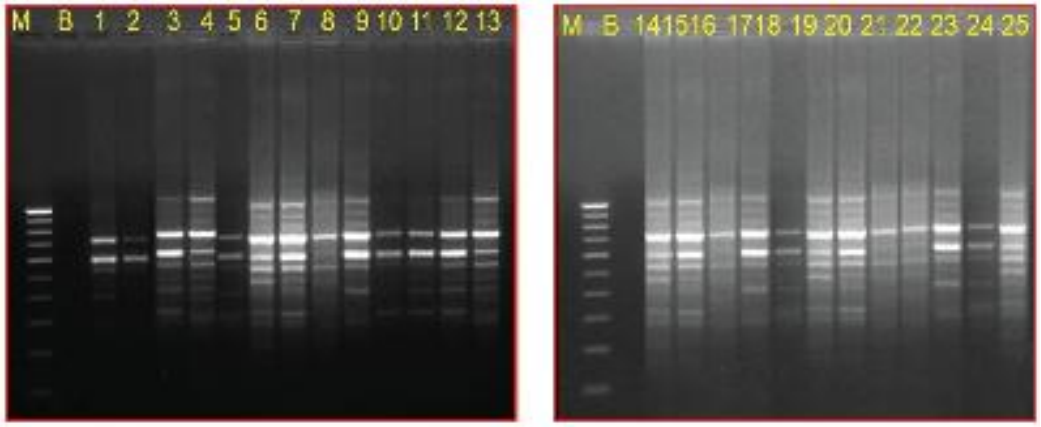


Plate 8b. Amplification with primer OPC 2

Lane-Marker 100 bp. B-blank

1-OD-1	6-OD-9	11-OD-14	16-OD-20	21-OD-28
2-0D-2	7-OD-10	12-OD-15	17-OD-21	22-OD- 29
3-OD-4	8-OD-11	13-OD-16	18-OD-23	23-OD-39
4-OD-7	9-OD-12	14-OD-17	19-OD-24	24-OD-40
5-OD-8	10-OD-13	15-OD-18	20-OD-25	25-OD-320

Plate 8. Amplification pattern of 25 accessions of East Indian Lemongrass with RAPD primers OPC 1 and OPC 2

(ix) OPC 14

The amplification pattern of the agarose gel profile was observed in the 25 accessions of lemongrass with primer OPC 14 (Plate 9a). A total of 13 clear distinct loci were observed out of which 7 were polymorphic and 6 were monomorphic. The percentage of polymorphism was 53 and the amplicons ranged from 300bp to 1600bp.

(x) OPD 15

The amplification pattern of the agarose gel profile was observed in the 25 accessions of lemongrass with primer OPD 15 (Plate 9b). A total of 7 clear distinct loci were observed out of which 3 were polymorphic and 4 were monomorphic. The percentage of polymorphism was 57 and the amplicons ranged from 250bp to 800bp.

The amplification details of these selected 10 primes are shown in Table 8.

4. 3 Inter Simple Sequences Repeats (ISSR) analysis

Good quality genomic DNA from 25 accessions of lemongrass were subjected to ISSR assay. The various experiments carried out under this included screening of ISSR primers and ISSR analysis with selected primers.

4.3.1 Screening of primers for ISSR analysis

Thirty one primers belonging to series UBC, ISSR, and SPP were tested for amplification of genomic DNA of lemongrass accessions. The amplification patterns of some these primers are shown in Plate 10 and Table 9.

Out of twenty one UBC series primer screened seven were selected *viz.*, UBC 7 UBC 15, UBC 26, UBC 40, UBC 868, UBC 890, UBC S2 for the final amplification with the 25 accessions of the lemongrass. Out of eight ISSR series primer screened three viz., ISSR 5, ISSR 7, ISSR 8 were selected for the final amplification with the 25 accessions of the lemongrass. Two primers from series

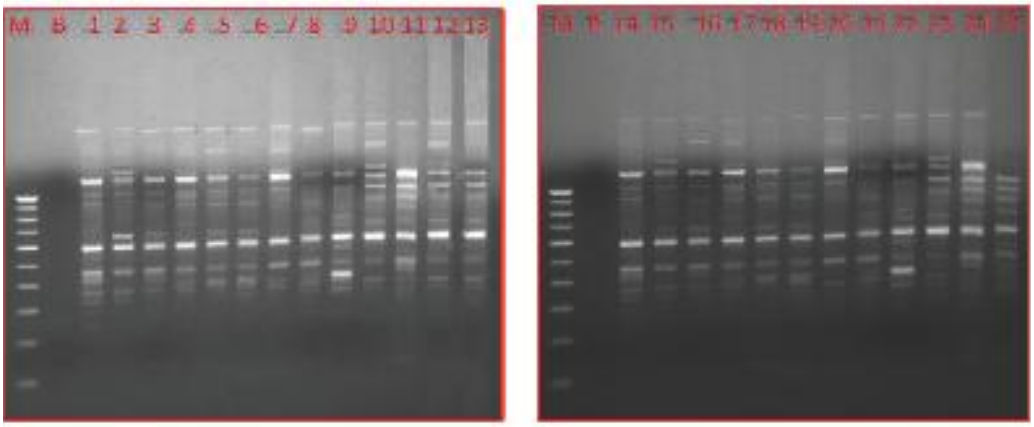


Plate 9a. Amplification with primer 14

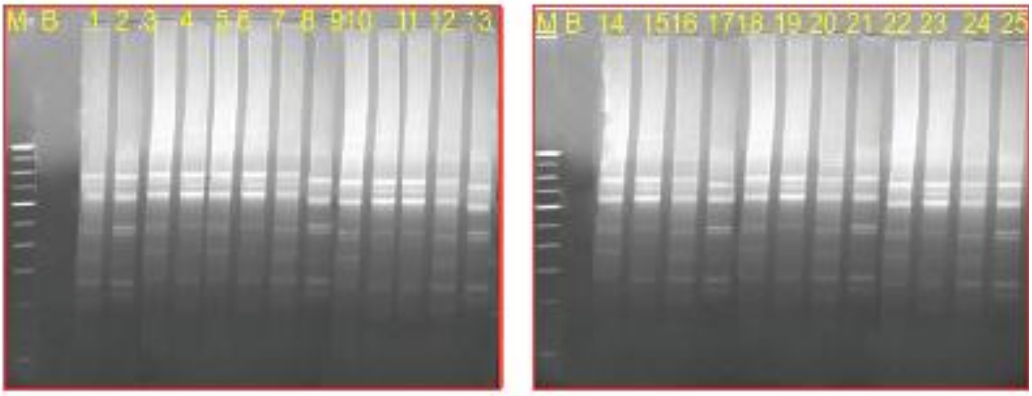


Plate 9b. Amplification with primer OPD 15

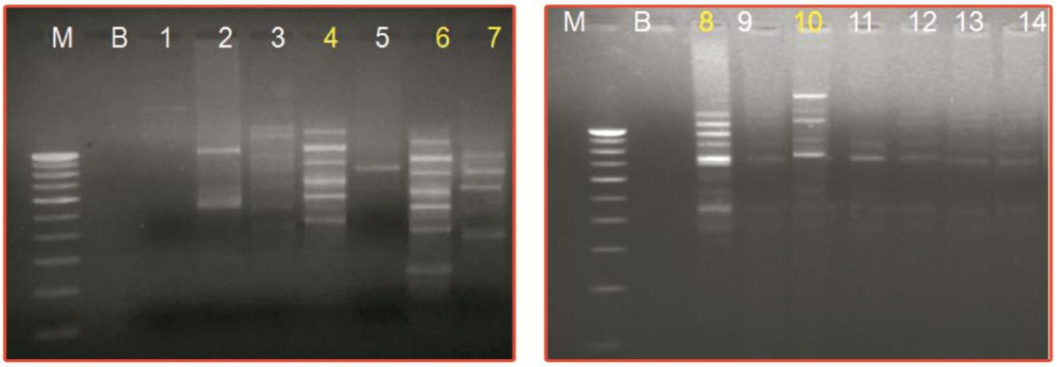
Lane-Marker 100 bp. B-blank

1-OD-1	6-OD-9	11-OD-14	16-OD-20	21-OD-28
2-0D-2	7-OD-10	12-OD-15	17-OD-21	22-OD- 29
3-OD-4	8-OD-11	13-OD-16	18-OD-23	23-OD-39
4-OD-7	9-OD-12	14-OD-17	19-OD-24	24-OD-40
5-OD-8	10-OD-13	15-OD-18	20-OD-25	25-OD-320

Plate 9. Amplification pattern of 25 accessions of East Indian Lemongrass with RAPD primers OPC 14 AND OPD 15

Table 8. Details of amplification with selected primers for RAPD assay in lemongrass

Sl No	Primer	Total no of amplicons	No of polymorphic amplicons	No of monomorphic amplicons	Polymorphism (%)
1	OPA 2	9	7	5	53
2	OPA 4	11	5	6	45
3	OPA 10	7	4	3	57
4	OPA 12	8	5	3	62
5	OPAH 3	11	8	3	72
6	OPA 27	10	5	5	50
7	OPC 1	12	6	6	50
8	OPC 2	9	7	2	77
9	OPC 14	3	7	6	53
10	OPD 15	7	4	3	57
Total		101	58	43	57
Average		10.1	5.8	4.3	



Lane: M-100bp.marker.B-Blank, 1-UBC 818, 2-UBC 820, 3-UBC 834, 4-UBC 890, 5-UBC 848, 6-UBC S2, 7-UBC 826, 8-ISSR 5, 9-ISSR 4, 10-ISSR 7, 11-ISSR 2, 12-ISSR-3, 13-ISSR-9, 14-ISSR 15

Plate 10. Screening of ISSR primers for amplification of lemongrass DNA

Table 9. Screening of 31 primers for ISSR assay in lemongrass accessions

SI. No	Primers	Amplification pattern			
		No. of Bands	Type of bands		Remarks
			Distinct	Faint	
1	UBC 811	2	1	1	----
2	UBC 813	1	1	0	----
3	UBC 814	0	0	0	----
4	UBC 815	7	5	2	Selected
5	UBC 834	4	1	3	----
6	UBC 835	4	2	2	----
7	UBC 836	3	0	3	----
8	UBC 840	10	8	2	Selected
9	UBC 844	5	1	4	----
10	UBC 890	10	8	2	Selected
11	UBC 866	7	5	2	----
12	UBC 807	17	12	5	Selected
13	UBC 843	0	0	0	----
14	UBC S2	10	8	2	Selected
15	UBC 820	3	2	1	----
16	UBC 854	7	1	6	----
17	UBC 845	1	0	1	----
18	UBC 817	0	0	0	----
19	UBC 826	6	4	2	Selected
20	UBC 818	2	0	2	----
21	ISSR 04	7	2	5	----
22	ISSR 05	11	8	3	Selected
23	ISSR 06	7	2	5	----
24	ISSR 07	7	4	3	Selected
25	ISSR 08	10	7	3	Selected
26	ISSR 09	4	1	3	----
27	ISSR 10	6	2	4	----
28	ISSR 15	3	0	3	----
29	SPS 03	2	0	2	----
30	SPS 08	2	0	2	----
31	UBC 868	13	10	3	Selected

Table 10. List of ISSR primers selected for molecular characterization of lemongrass accessions

Sl. No.	Name of Primer	Nucleotide Sequence
1	UBC 807	5'AGAGAGAGAGAGAGAGT3'
2	ISSR 15	5'TCCTCCTCCTCCTCC3'
3	UBC 826	5'ACACACACACACACACC3'
4	UBC 840	5'GAGAGAGAGAGAGAGAYT3'
5	UBC 868	5'GAAGAAGAAGAAGAAGA3'
6	UBC 890	5'VHVGTGTGTGTGTGTGT3'
7	UBC S 2	5'CTCTCTCTCGTGTGTGTG3'
8	ISSR 05	5'CTCTCTCTCTCTCTTG3'
9	ISSR 07	5'CTCTCTCTCTCTCTTG3'
10	ISSR 08	5'GAGAGAGAGAGAGAGAT3'

SPP screened the amplification but no amplification was found and hence these primers were not selected for the final amplification of the 25 accessions of the lemongrass.

ISSR analysis with the thermal settings identified gave good amplification. The amplification pattern was different corresponding to the ISSR primer used

4.6.3 Amplification with selected ISSR primers

The ISSR primer sets UBC, ISSR were selected for amplifying DNA of 25 accessions of lemongrass.

(ii) UBC 807

The agarose gel profile for the amplification pattern was observed in the 25 accessions of lemongrass with primer UBC 815 (Plate 11b). A total of 17 clear distinct loci were observed out of which 7 were polymorphic and 10 monomorphic. The percentage of polymorphism was 41 and the amplicons ranged from 350bp to 1300bp.

(ii) UBC 815

The agarose gel profile for the amplification pattern was observed in the 25 accessions of lemongrass with primer UBC 815 (Plate 11b). A total of 9 clear distinct loci were observed out of which 4 were polymorphic and 5 were monomorphic. The percentage of polymorphism was 44 and the amplicons ranged from 350bp to 1250bp.

(iii) UBC 826

The agarose gel profile for the amplification pattern was observed in the 25 accessions of lemongrass with primer UBC 826 (Plate 12a). A total of 11 clear

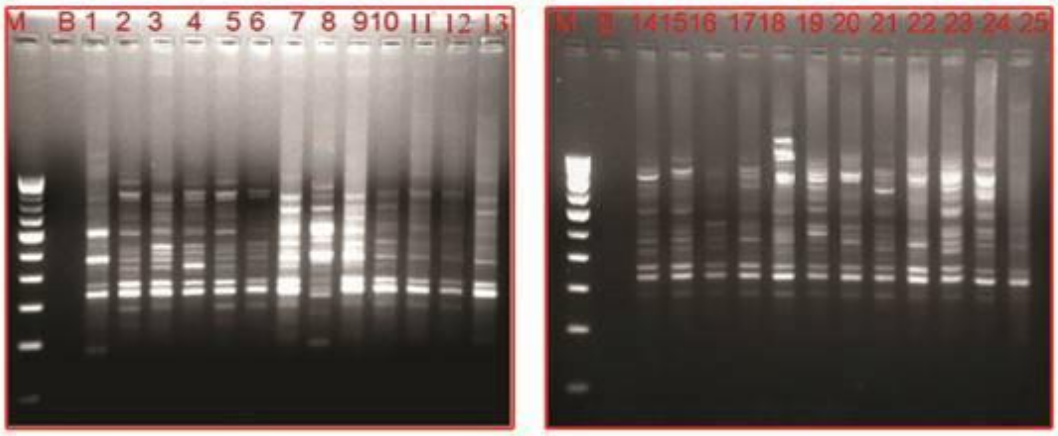


Plate 11a. Amplification with primer UBC 807

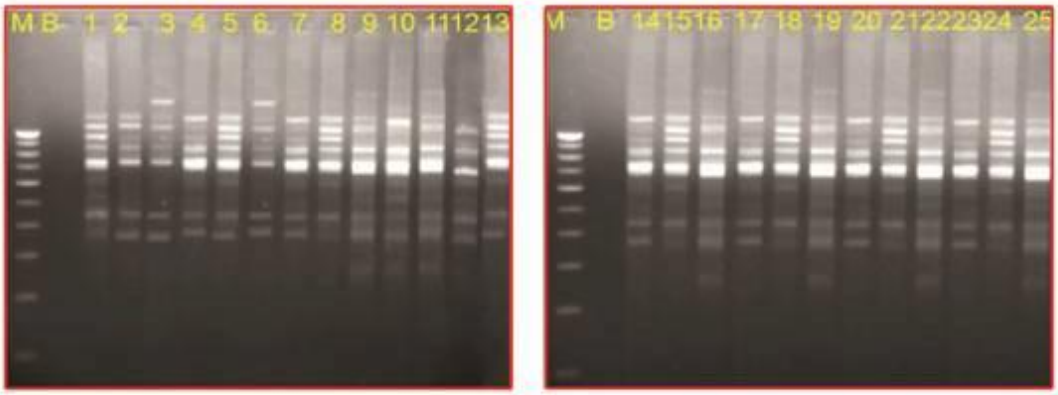


Plate 11b. Amplification with primer UBC 815

Lane-Marker 100 bp. B-blank

1-OD-1	6-OD-9	11-OD-14	16-OD-20	21-OD-28
2-0D-2	7-OD-10	12-OD-15	17-OD-21	22-OD- 29
3-OD-4	8-OD-11	13-OD-16	18-OD-23	23-OD-39
4-OD-7	9-OD-12	14-OD-17	19-OD-24	24-OD-40
5-OD-8	10-OD-13	15-OD-18	20-OD-25	25-OD-320

Plate 11. Amplification pattern of 25 accessions of East Indian lemongrass with ISSR primers UBC 807 and UBC 815

distinct loci were observed out of which 3 were polymorphic and 10 were monomorphic. The percentage of polymorphism was 27 and the amplicons ranged from 220bp to To 1600bp.

(iv) UBC 840

The agarose gel profile for the amplification pattern was observed in the 25 accessions of lemongrass with primer UBC 840 (Plate 12b). A total of 10 clear distinct loci were observed out of which 4 were polymorphic 6 were monomorphic. The percentage of polymorphism was 40 and the amplicons ranged from 210bp to 2100bp.

(v) UBC 868

The agarose gel profile for the amplification pattern was observed in the 25 accessions of lemongrass with primer UBC 868 (Plate 13a). A total of 13 clear distinct loci were observed out of which 6 were polymorphic and 7 were monomorphic. The percentage of polymorphism was 46 and the amplicons ranged from 600bp to 1800bp.

(vi) UBC 890

The agarose gel profile for the amplification pattern was observed in the 25 accessions of lemongrass with primer UBC 890 (Plate 13b). A total of 10 clear distinct loci were observed out of which 3 were polymorphic and 5 were monomorphic. The percentage of polymorphism was 44 and the amplicons ranged from 400bp to 1600bp.

(vii) UBC S2

The agarose gel profile for the amplification pattern was observed in the 25 accessions of lemongrass with primer UBC S2 (Plate 14a). A total of 9 clear distinct loci were observed out of which 4 were polymorphic and 7 were monomorphic. The percentage of polymorphism was 30 and the amplicons ranged from 400bp to 1800bp.

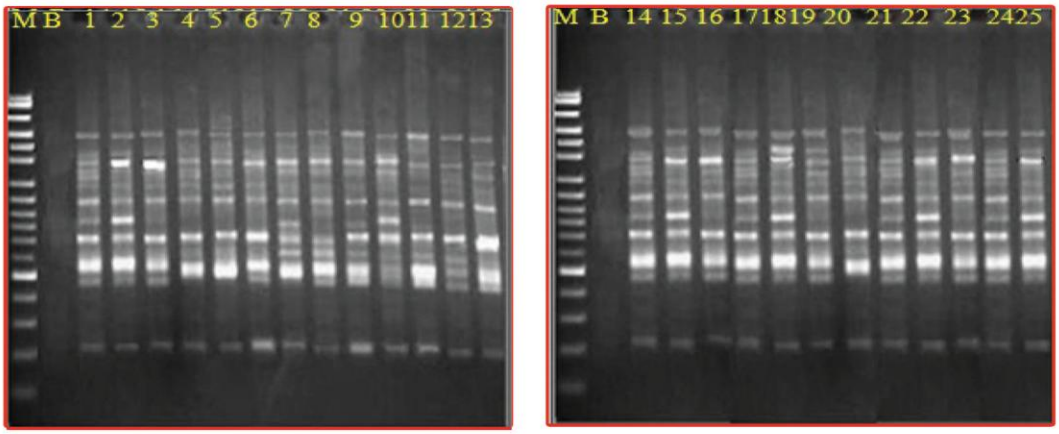


Plate 12a. Amplification with primer UBC 826

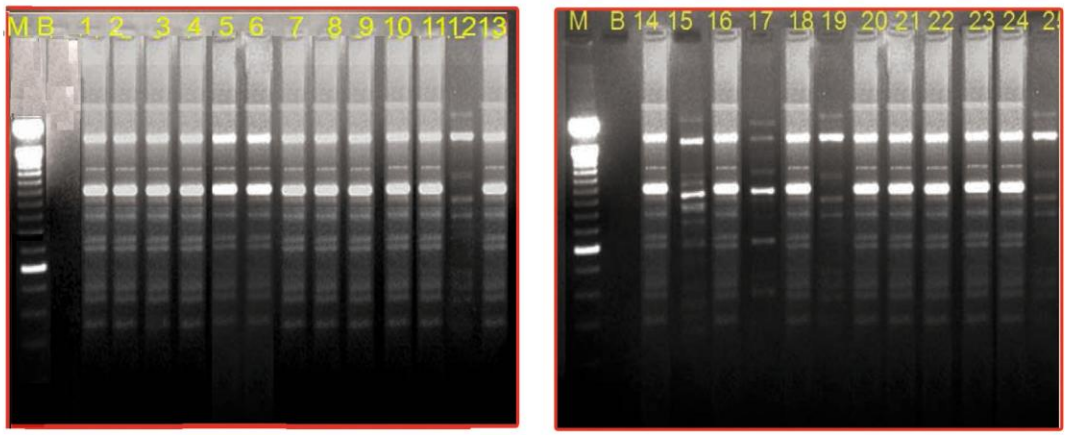


Plate 12b. Amplification with primer UBC 840

Lane-Marker 100 bp. B-blank

1-OD-1	6-OD-9	11-OD-14	16-OD-20	21-OD-28
2-0D-2	7-OD-10	12-OD-15	17-OD-21	22-OD- 29
3-OD-4	8-OD-11	13-OD-16	18-OD-23	23-OD-39
4-OD-7	9-OD-12	14-OD-17	19-OD-24	24-OD-40
5-OD-8	10-OD-13	15-OD-18	20-OD-25	25-OD-320

Plate 12. Amplification pattern of 25 accessions of East Indian lemongrass with ISSR primers UBC 826 and UBC 840

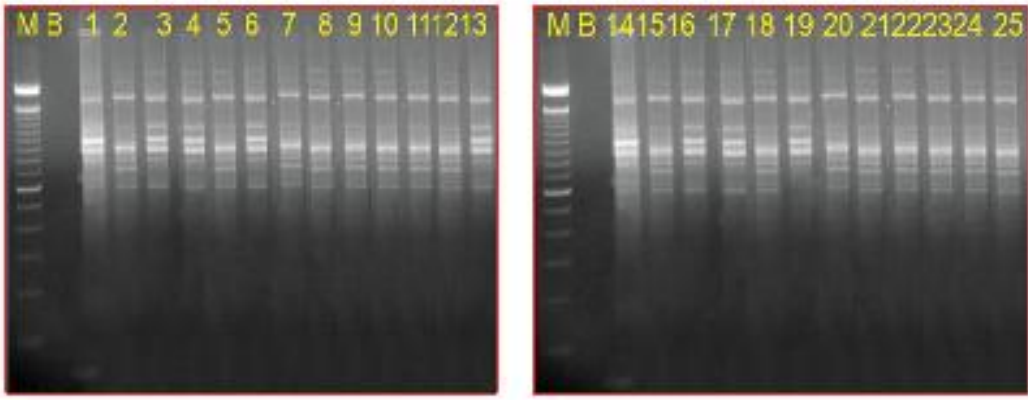


Plate 13a. Amplification with primer UBC 868

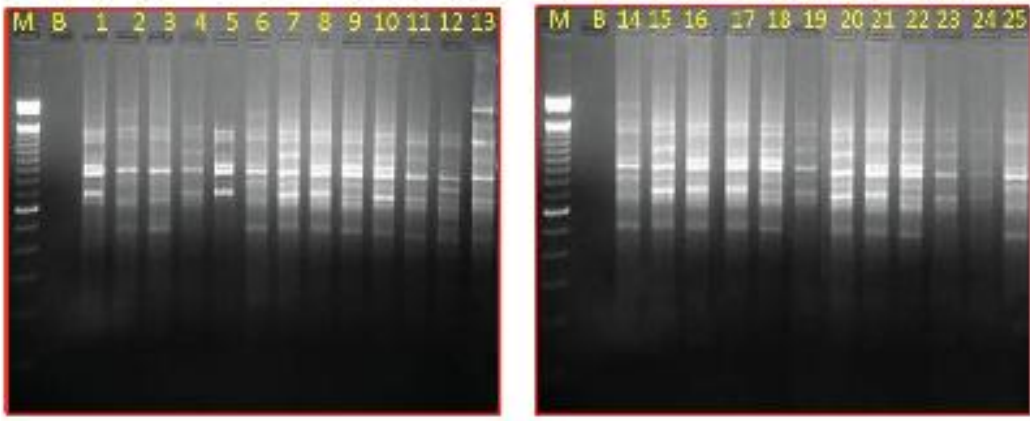


Plate 13b. Amplification with primer UBC 890

Lane-Marker 100 bp. B-blank

1-OD-1	6-OD-9	11-OD-14	16-OD-20	21-OD-28
2-0D-2	7-OD-10	12-OD-15	17-OD-21	22-OD- 29
3-OD-4	8-OD-11	13-OD-16	18-OD-23	23-OD-39
4-OD-7	9-OD-12	14-OD-17	19-OD-24	24-OD-40
5-OD-8	10-OD-13	15-OD-18	20-OD-25	25-OD-320

Plate 13. Amplification pattern of 25 accessions of East Indian lemongrass with ISSR primers UBC 868 and UBC 890

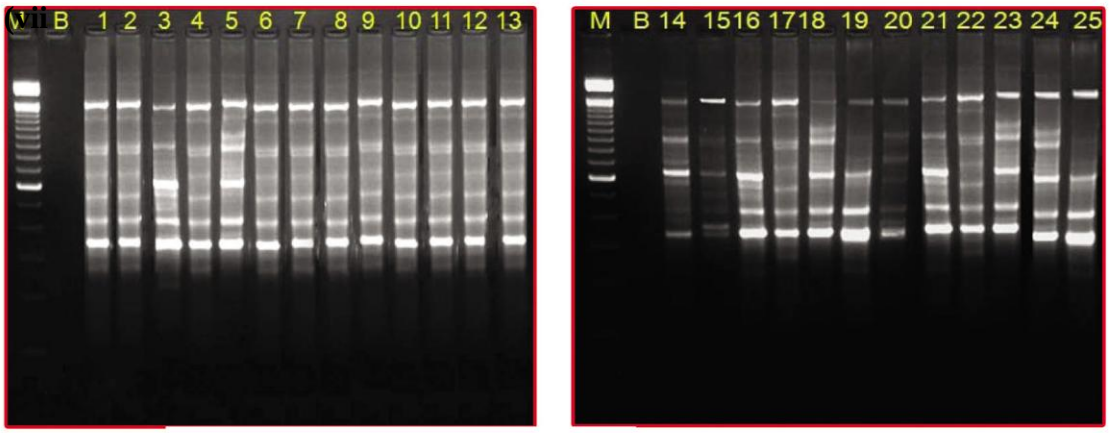


Plate 14a. Amplification with primer UBC S2

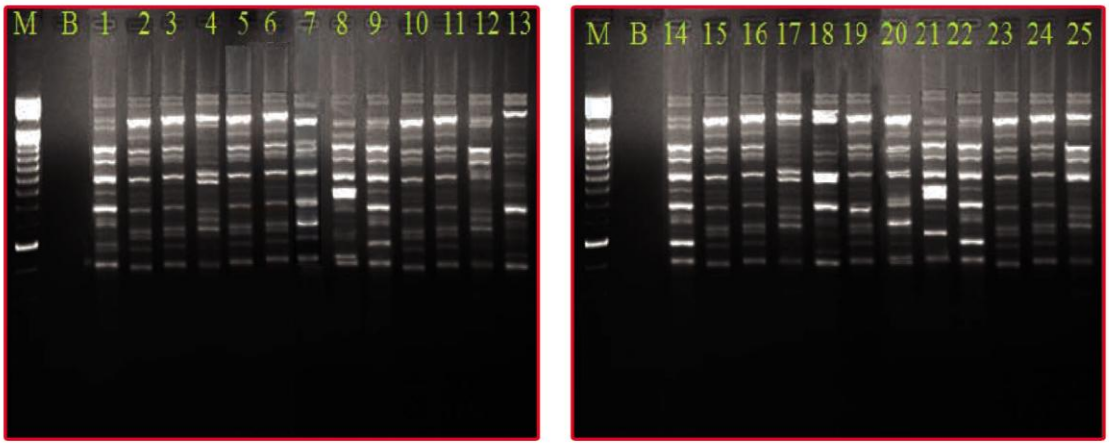


Plate 14b. Amplification with primer ISSR 5

Lane-Marker 100 bp. B-blank

1-OD-1	6-OD-9	11-OD-14	16-OD-20	21-OD-28
2-0D-2	7-OD-10	12-OD-15	17-OD-21	22-OD- 29
3-OD-4	8-OD-11	13-OD-16	18-OD-23	23-OD-39
4-OD-7	9-OD-12	14-OD-17	19-OD-24	24-OD-40
5-OD-8	10-OD-13	15-OD-18	20-OD-25	25-OD-320

Plate 14. Amplification pattern of 25 accessions of East Indian lemongrass with ISSR primers UBC S2 and ISSR 5

i) ISSR 5

The agarose gel profile for the amplification pattern was observed in 25 accessions of lemongrass with primer ISSR 5 (Plate 14b). A total of 17 clear distinct loci were observed out of which 10 were polymorphic and 7 were monomorphic.The percentage of polymorphism was 58 and the amplicons ranged from 400bp to 200bp.

(ix) ISSR 7

The agarose gel profile for the amplification pattern was observed in the 25 accessions of lemongrass with primer ISSR 7 (Plate 15a). A total of 11 clear distinct loci were observed out of which 4 were polymorphic and 7 were monomorphic. The percentage of polymorphism was 36 and the amplicons ranged from 250bp to 1200bp.

(x) ISSR 8

The agarose gel profile for the amplification pattern was observed in the 25 accessions of lemongrass with primer ISSR 8 (Plate 15b). A total of 10 clear distinct loci were observed out of which 3 were polymorphic and 7 were monomorphic. The percentage of polymorphism was 30 and the amplicons ranged from 400bp to 1300bp.

The amplification details of the selected primers are shown in Table 11.

4.4.1 Resolving power of selected RAPD and ISSR markers

The Resolving power (Rp) calculated for the random primers are depicted in Fig. 1a and Table 12. It ranged between 10.80 (OPA 10) and 19.30 (OPA 2) with an average of 16.24 for RAPD primers. ISSR primers recorded Rp values ranged between 12.4 (UBC 840) and 23.4 (UBC 807) with an average 16.27 (Fig.1b and Table 13).

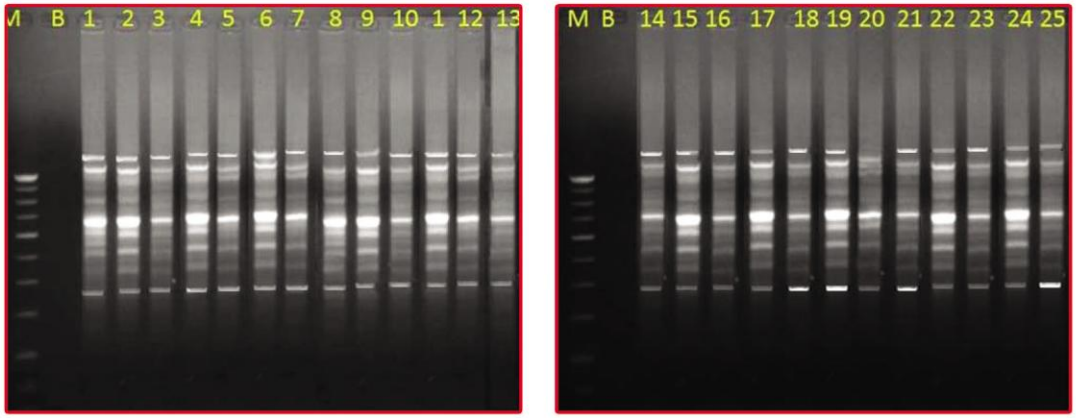


Plate 15 a. Amplification with primer ISSR 7

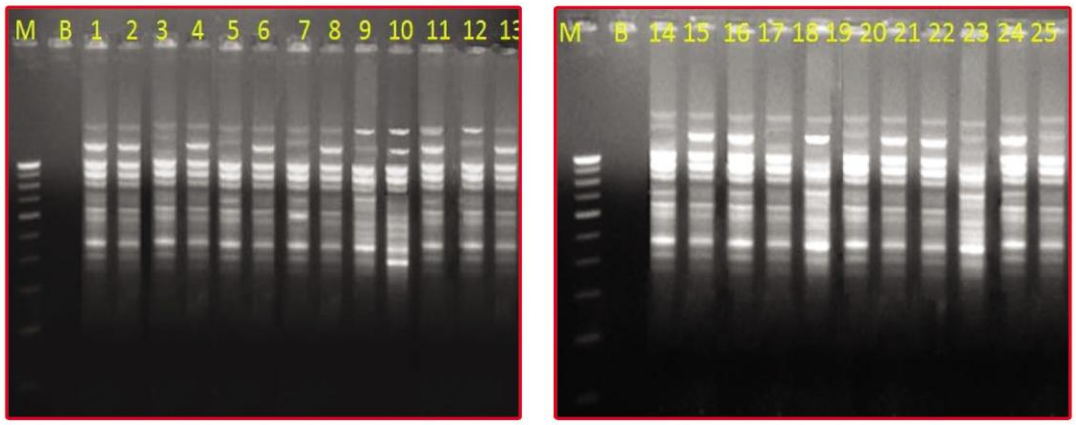


Plate 15 b. Amplification with primer ISSR 8

Lane-Marker 100 bp. B-blank

1-OD-1	6-OD-9	11-OD-14	16-OD-20	21-OD-28
2-0D-2	7-OD-10	12-OD-15	17-OD-21	22-OD- 29
3-OD-4	8-OD-11	13-OD-16	18-OD-23	23-OD-39
4-OD-7	9-OD-12	14-OD-17	19-OD-24	24-OD-40
5-OD-8	10-OD-13	15-OD-18	20-OD-25	25-OD-320

Plate 15. Amplification pattern of 25 accessions of East Indian lemongrass with ISSR primers ISSR 7 and ISSR 8

Table 11. Details of amplification with selected primers for ISSR assay in lemongrass

Sl No	Primer	Total no of amplicons	No of polymorphic amplicons	No of monomorphic amplicons	Polymorphism (%)
1	UBC 807	17	7	10	41
2	UBC 815	9	4	5	44
3	UBC 826	10	3	8	27
4	UBC 840	11	4	6	40
5	UBC 868	13	6	7	46
6	UBC 890	10	3	7	30
7	UBC S2	9	4	5	44
8	ISSR 5	17	10	7	58
9	ISSR 7	11	4	7	36
10	ISSR 8	10	3	7	30
Total		117	48	69	41
Average		11.7	4.8	6.9	

Fig. 1a. Rp value of 10 selected RAPD primers

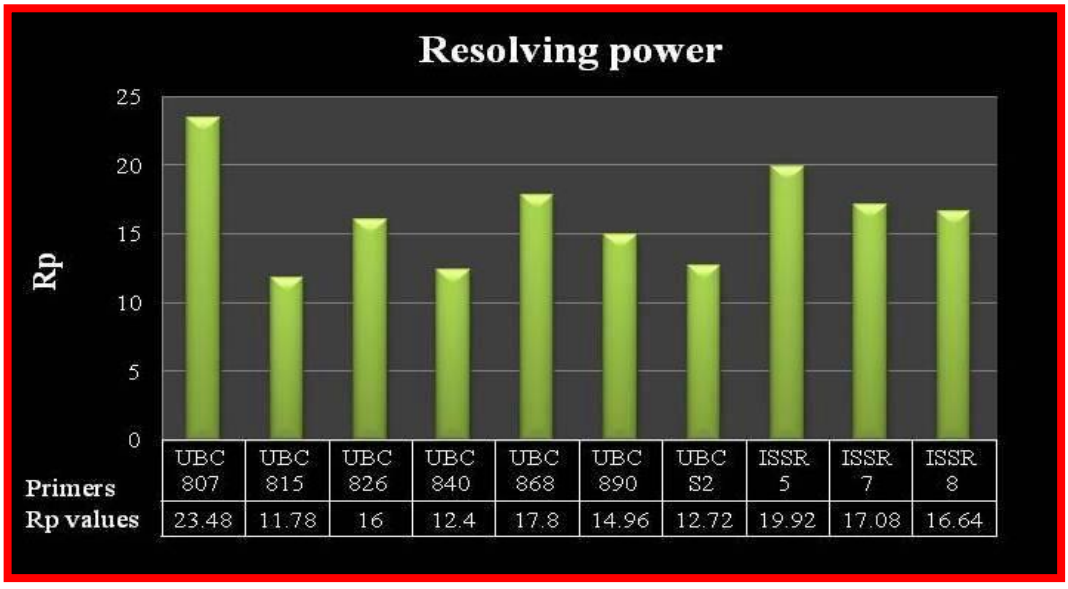


Fig.1b. Rp value of 10 selected ISSR primers

Fig. 1. Resolving power of for selected RAPD and ISSR primers

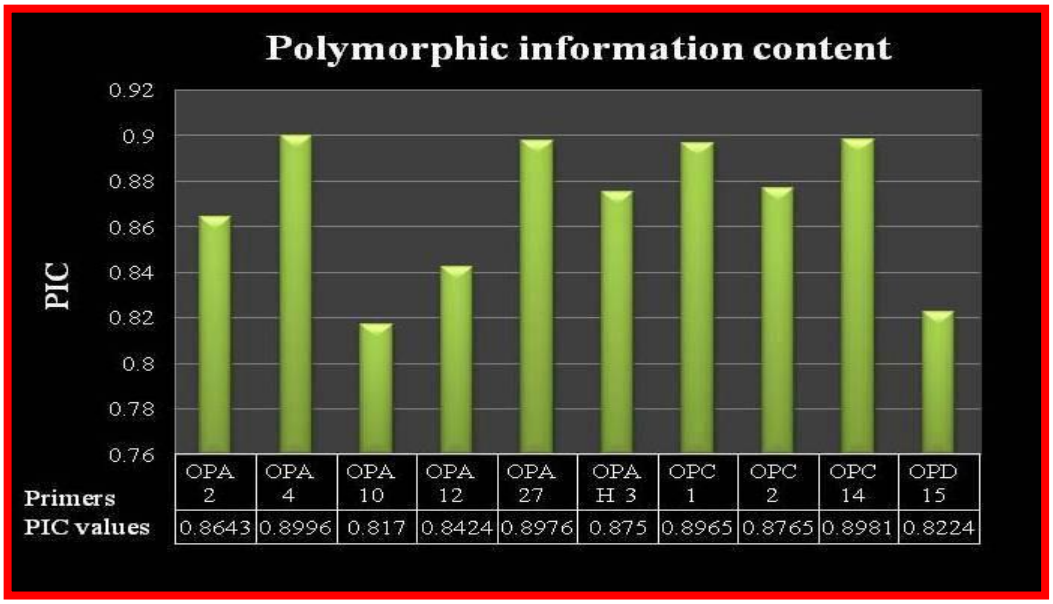


Fig. 2a. PIC value of 10 selected RAPD primers

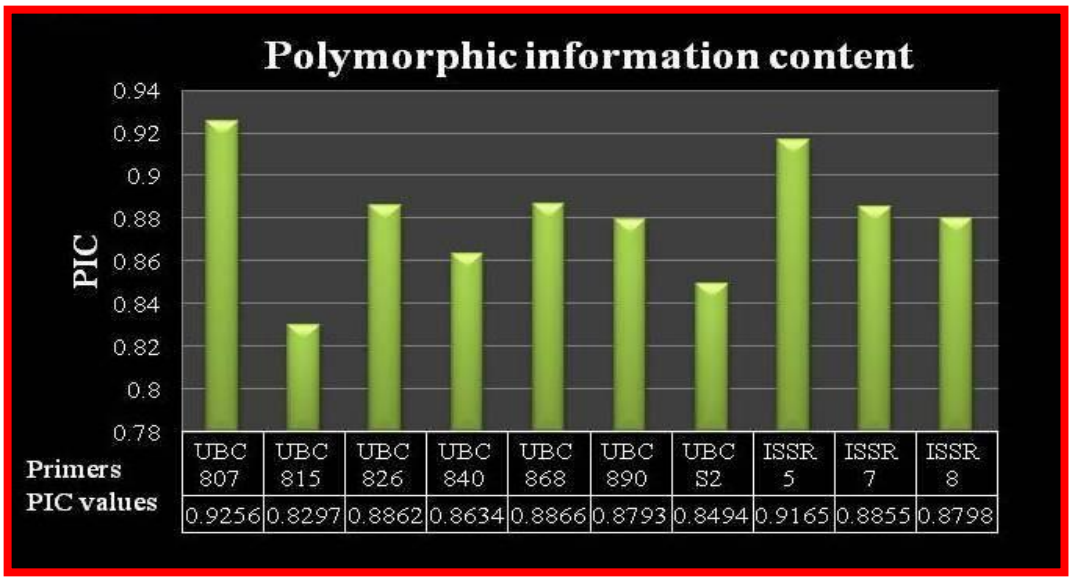


Fig. 2b. PIC value of 10 selected ISSR primers

Fig. 2. Polymorphic Information Content (PIC) for selected RAPD and ISSR primers

Table 12. Plymorphic information content (PIC) value and Resolving power (Rp) of selected RAPD primer

Sl. No.	Name of primers	PIC values	Resolving power
1	OPA 2	0.8643	14.36
2	OPA 4	0.8996	20.16
3	OPA 10	0.817	10.80
4	OPA 12	0.8424	12.88
5	OPA 27	0.8976	17.76
6	OPAH 3	0.875	15.84
7	OPC 1	0.8965	19.92
8	OPC 2	0.8765	15.6
9	OPC 14	0.8981	18.72
10	OPD 12	0.8224	11.44

Table 13. Polymorphic information content (PIC) value and Resolving power of selected ISSR primer

Sl. No.	Name of primers	PIC values	Resolving power
1	UBC 807	0.9256	23.48
2	UBC 815	0.8297	11.78
3	UBC 826	0.8862	16
4	UBC 840	0.8634	12.4
5	UBC 868	0.8866	17.8
6	UBC 890	0.8793	14.96
7	UBC S2	0.8494	12.72
8	ISSR 5	0.9165	19.92
9	ISSR 7	0.8855	17.08
10	ISSR 8	0.8798	16.64

4.4.2 Polymorphic Information Content (PIC) value for the selected RAPD and ISSR primers

The Polymorphic Information Content (PIC) value calculated for the 10 selected RAPD primers are depicted in Fig. 2a and Table 12. It ranged between 0.82 (UBC 815) to 0.90 (UBC 807) with an average of 0.82. The 10 selected ISSR primers recorded PIC values ranging from 0.82 (UBC 815) to 0.92 (UBC 807) with an average of 0.87 (Fig. 2b and Table 13).

4.5 Data analysis

4.5.1 RAPD data analysis

Band fragments were scored and each scorable band was scored as presence (1) or absence (0). The total numbers of markers observed among 25 accessions on RAPD analysis with ten decamer primers were 101. The number of scorable markers produced per primer ranged from 7 to 13. The total number of polymorphic markers and the percentage of polymorphism were 57 and 56 respectively.

Dendrogram for RAPD (Fig. 3) cluster analysis was produced according to the unweighted pair-group mean arithmetic method (UPGMA) using NTSYS pc version 2.02i software. Jaccard similarity coefficient ranged from 0.71 to 0.94. Three main clusters were form. The first cluster involved 21 accessions i.e. OD-11,OD-2, OD-28, OD-320 ,OD-9, OD-10, OD-4, OD-39, OD-24, OD-15, OD-40, OD-16, OD-21, OD-18, OD-8, OD-14, OD-20, OD-12, OD-9, OD- 25 and OD-29. In this cluster, the accessions OD-4 and OD-39 were genetically closer (94% simlilarity) having high citral content (86.40 and 83.70% respectively) was collected from the same Kollam district of Kerala state. This indicates that their origin of occurrence may be same. This first cluster formed one subcluster with accession OD-13 showed only 5 per cent variation with the first cluster.

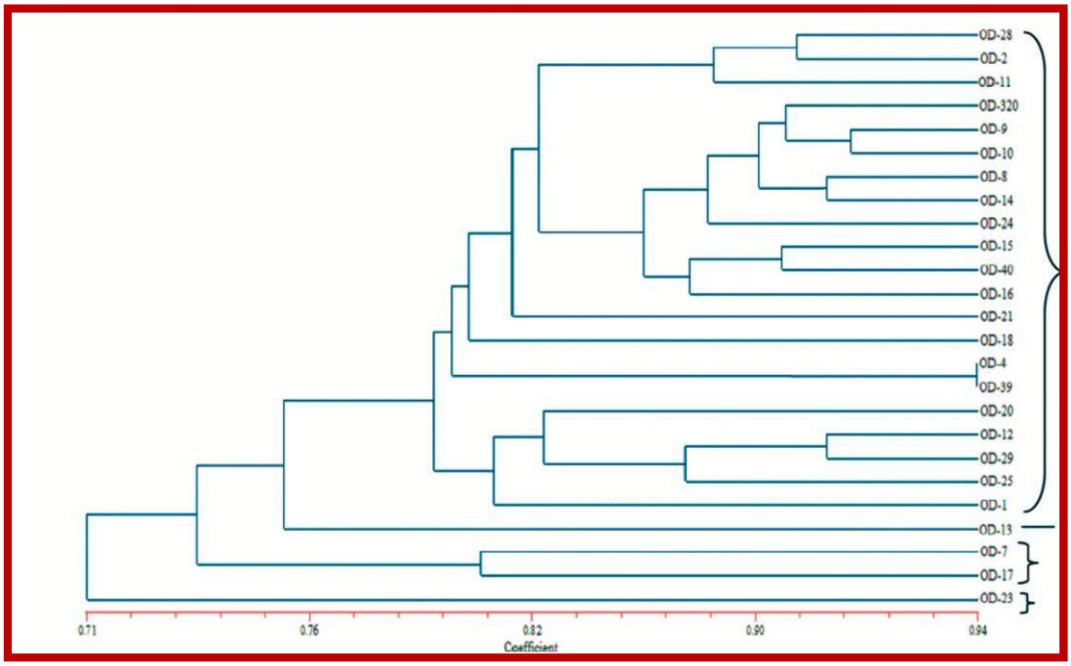


Fig. 3 Dendrogram of RAPD analysis in 25 accessions of lemongrass using NTSYS

The second cluster with 2 accessions i.e. OD-7 and OD-17 showed 81 per cent similarity to each other and has high citral content (84.40 and 83.20% respectively). The third cluster involved only one accession i.e. OD-23 and has high citral content i.e.88.50 per cent. This accession OD-23 showed more variation from rest of the accessions and was collected from Dehradun.

4.5.2 ISSR data analysis

Band fragments were scored and each scorable band was scored as presence (1) or absence (0). The total numbers of markers observed among 25 accessions on RAPD analysis with ten decamer primers were 101. The number of scorable markers produced per primer ranged from 9 to 17. The total number of polymorphic markers and the percentage of polymorphism were 48 and 41 respectively.

Dendrogram for ISSR (Fig. 4) was produced according to the unweighted pair-group mean arithmetic method (UPGMA) using NTSYS pc version 2.02i software. Jaccard similarity coefficient ranged from 0.79 to 0.97. Three main clusters were form. The first cluster involved 21 accessions i.e. OD-11,OD-2, OD-28, OD-320 ,OD-9, OD-10, OD-4, OD-39, OD-24, OD-15, OD-40, OD-16, OD-21, OD-18, OD-8, OD-14, OD-20, OD-12, OD-9, OD- 25 and OD-29. In this cluster, the accessions OD-4 and OD-39 were genetically closer (97 % similarity) having high citral content (86.40 and 83.70% respectively) was collected from the same Kollam district of Kerala state. These both accessions were similar in terms of red leaf sheath colour. This indicates that their origin of occurrence may be same. This first cluster formed one subcluster with accession OD-13 showed only 5 per cent variation with the first cluster.

The second cluster with 2 accessions i.e. OD-7 and OD-17 showed 85 per cent similarity to each other and has high cital content (84.40 and 83.20% respectively). The third cluster involved accession OD-23 and has high citral content i.e. 88.50 per cent. This accession OD-23 showed more variation from rest of the accessions and was collected from Dehradun.

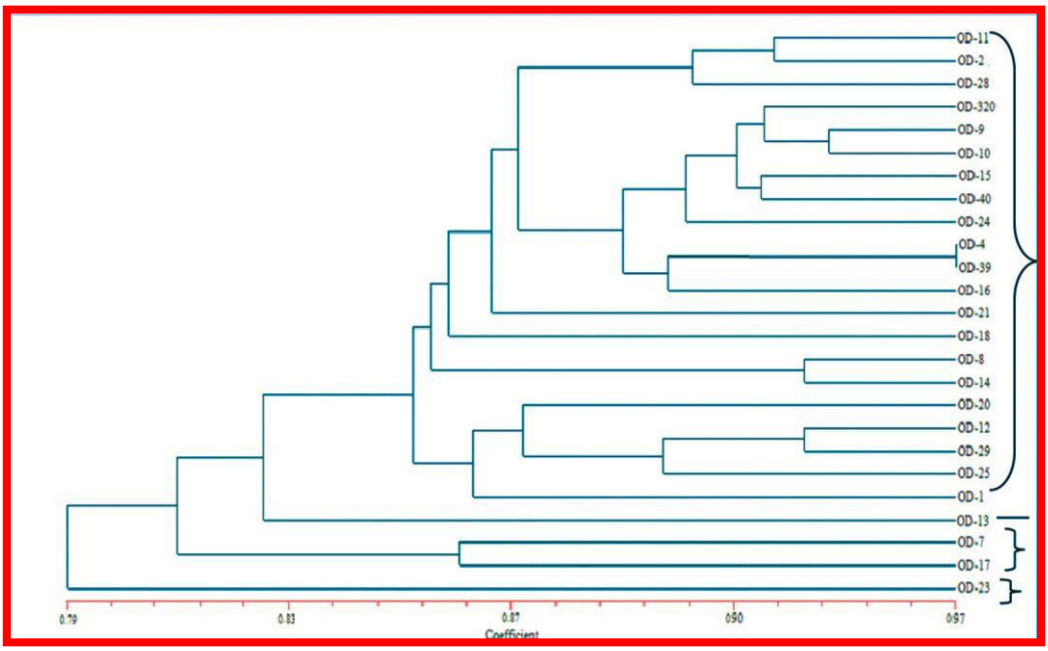


Fig. 4. Dendrogram of ISSR analysis in 25 accessions of lemongrass using NTSYS

4.5.4 Combined analysis of RAPD and ISSR data

The RAPD and ISSR data were combined; the NTSYS pc version 2.02i was used for UPGMA analysis.

A genetic similarity matrix (Table 14) as well as UPGMA dendrogram (Fig.5) was constructed based on Jaccard's similarity coefficients. The Jaccards coefficient values varied between 0.72 and 0.96. Three main clusters were formed. The first cluster involved 21 accessions OD-11, OD-2, OD-28, OD-320, OD-9, OD-10, OD-4, OD-39, OD-24, OD-15, OD-40, OD-16, OD-21, OD-18, OD-8, OD-14, OD-20, OD-12, OD-9, OD-25 and OD-29. In this cluster, the accessions OD-4 and OD-39 were genetically closer (96% similarity) having high citral content 86.40 and 83.70 per cent citral content and these accessions were collected from the same district Kollam. These both accessions were similar in terms of red leaf sheath colour. This indicates that their origin of occurrence may be same. This first cluster formed one subcluster with one accession OD-13 showed only 5 per cent variation with first cluster. In the first cluster most of the accessions showed same morphology in terms of leaf sheath colour but their biochemical content were different.

The second cluster with 2 accessions i.e. OD-7 and OD-17 showed 75 per cent similarity to each other and has high cital content (84.40 and 83.20% respectively). The third cluster involved accession OD-23 and has high citral content i.e. 88.50 per cent. This accession OD-23 showed more variation from rest of the accessions and was collected from Dehradun. The result of the dendrogram of RAPD and ISSR separately and combined gave almost similar result in dendrogram with slight difference. The genetic variation between the accessions by RAPD dendrogrm analysis was 29 per cent and by ISSR analysis was 21 per cent. The combined analysis of RAPD and ISSR gave a variation of 28 per cent.

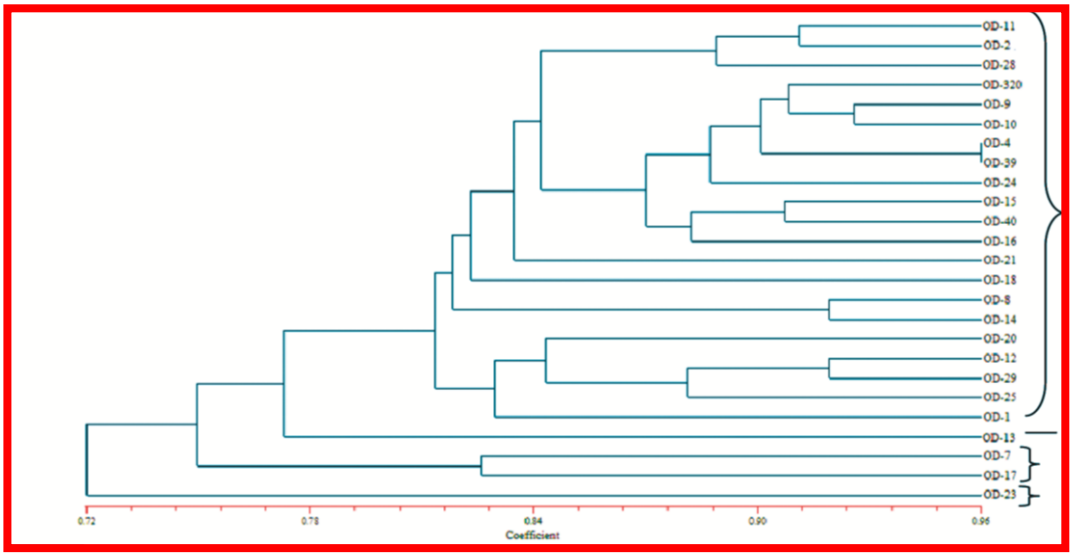


Fig. 5. Combined dendrogram of RAPD+ISSR analysis in 25 accessions of lemongrass using NTSYS

Table 14. Genetic similarity Matrix of lemon grass accessions based on the proportion of shared RAPD and ISSR fragments

	OD-1	OD-2	OD-4	OD-7	OD-8	OD-9	OD-10	OD-11	OD-12	OD-13	OD-14	OD-15	OD-16	OD-17	OD-18	OD-20	OD-21	OD-23	OD-24	OD-25	OD-28	OD-29	OD-39	OD-40	OD-320
OD-1	1.0000																								
OD-2	0.7971	1.0000																							
OD-4	0.8791	0.8889	1.0000																						
OD-7	0.8409	0.7500	0.7527	1.0000																					
OD-8	0.8764	0.8652	0.8876	0.8488	1.0000																				
OD-9	0.8387	0.8889	0.9111	0.7340	0.8462	1.0000																			
OD-10	0.8444	0.7188	0.7396	0.7753	0.7340	0.7216	1.0000																		
OD-11	0.6875	0.7889	0.7527	0.5876	0.6915	0.7717	0.6809	1.0000																	
OD-12	0.8901	0.9213	0.9011	0.7826	0.8989	0.9222	0.7684	0.7447	1.0000																
OD-13	0.8876	0.8152	0.7979	0.8391	0.7935	0.7979	0.8222	0.6667	0.8280	1.0000															
OD-14	0.8000	0.7889	0.8111	0.7907	0.9157	0.7717	0.7363	0.6923	0.8427	0.7204	1.0000														
OD-15	0.8404	0.8901	0.8913	0.7368	0.8478	0.8710	0.7604	0.7742	0.8817	0.8000	0.7935	1.0000													
OD-16	0.8617	0.8511	0.8723	0.7579	0.8495	0.8144	0.8387	0.7216	0.8632	0.8404	0.7766	0.8936	1.0000												
OD-17	0.7701	0.7791	0.7222	0.8250	0.8193	0.7416	0.6854	0.6222	0.7931	0.7471	0.7805	0.6882	0.6915	1.0000											
OD-18	0.8090	0.8182	0.8409	0.7191	0.8161	0.8202	0.7253	0.7191	0.8523	0.7667	0.7386	0.8222	0.8043	0.7059	1.0000										
OD-20	0.7766	0.8043	0.8065	0.6737	0.7826	0.7872	0.7717	0.8276	0.8571	0.7553	0.8276	0.8280	0.8105	0.6966	0.7174	1.0000									
OD-21	0.8556	0.8864	0.8261	0.7865	0.8022	0.8065	0.7912	0.6915	0.8571	0.8132	0.7283	0.7895	0.8298	0.7765	0.8161	0.7263	1.0000								
OD-23	0.7826	0.8315	0.8132	0.7333	0.7889	0.7935	0.7978	0.7333	0.8652	0.7802	0.8140	0.7957	0.7979	0.7011	0.7816	0.8506	0.7889	1.0000							
OD-24	0.8667	0.8764	0.8989	0.7778	0.8750	0.8571	0.7826	0.7204	0.9101	0.8242	0.8182	0.8387	0.8602	0.7882	0.8488	0.8333	0.8539	0.8409	1.0000						
OD-25	0.8462	0.8556	0.8571	0.7582	0.8333	0.8172	0.8022	0.7391	0.8681	0.8043	0.8391	0.8387	0.8602	0.6703	0.8068	0.8333	0.8333	0.8621	0.8242	1.0000					
OD-28	0.9080	0.8333	0.8556	0.8161	0.8315	0.8352	0.8000	0.6809	0.8667	0.8851	0.7556	0.8172	0.8000	0.7647	0.8256	0.7717	0.8111	0.8182	0.8851	0.8022	1.0000				
OD-29	0.8132	0.8427	0.8242	0.7253	0.8000	0.8043	0.7889	0.7253	0.8556	0.7912	0.8256	0.8065	0.7895	0.7126	0.8140	0.8409	0.8202	0.9157	0.8736	0.8953	0.8506	1.0000			
OD-39	0.8817	0.8913	0.9556	0.7766	0.8901	0.8925	0.7629	0.7396	0.9032	0.8404	0.8152	0.8737	0.9149	0.7283	0.8444	0.7917	0.8495	0.8172	0.8804	0.8804	0.8387	0.8280	1.0000		
OD-40	0.8333	0.8617	0.8438	0.7684	0.8404	0.8438	0.7917	0.7500	0.9140	0.8125	0.8065	0.9043	0.8660	0.7582	0.8152	0.8602	0.8211	0.8478	0.8913	0.8125	0.8495	0.8387	0.8469	1.0000	
OD-320	0.7979	0.8667	0.8280	0.6598	0.7849	0.8280	0.7368	0.8090	0.8387	0.7216	0.8090	0.7917	0.7576	0.7000	0.7204	0.8043	0.8043	0.8315	0.7957	0.8352	0.7742	0.8427	0.8316	0.8229	1.0000

Discussion

5. DISCUSSION

East Indian lemongrass (*Cymbopogan flexuosus* Stapf.) is a perennial aromatic grass (family: Poaceae) which grows in many parts of tropical and sub-tropical South East Asia and Africa. In India, it is cultivated along Western Ghats (Maharashtra and Kerala), Karnataka and Tamil Nadu states besides foot-hills of Arunachal Pradesh and Sikkim. It was introduced in India about a century back and is now commercially cultivated in these states. Most of the species of lemongrass are native to South Asia, South-East Asia, Australia, India, Sri Lanka, Burma and Thailand. The lemongrass cultivated in Kerala is also known as Malabar or Cochin grass. Lemongrass oil is rich in active ingredients such as citral (75-85%) and possesses a lemon like odour which is widely used as herbal tea and in pharmaceutical and perfume industry. Lemongrass oil is popularly known as "Cochin oil" in the world trade (Joy *et al.,* 2001).

Cymbopogons are highly heterozygous plants due to its cross pollination. Thus profound genetic variations are prevalent in the species (Sreenath and Jagadishchandra, 1991) that always demands better germplasm management and conservation practices. Knowledge of the genetic relationships among different accessions is essential for developing appropriate strategies for breeding, germplasm management and utilization of genetic resources (Paterson *et al*., 1991). Many previous workers reported the genetic diversity among different taxa of *Cymbopogon* species based on oil constituents as well as molecular analysis (Sangwan *et al*., 2001a). The Aromatic and Medicinal plants Research Station (AMPRS), Odakkali has maintained around 400 accessions of lemongrass, which is collected from different parts of Kerala and other states and has been morphologically and biochemically characterized and no molecular data has been developed so far. Therefore 25 accessions of East Indian lemongrass diverse in oil and citral content are selected for the molecular characterization from the AMPRS, Odakkali.

Assessment of genetic diversity is one of the key steps in any plant breeding programmes. Therefore by molecular characterization of these accessions, it is possible to study the level of diversity existing within a species and to establish an index of genetic similarities among different populations and varieties. Molecular markers have a number of perceived advantages over the morphological characters assessment of genetic diversity. Most of the morphological characters are sensitive to environmental conditions and growth stages where as molecular markers are insensitive to such factors and are abundantly present. Marker assisted selection (MAS) has been the mainstay of any modern breeding.

The selected accessions of the lemongrass were diverse in essential oil and citral content. Data were collected from AMPRS, Odakkali. The genetic diversity on the basis of the oil content is an important tool for further studies.

5.1 Molecular characterization

The cultivated lemongrass appears to be an ideal candidate for characterization using molecular markers and variability available in the lemongrass is very high. The assessment of intra and inter-varietal variation using the present system of characterization based on morphological descriptor alone is not sufficient. The reasons are it is time consuming as both vegetative and reproductive characters have to be recorded, is influenced by environment and also, the existing morphological keys are not sufficient to identify good accessions. Molecular markers in conjugation with morphological markers will be the ideal method to characterize any accession or variety.

5.2.1 DNA isolation

In the present study pale green tender leaves (1^{st} or 2^{nd} leaf from tip) were used for DNA extraction from 25 accessions of lemongrass. The protocol suggested by Rogers and Bendich (1994) modified by using 2X CTAB extraction buffer yielded good quality DNA. Many workers (Adhikari *et al*., 2013;

Bhattacharya *et al*., 2010a; Sangwan *et al*., 2001a, 2003; Khanuja *et al*., 2005) followed CTAB method in *Cymbopogon* genus for isolation of genomic DNA. The electrophoresed DNA showed distinct bands without shearing. Detergents like CTAB and PVP remove polyphenols and polysaccharides, while the β-mercaptoethanol reduces oxidation (Cruz *et al*., 1997). PVP has been used to remove polyphenols from leaf tissues in several protocols (Lodhi *et al.*, 1994; Doyle and Doyle, 1987; Aljanabi *et al.*, 1999; Kim *et al.*, 1997). PVP or PVPP (polyvinylpoly-pyrrolidone) efficiently forms complex hydrogen bonds with polyphenolic compounds and alkaloids, which can be separated from DNA by centrifugation (Maliyakal, 1992). The major problem encountered in the isolation and purification of high molecular weight DNA from plants species is the protein contamination. To avoid this problem, samples were collected early morning. DNA was isolated 2-3 times up to getting good quality.

5.2.2. Purification and Quantification of DNA

The quality of the DNA was tested by subjected to agarose gel electrophoresis and Nanodrop spectrophotometer analysis. DNA was visualized on 0.8 per cent agarose gel under UV light by ethidium bromide staining. The DNA extracted samples showed high quantity with respect to of high molecular weight with little smearing and a low amount of RNA at bottom of gel profile. RNA was removed from samples by RNase A treatment. Use of RNase A was reported by several workers (Raval *et al*., 1998; Wettasingf and Peffley, 1998; Gallego and Martinez, 1996). The RNase treated DNA sample on electrophoresis showed a high molecular weight DNA, which formed a single band just below the well. This indicted that the DNA under test was of good quality.

In Nanodrop spectrophotometer analysis, the ratio of optical density at 260 and 280 nm was worked out to test the quality. The absorbance ratio was calculated as OD at 260/280, for the various samples ratio between 1.8 and 2.0 were considered to be of high quality and same ratio recoded in the entire sample.

5.3 Molecular Marker Analysis

Molecular markers have been proved to be a fundamental and reliable tool for fingerprinting varieties, establishing the fidelity of progenies, germplasm characterization, diversity and population structure analysis etc. Molecular markers provide an important technology for evaluating levels and patterns of genetic diversity. Molecular markers successfully developed during the last two decades have largely overcome the problems that are associated with phenotype-based marker. Molecular markers are independent of developmental stages of the crop and are not influenced by the varying environmental conditions. Hence it is preferred for diversity analysis than traditional morphological markers (Mohapatra, 2007; Krishna and Singh, 2007; Spooner *et al*., 2005).

Most of the molecular marker are developed by PCR (Polymerase Chain Reaction) technology and amplifies unique regions on the genomic DNA based on the primers designed for DNA amplification. In the present study two PCR based markers systems *viz*., RAPD and ISSR were utilized for molecular characterization of East Indian lemongrass on the basis of essential oil content.

5.3.1 RAPD analysis

The RAPD technique was developed by Williams *et al*. (1990) and the technique relies on the differential enzymatic amplification of DNA fragments using PCR with arbitrary decamer primers. RAPD marker has been reported in Cymbopogon species (Adhikari *et al*., 2013; Bhattacharya *et al*., 2010a; Sangwan *et al*., 2001a, 2003; Nayak *et al*., 2003; Khanuja *et al*., 2002, 2005; Kumar *et al*., 2007, 2009; Ganjewala., 2008) for molecular characterization. In RAPD markers, polymorphism results from the changes in the sequence of the primer binding site. Usually, RAPD markers are dominant in nature (Waugh and Powell, 1992) because polymorphisms are detected as the presence or absence of bands. RAPD patterns are very sensitive to slight changes in amplification conditions giving problems of reproducibility and necessity for extensive standardization to obtain reproducible results (Penner *et al*., 1993). Technical problems associated with

application of RAPD technique in the field of genetic variation research have been reported by many workers (Lynch and Milligan, 1994; Rajput *et al*., 2006). Use of high quality DNA is shown to be a key factor in obtaining reproducible RAPDs bands (Penner *et al*., 1993). In the present study, the use of high quality DNA helped in getting reproducible bands using the standardized conditions for the thermal cycler.

However the advantages of RAPD include simplicity, rapidity, requirement for only a small quantity of DNA and ability to generate polymorphisms.

5.3.1.1 DNA amplification conditions for RAPD

The thermal cycler used for the PCR also influence the amplification pattern (Babu, 2000). The amplification conditions standardized in the present study were suited to Mastercycler personal thermalcycler from Eppendorf (USA). Also, the annealing temperature was identified as the most critical aspect with respect to number of amplified fragments and reproducibility of result. Cipriani *et al.* (1996) and Erlich *et al*. (1991) suggested 37ºC as the best, and hence only time was varied for this step. However in present study annealing temperature of the primer ranged between 32ºC to 34ºC were used for further analysis. For RAPD PCR programmes 39 cycles were applied which gave good amplification.

The amplification pattern produced by the different combinations of the ingredients of the reaction mixture indicated that the most important factor affecting the specificity and yield of amplification were concetration of $MgCl_2$ in the buffer as well as concentration and type of *Taq* DNA polymerase enzyme. In the present investigation, the enzyme used was *Taq* DNA polymerase supplied by Genei, Bangalore. The molarities of primers as well as dNTPs were also found to affect the intensity and number of amplifications. All reactions were performed in 20 µl final volume.

5.3.1.2. Primer Screening for RAPD

Out of 35 random decamer primers screened, 10 primers were selected on the basis of screening test considering the number, intensity and consistency of bands, were found useful for screening the lemongrass accessions. Bhattacharya *et al*.(2010a) screened 40 RAPD primers and out of which 10 primers were selected for further studies on *Cymbopogon.* All these 10 primers produced polymorphic bands between 25 accessions of lemongrass. The RAPD markers generated using the selected primers were visualised by electrophoresis, in a 1.8 per cent agarose gel stained with ethidium bromide.

5.3.1.3. Characterization using RAPD

In the present study the total numbers of markers observed among 25 accessions on RAPD analysis with 10 decamer primers were 101. Molecular characterization of *Cymbopogon* species showed more polymorphism with RAPD primers (Agarwal *et al.*, 2013). The number of scorable markers produced per primer ranged from 7 to 13. Kumar *et al.* (2009) reported that RAPD producing 119 fragments and the scorable marker were in the range of 6 to 9. The total number of polymorphic markers and the percentage of polymorphism was 58 and 57 respectively. In the present study RAPD markers showed more polymorphism (57%). The characterization of *Cymbopogon* genus using molecular markers, RAPD markers showed more polymorphism (Bhattacharya *et al*., 2010a; Kumar *et al*., 2007; sangwan *et al.*, 2003; Nayak *et al*., 2003).

In the present study RAPD marker was found to be more efficient in estimation of molecular diversity of different accessions of *Cymbopogon flexuosus* than ISSR marker in terms of average number of polymorphic bands The Polymorphic Information Content (PIC) was originally introduced into human genetics by Bostein *et al*. (1980) and it is refers to the value of the detecting polymorphism within a population depend upon number of detectable allele and their frequency. In the present study the PIC value calculated for the 10 selected RAPD primers varied from 0.82 (UBC 815) to 0.90 (UBC 807) with an

average of 0.82.Whereas Bhattacharya *et al*. (2010a) in his work reported PIC value for RAPD primers was in the range of 0-0.24. Adhikari *et al*. (2013) on the *Cymbopogon flexuosus* reported PIC value for the RAPD primers with an average of 0.42.

The resolving Power (Rp) provides indication of the ability of the primers to distinguish between cultivar (Prevost and wilkson, 1990). In the present study Rp value was ranging between 10.80 (OPA 10) and 19.30 (OPA 2) with an average of 16.24. Bhattacharya *et al*. (2010a) reported Rp value for the RAPD primers with an average of 7.87.

The dendrogram was constructed using UPGMA method. In the present study the jaccard similarity coefficient for RAPD marker in lemongrass accessions ranged from 0.79 to 0.97. Twenty nine per cent variation was observed among the 25 accessions. Santos *et al.* (2010) also reported the range of similarity coefficient between the range of 0.72 to 0.98 in sorghum with 28 per cent variation. Kumar *et al.* (2009) reported similarity coefficient between the range of 0.64 to 0.87 in *Cymbopogon* with 38 per cent variation.

5.3.2 ISSR analysis

The marker system called ISSR (Inter Simple Sequence Repeats) is a PCR based method that assesses variation in the numerous microsatellite regions dispersed throughout the genome. In this technique reported by Zietkiewicz *et al*. (1994), primers based on microsatellites are utilized to amplify inter simple sequence repeat sequences in the DNA. When the primer successfully locates two microsatellite regions within an amplifiable distance away on the two strands of the template DNA, the PCR reaction will generate a band of a particular molecular weight for that locus representing the intervening stretch of DNA between the microsatellites. The method uses a single oligonucleotide primer composed of 4 to 10 tri or di nucleotide repeats and ending with 3’- or 5’- anchor sequence. ISSR is a multilocus molecular technique based on PCR that identifies insertions and deletions in DNA. It is highly sensitive, highly reproducible,

provides Mendelian segregation, and has been successfully applied in genetic and evolutionary studies of many species, including Poaceae family members like wheat (Sozen *et al.,* 2010), maize (Liet *et al.,* 2011), lemongrass (Bhattacharya *et al.,* 2009), sugarcane (kalwade *et al.,* 2012), burmudagrass (Farsani *et al.,* 2012), sorghum (Turki *et al*., 2011) etc.

5.3.2.2. DNA amplification conditions for ISSR

Optimization of concentration of different reagents and temperature regimes used in polymerase chain reaction (PCR) is necessary for conducting any genome characterization study. However, annealing temperature and concentration of $MgCl_2$ are important parameters for any marker system, which need optimization. The genomic DNA of lemongrass accessions was used to optimize the PCR conditions. Amount of template DNA strongly influences the outcome of the reaction. More than 30 ng/20 µl give the premium amplification (Henegariu *et al*., 1997), also in the present study; 40 ng/20 µl was found optimum. Optimization of $MgCl_2$ is an important factor for precise amplification. In these experimental studies, 2 mM of $MgCl_2$ was found optimum in 20 µl final reaction volume. The Mg ions binds tightly to the phosphate sugar backbone of nucleic acids and variation in the $MgCl_2$ concentration has strong effects on nucleic acid interactions (Ely *et al*., 1998). Variations in $MgCl_2$ concentration below 4 mM can improve performance of PCR by affecting specificity (higher concentration lowers the specificity, lower concentration raise specificity) (Blanchard *et al*., 1993). Moreover, concentration of dNTPs in reaction mixture is also strongly correlated to the Mg ions concentration due to the interaction between mononucleotides and the Mg^{2+}. A higher concentration of Mg^{2+} allows amplification with a higher concentration of dNTPs that is not seen at lower Mg^{2+} concentrations (Blanchard *et al*., 1993).

In Polymerase Chain Reaction, 1 unit of *Taq* DNA polymerase is normally used in 20µl final volume. Higher *Taq* DNA polymerase concentration (above 4 units/25 µl) can generate nonspecific products and may reduce the yield of the

desired product (Saiki, 1989). However, in the present study, 1 unit/20 µl reaction was used to amplify the loci without non-specific products. Annealing temperature is one of the most important parameters that need adjustment in the PCR. The normal range of annealing temperature for ISSR is 52-58°C. Optimal annealing temperature (T_a) of primers was determined by testing in the range of ±3°C from the theoretical annealing temperature. For ISSR PCR programmes 35 cycles were found to give good amplification.

5.3.2.3. Primer Screening for ISSR

In the present study, 31 primers were used to screen the 25 accessions of the lemongrass. The amplification pattern of selected primers were visualised by electrophoresis, in a high resolution 2 per cent agarose gel stained with ethidium bromide. The most of the primers were fom UBC series. Tuvesson *et al*. (2002) also used the UBC series of ISSR primers in his work on *L. perenne.* Yu *et al.* (2002) also used same series of primes for sugarcane.

5.3.3. Characterization using ISSR

In the present study the total numbers of markers observed among 25 accessions on ISSR analysis with 10 selected primers were 117 with average of 11.7 bands per primer. The number of scorable markers produced per primer ranged from 7 to 13. The percentage of polymorphism was 49 with an average of 4.9 bands per primer. Yang *et al.* (2012) in his work in bamboo reported 155 fragments of ISSR primers with 10 selected ISSR primes and the percentage of polymorphism was 98. Rizkalla *et al.* (2012) observed 75 bands with 10 selected ISSR primers in wheat and the percentage of polymorphism was 54.66. Agarwal *et al.* (2012) observed 164 bands with 13 selected ISSR primes with 75 per cent of polymorphism in his work on wheat.

ISSR primers found to be good in PIC and Rp value. The PIC value of ISSR primes were ranged between 0.82 and 0.92. Liet *et al*. (2011) reported the

range of PIC value between 0.10 and 0.39. This report correlates with the reports on sorghum by Turki *et al*. (2011).

The dendrogram was constructed using UPGMA method. In the present study the jaccard similarity coefficient for ISSR marker in lemongrass accessions ranged from 0.79 to 0.97. Twenty one per cent variation was observed among the 25 accessios of lemongrass. Liet *et al*. (2011) reported similarity coefficient in the range of 0.52 to 0.90 in maize. Devarumath *et al.* (2012) reported similarity coefficient in the range of 0.23 to 0.95 in sugarcane.

5.4 Combined analysis of RAPD and ISSR data

In the present study RAPD marker proved to be efficient to bring out more variability in the selected accessions, while ISSR marker comparatively showing less polymorphism.This correlates with previous reports by Bhattacharya *et al* (2010a) in the *Cymbopogon* genus. The resolving power and Polymorphic information content value of the ISSR marker was high. This is due to more number of bands produced by the ISSR primers. The PIC and Rp value of the selected RAPD and ISSR primers were ranging from 8.1 to 9.2 and 10 to 23 respectively suggesting that the selected primer was more informative.

The dendrogram was constructed using UPGMA (Unweighed pair group method) method. So many previous workers (Bhattacharya *et al.*, 2010a; Adhikari *et al*., 2013; Sangwan *et al*., 2003, 2005; Kumar *et al.*, 2005) used same method for cluster analysis. In the present study combined cluster analysis of RAPD and ISSR marker showed 28 per cent variation among the 25 accessions. There was slight difference in combined and separate cluster analysis of RAPD and ISSR markers. In the previous report of Bhattacharya *et al*. (2010a) on *Cymbopogon winterianus* showed about a 64 per cent variation. Adhikari *et al.* (2013) reported 53 per cent variations in his work on lemongrass. The genetic similarity matrix was calculated using Jaccards Coefficient ranged between 0.72 to 0.96 per cent. So many previous workers used same coefficient for calculating

genetic similarity matrix. Ganjewala *et al.* (2008) in his work on *Cymbopogon* reported similarity coefficient in the range of 0 to 1.

The first cluster grouped together with 21 accessions of lemongrass i.e. OD-11, OD-2, OD-28, OD-320, OD-9, OD-10, OD-4, OD-39, OD-24, OD-15, OD-40, OD-16, OD-21, OD-18, OD-8, OD-14, OD-20, OD-12, OD-29, OD-25 and OD-29. Citral content ranged from 71 to 86 per cent. In this cluster, the accessions OD-4 and OD-39 are genetically similar which yielded 86.40 to 83.70 per cent citral content and these accessions are from the Kollam district of Kerala. This indicates that accessions of same geographic origin are genetically and biochemically more or less similar. These two accessions are also similar in terms of same leaf sheath colour. The accession OD-13 showed only 5 per cent variation from the accessions in the first cluster. The second cluster having 2 accessions i.e. OD-7 and OD-17 showed 75 per cent similarity to each other with 84 per cent citral content. The third cluster having accession OD-23 showed more variation from rest of the accessions was collected from Deradun unlike other accessions which were collected from Kerala. The percentage of citral content was also very high (88.5. %) in this accession.

The characterization of lemongrass accession using molecular markers and analysis of data using NTSYS software showed correlation to biochemical and morphological data. The accessions OD-4 and OD-39 which was collected from Kollam district of Kerala showed 96% similarity in morphological biochemical and molecular analysis. Most of the accessions collected from Kerala showed only 28% genetic variation and there was no correlation between morphological and biochemical data. Because in the first cluster 21 accessions were grouped together with same morphology in terms of leaf sheath colour but their oil content and citral content are different from each other.

Molecular characterization of remaining germplasms to identify divergent and genetically distinct accessions with useful traits like high oil yield, high citral content etc are required to use them for crop improvement programmes, and for

plant varietal protection and to avoid duplication of accessions in germplasm. Genetic variation between different cultivated *Cymbopogon* species needs to be studied. Characterization of accessions within and between the species can be exploited for germplasm management though molecular breeding programme. DNA markers associated with oil quality i.e. citral content must be identified to utilize the information in marker assisted selection. Possibility of using other advanced PCR based markers also can be exploited in further studies.

Summary

6. SUMMARY

The study entitled "Molecular characterization of East Indian lemongrass (*Cymbopogon flexuosus* Stapf) germplasm accessions" was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara during the period 2011-2013 using molecular markers, RAPD and ISSR. Twenty five lemongrass accessions were collected from Aromatic and Medicinal Plant Research Station (AMPRS) Odakkali and maintained at CPBMB, College of horticulture, Vellanikkara were used for the study.

The salient findings of the study are stated below:

1. Morphological and biochemical data of 25 accessions were collected for 25 accessions from AMPRS, odakkali..

2. The protocol for genomic DNA isolation was standardized. The protocol suggested by Rogers and Bendich (1994) with slight modification was found to be the most appropriate for isolation of DNA from lemongrass. The RNA contamination was completely removed through RNase treatment.

3. The quality and quantity of DNA was analysed by NanoDrop^{R} ND-1000 spectrophometer. The absorbance ratio ranged from 1.82-1.95, which indicated good quality DNA and the recovery was high. This DNA was suitable for RAPD and ISSR analysis.

4. Protocol for RAPD and ISSR assay in lemongrass were standardized with the various quantities of DNA, PCR mixtures and conditions for DNA amplification.

5. Thirty five RAPD primers and 31 ISSR primer pairs were screened for the ability to amplify DNA fragments. Out of these 10 RAPD primers and 10 ISSR primer pairs were selected based on the number of bands and nature of amplicons.

6. The Resolving power (Rp) of the RAPD and ISSR primers was calculated and the values ranged between 10.80 to 20.16 and 11.78 to 23.48 for RAPD and ISSR primers respectively. The RAPD primer OPA 4 and ISSR primer UBC 807 showed high resolving power.

7. The Polymorphic Information Content (PIC) for RAPD and ISSR primer were calculated and ranged between 0.81 to 0.89 and 0.82 to 0.92 respectively indicating the suitability of primers to detect polymorphism. The RAPD primer OPA 4 and ISSR primer UBC 807 showed highest PIC values.

8. Clear RAPD and ISSR bands were scored for presence (1) and absence (0).

9. The scored data based on RAPD and ISSR banding was used to construct a dendrogram using the NTSYS pc (version 2.02i) software. Three main clusters were found. Similarity coefficient ranged from 0.72 to 0.96. The highest similarity (96 per cent) was observed between lemongrass accessions OD-4 and OD-39. The accession OD-23 was the most distinct one from other accessions with 28per cent variability.

10. The information generated will be useful in designing future breeding programmes involving the selected accessions.

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Annexures

ANNEXURE I

Details of laboratory equipment items used for the study

High speed refrigerated centrifuge	: Kubota, Japan
Horizontal electrophoresis system	: BIO-RAD
Thermal cycler	: Master cycler personal, Eppendorf
Gel documentation system	: BIO- RAD, USA UVP (Inc. CA)
Nanodrop^{R} ND-1000 Spectrophotometer	: Nanodrop^{R} Technologies Inc. USA

ANNEXURE II

Reagent required for DNA isolation

Reagents:

1. 2x CTAB extraction buffer (100 ml)

CTAB (2%)	:	2 g
(Cetyl trimethyl ammonium bromide)		
Tris HCl (100mM)	:	1.21 g
EDTA (20mM)	:	0.745 g
NACl (1.4M)	:	8.18 g
PVP (1%)	:	1.0 g

Adjusted the pH to 8 and made up final volume up to 100 ml.

2. CTAB (10 per cent, 100 ml)

CTAB (10%)	:	10 g
NACl (0.7M)	:	4.09 g

3. Chloroform- isoamylalcohol (24:1)

To chloroform (24 parts), isoamyl alcohol (1 part) was added and mixed properly.

4. Chilled isopropanol

Isopropanol was stored in refrigerator at -20ºC and was used for the study.

5. Ethanol (70 per cent)

To the 70 parts of absolute ethanol (100 per cent), 30 parts of sterile distilled water was added to make 70 per cent ethanol.

6. TE buffer (pH8, 100 ml)

Tris HCl (10 mM)	:	0.1576 g
EDTA (1 mM)	:	0.0372 g

The solution was prepared with sterile distilled water, autoclaved and stored at room temperature.

ANNEXURE III

Composition of Buffers and Dyes used for Gel electrophoresis

1. TAE Buffer 50X

Tris base	:	242 g
Glacial acetic acid	:	57.1 ml
0.5M EDTA (pH 8.0)	:	100 ml

2. Loading Dye (6X)

0.25% bromophenol blue

0.25% xylene cyanol

30% glycerol in water

3. Ethidium bromide

The dye was prepared as a stock solution of 10 mg/L in water and was stored at room temperature in a dark bottle.

MOLECULAR CHARACTERIZATION OF EAST INDIAN LEMONGRASS (*Cymbopogon flexuosus* Stapf.) GERMPLASM ACCESSIONS

By

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ABSTRACT OF THE THESIS

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ABSTRACT

East Indian lemongrass (*Cymbopogon flexuosus* Stapf.) is commonly known as Malabar or Cochin grass. It is an aromatic crop cultivated for its commercial essential oil and oil exported from India is known as "Cochin oil" in the world trade. The major component of this oil is citral which is widely used in pharmaceutical and perfumery industry. The Aromatic Medicinal plants Research Station, Odakkali has maintained around 400 accessions of East Indian lemongrass. Among them twenty five elite accessions good in oil and citral content were selected for the molecular characterization. The study elucidated genetic variation among the selected accessions.

The present investigations on "Molecular characterization of East Indian lemongrass (*Cymbopogon flexuosus* Stapf.) germplasm accessions" were carried out at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Kerala Agricultural University during the period 2011-2013. The objective of the investigation was to characterize twenty five accessions of East Indian lemongrass using molecular markers, Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeats (ISSR).

For molecular characterization, good quality genomic DNA was isolated from East Indian lemongrass accessions using CTAB method (Rogers and Benedich, 1994). Thirty five RAPD and thirty one ISSR primers were screened for amplification of genomic DNA and ten RAPD and ten ISSR primers were selected for further analysis based on the amplification pattern. RAPD analysis using selected primers produced 101 amplicons, 58 were polymorphic with an average of 5.8 polymorphic bands/primer and polymorphism percentage was 57. In ISSR, selected primers produced 117 amplicons, 48 were polymorphic with average of 4.8 polymorphic bands/primer and a polymorphism percentage was 41.

The RAPD markers found to be more effective to bring out variability among the accessions. The RAPD primer OPC 2 gave maximum polymorphism

i.e. 77 per cent. In ISSR, the primer ISSR 5 gave maximum polymorphism i.e. 58 per cent. The polymorphic information content and resolving power of ISSR primers was higher than RAPD primers.

The dendrogram generated based on RAPD and ISSR profiles grouped twenty five accessions of East Indian lemongrass into 3 main clusters. The first cluster grouped 21 accessions of lemongrass together. This cluster includes the accessions OD-11, OD-2, OD-28, OD-320, OD-9, OD-10, OD-4, OD-39, OD-24, OD-15, OD-40, OD-16, OD-21, OD-18, OD-8, OD-14, OD-20, OD-12, OD-29, OD-25 and OD-29. In this cluster, the accessions OD-4 and OD-39 were genetically similar which yielded 86.40 to 83.70 per cent citral content and these accessions were collected form Kollam district of Kerala state. This indicates that accessions of same geographic origin are genetically and biochemically similar. These 2 accessions were similar in terms of same leaf sheath colour. This first cluster formed one subcluster with accession OD-13 showed only 5 per cent variation with first cluster. The second cluster involved 2 accessions i.e. OD-7 and OD-17 showed 75 per cent similarity with each other with 84.40 and 83.20 percent citral content. The third cluster involved only one accession i.e. OD-23 with high citral content i.e. 88.50 percent was collected from Dehradun. The information generated will be useful in designing future breeding programmes involving the selected accessions.