DNA FINGERPRINTING OF RELEASED VARIETIES AND SELECTED SUPERIOR SOMACLONES OF GINGER (*Zingiber officinale* Rosc.)

By

PUJAITA GHOSH
(2011 - 11 - 108)

THESIS

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CENTRE FOR PLANT BIOTECHNOLOGY AND MOLECULAR BIOLOGY
COLLEGE OF HORTICULTURE
VELLANIKKARA, THRISSUR - 680 656
KERALA, INDIA

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DECLARATION

I, hereby declare that this thesis entitled **"DNA fingerprinting of released varieties and selected superior somaclones in ginger (*Zingiber officinale* Rosc.)"** is a bonafide record of research work done by me during the course of research and that it has not been previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other University or Society.

Vellanikkara
Date: 7/8/2013

Pujaita Ghosh
(2011-11-108)

CERTIFICATE

Certified that this thesis entitled **"DNA fingerprinting of released varieties and selected superior somaclones in ginger (*Zingiber officinale* Rosc.)"** is a bonafide record of research work done independently by **Ms. Pujaita Ghosh (2011-11-108)** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

Vellanikkara
Date: 7/8/2013

Dr. M.R. Shylaja
(Chairman, Advisory Committee)
Professor
Centre for Plant Biotechnology and Molecular Biology
College of Horticulture
Kerala Agricultural University
Vellanikkara,
Thrissur- 680656.

CERTIFICATE

We, the undersigned members of the Advisory Committee of **Ms. Pujaita Ghosh (2011-11-108)**, a candidate for the degree of **Master of Science in Agriculture** with major field in **Plant Biotechnology,** agree that the thesis entitled **"DNA fingerprinting of released varieties and selected superior somaclones in ginger (*Zingiber officinale* Rosc.)"** may be submitted by **Ms. Pujaita Ghosh** in partial fulfillment of the requirements for the degree.

31/8/2013

Dr M.R. Shylaja
(Chairman, Advisory Committee)
Professor
Centre for Plant Biotechnology and
Molecular Biology
College of Horticulture
Kerala Agricultural University, Thrissur.

31/8/2013

Dr. P.A.Valsala,
(Member, Advisory committee)
Professor and Head
Centre for Plant Biotechnology and
Molecular Biology,
College of Horticulture
Vellanikkara

Dr. P. A. Nazeem
(Member, Advisory committee)
Professor and Coordinator (DIC)
Centre for Plant Biotechnology and
Molecular Biology,
College of Horticulture
Vellanikkara

R. Keshavachandran

Dr. R. Keshavachandran
(Member, Advisory committee)
Professor and Coordinator (DIC) Retd.
Centre for Plant Biotechnology and
Molecular Biology,
College of Horticulture,
Vellanikkara

Dr. E. V. Nybe
(Member, Advisory committee)
Professor and Head,
Department of Plantation Crops and Spices
and Director (Acad. and PG studies)
College of Horticulture,
Vellanikkara

[Illegible]
Dr N. Senthil
Professor & Head Plant Molecular Biology and Biotechnology CPMB
TNAU Coimbatore 641003

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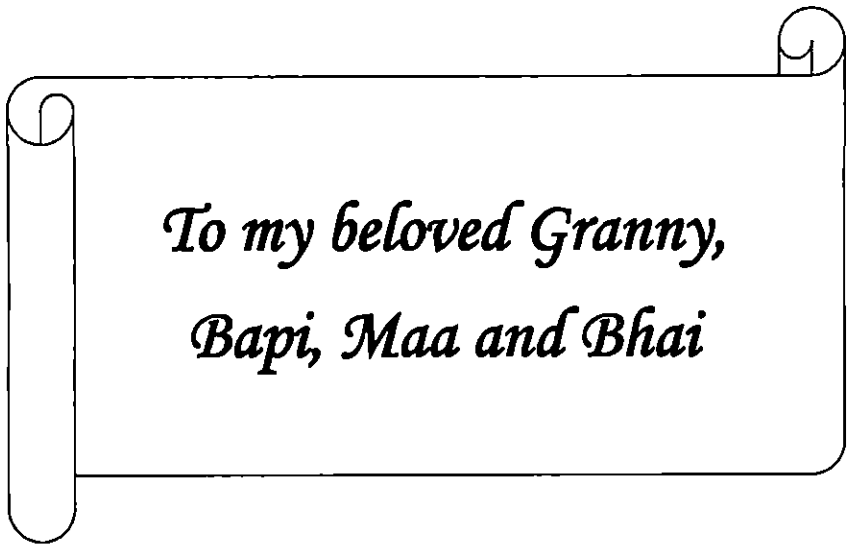
To my beloved Granny,
Bapi, Maa and Bhai

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ABBREVIATIONS

A	Adenine
AFLP	Amplified Fragment Length Polymorphism
AP-PCR	Arbitrarily Primed Polymerase Chain Reaction
Bp	Base pair
β	Beta
C	Cytosine
CPBMB	Centre for Plant Biotechnology and Molecular biology
CTAB	Cetyl Trimethyl Ammonium Bromide
^{0}C	Degree Celsius
cm	Centimeter
DNA	Deoxyribo Nucleic Acid
dNTPs	Deoxyribo Nucleoside Triphosphate
EDTA	Ethylene Diamine TetraAcetic Acid
g	Gram
G	Guanine
HCl	Hydrochloric Acid
ISSR	Inter Simple Sequence Repeat
kb	Kilo basepairs
KAU	Kerala Agricultural University
l	Litre
M	Molar
MAS	Marker-Assisted Selection
mg	Milligram
mA	Milli Ampere
µl	Micro litre
µM	Micro molar
ml	Millilitre

Mg	Magnesium
$MgCl_2$	Magnesium Chloride
mM	Milli mole
µg	Microgram
µl	Microlitre
NaCl	Sodium Chloride
ng/µl	Nanogram per micro litre
OD	Optical Density
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
pH	Hydrogen ion concentration
%	Percentage
PVP	Poly vinyl pyrrolidone
RAPD	Random Amplified Polymorphic DNA
RNA	Ribo Nucleic acid
RNase	Ribonuclease
RFLP	Restriction Fragment Length Polymorphism
rpm	Revolutions per minute
SSR	Simple Sequence Repeat
T	Thymine
TAE	Tris Acetate EDTA
TE	Tris EDTA
U	Unit
UV	Ultra violet
V	Volts
v/v	Volume by Volume
w/v	Weight by Volume

Introduction

1. INTRODUCTION

India, 'the spice bowl of the world,' enjoys a unique position in the production and export of ginger (*Zingiber officinale* Rosc.) from time immemorial. The crop is much valued as a spice, medicine and vegetable. Ginger is used as carminative, stimulant, anti-inflammatory, antiemetic and aphrodisiac in ayurvedic system of medicine. The ginger plant has a long history of cultivation believed to be originated in South East Asia. In India, ginger is cultivated in an area of 1,55,063 ha with a production of 755618 t as per 2011-2012 statistics (www.indianspices.com). During the year 2012-2013, 19,850 t of ginger was exported, fetching a foreign exchange of Rs. 16863.10 lakhs (www.indianspices.com). Cochin and Calicut ginger produced in Kerala are famous in the international market owing to its high intrinsic quality.

Breeding of ginger through selection and hybridization is seriously handicapped by lack of variability, absence of natural seed set and exclusive vegetative propagation. As natural variability stands limited, broadening the genetic base through mutagenesis or tissue culture techniques pave way for exploitation of induced variability for isolation of plant types with desirable traits.

The induction of variability through tissue culture techniques was initiated at College of Horticulture, Kerala Agricultural University from 1996 onwards and *in vitro* pollination (Valsala, 1994) and *in vitro* induction of variation and evaluation of somaclones (Paul, 2006) were attempted. Somaclonal variation was induced in the two cultivars viz., Maran and Rio-de-Janeiro through various modes of regeneration and through *in vitro* mutagenesis. The somaclones were subjected to single plant evaluation and they were further evaluated for yield, quality and reaction to pests and diseases (Paul and Shylaja, 2009; Paul *et al.*, 2009; Paul *et al.*, 2011; Paul and Shylaja, 2012). After conducting Initial Evaluation Trials, Advanced Varietal Trials, On Farm Evaluation Trials and Multi

Locational Trials, the two superior somaclones, were released as varieties under the names "Athira" and "Karthika" during 2010 (Shylaja *et al.*, 2010) and somaclones viz., 292R, 478R, 88R and B3 were selected as superior clones after the evaluation. The varieties Athira, Karthika and somaclone B3 were derived from the cultivar Maran and the clones 292R, 478R and 88R were from the cultivar Rio-de-Janeiro.

DNA fingerprinting is an efficient tool for genotype identification, assessing genetic diversity and protecting plant varieties. DNA fingerprinting was also attempted in ginger by several workers (Nayak *et al.*, 2005; Harisaranraj *et al.*, 2009; Palai and Rout, 2007; Prem *et al.*, 2008 and Sajeev *et al.*, 2011). Central Seed Committee established under the Seed Act, 1996 stipulates the necessity of DNA fingerprint data for the varieties released or proposed to be released.

For the newly released ginger varieties and selected superior somaclones in pipeline for release, no fingerprint data are available. The proposed study will help to make fingerprint data for the two newly released varieties and four selected superior somaclones of ginger. The specific fingerprint data will serve as a mark for identifying the varieties / clones and could be utilized for registration and documentation of varieties, settling IPR issues and to avoid biopiracy. Comparison of fingerprint data of varieties / somaclones with source parent cultivars will help to assess the extent of somaclonal variability in the varieties or clones and the extent of variability from the source cultivars.

In this context, the present study was taken up at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Kerala Agricultural University, Thrissur to characterize two released varieties and four selected superior somaclones using molecular markers and to develop a DNA fingerprint specific to each variety / somaclone.

Review of literature

2. REVIEW OF LITERATURE

The investigations on "DNA fingerprinting of released varieties and selected superior somaclones of ginger (*Zingiber officinale* Rosc.)" were carried out at Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Kerala Agricultural University, Thrissur, with the objective of characterising two released varieties, four selected superior somaclones and source parent cultivars of ginger using molecular markers and to develop a DNA fingerprint specific to each variety / somaclone.

The research works included mainly the morphological and molecular characterization of two released varieties of KAU (Athira and Karthika), four superior selected somaclones (292R, 478R, 88R and B3) and source parent cultivars (Maran and Rio-de-Janeiro). Two molecular marker systems viz. RAPD and ISSR were used to develop DNA fingerprints of the varieties / somaclones.

The relevant literatures on various aspects of the investigation are reviewed in this chapter under various heads.

2.1 Origin and History

Ginger has originated in South- East Asia, probably India or China (Bailey, 1949 and Purseglove, 1972). The Sanskrit name 'Singabera' has given rise to Greek '*Zingiberi*' and later the generic name *Zingiber*. The Indo-Malayan region is a centre of diversity of cultivated ginger. Traders brought it to Mediterranean region from India during first century A.D. (Burkill, 1966). During the 13^{th} century A.D. the Arabs took ginger to east Africa from India. Later, Portuguese spread it to West Africa for commercial cultivation during 16^{th} century.

2.2 Botany

Purseglove (1975) described *Zingiber officinale* as herbaceous perennial. The plant is erect with fibrous roots, aerial shoots (pseudostem) with leaves and underground stem (rhizome). As the ginger grows further, several fleshy roots are also produced from rhizomes. During initial growth, apical bud of rhizome bit grows out as mother tiller and as it grows, the mother rhizome develops at the base. Aerial shoots have many narrow leaves with long and narrow leaf sheaths. Inflorescence is a spike, which springs directly from the rhizomes, oval or conical in shape. Flowers are trimerous, bisexual, irregular and yellowish with dark purplish spots. Fruits, which are seldom produced, are an oblong capsule. Leaves, petiole, rhizomes and roots contain oil cells. The somatic chromosome number in *Zingiber* spp. is 2n= 22.

2.3 Genetic Diversity

The extent of genetic diversity in ginger is low, and the diversity is attributed to arise mainly from differences in agro-ecological conditions. Little attention has been paid to their characterization except for sporadic reports on germplasm evaluation and genetics of some quality trait (Nayak et al., 2005). Moreover, continuous domestication of preferred genotypes has eroded the genetic base of these plants, thereby making them equally susceptible to all major bacterial and fungal pathogens. This marvelous spice and medicinal plant is constrained severely by the absence of seed set and the breeder is left with the alternative of clonal selection or induced mutations with all its uncertainties and limitations. Experiments were conducted to standardize *in vitro* pollination and fertilization techniques in Zingiberaceous spice crops (Valsala, 1994). Through *in vitro* placental pollination, seed set could be achieved in ginger but the germination of seeds was yet to be refined. Under these circumstances, biotechnology opened up many potential avenues such as tissue culture, exploitation of somalonal variation, *in vitro* mutagenesis and selection,

molecular fingerprinting, recombinant DNA technology and genetic modification through transgenics for creating disease-resistant and high yielding lines.

Largest variability of ginger is reported from China, followed by India. This crop is known under cultivation and use in India and China for the last 2000 years or even more. Because of prevalence of sterility and asexual method of propagation, only local cultivars are in vogue. Most of the ginger cultivars are named in vernacular or after a particular trait of the cultivar or a place. Ravindran *et al.*, (1994) opined that geographical spread accompanied by genetic differentiation into locally adapted populations caused by mutations could be the main factor responsible for variations encountered in ginger. In India the diversity is more in Kerala and North East region.

2.5 Markers to identify genotypes

2.5.1 Morphological markers

According to Bhat *et al.*, (2010) morphological markers are those traits that are scored visually, or morphological markers are those genetic markers whose inheritance can be followed with the naked eye. The traits included in this group are plant height, disease response, photoperiod, sensitivity, shape or color of flowers, fruits or seeds etc. Although they are generally scored quickly, simply and without laboratory equipments, such markers are not put too much use because of the following reasons: genotypes can be ascertained generally at whole plant or plant organ level and frequently the mature plant is used. Such markers frequently cause major alternations in the phenotype which is undesirable in breeding programs. Dominant, recessive interactions frequently prevent distinguishing all genotypes associated with morphological traits. Morphological markers mask the effect of linked minor gene, making it nearly impossible to identify desirable linkages for

selection and are limited in number, influenced by environment and also specific stage of the analysis.

In an evaluation trial with 100 accessions, Ravindran *et al.*, (1994) observed moderate variability for many yield and quality traits in ginger. Tillers / plant had the highest variability followed by rhizome yield / plant. Correlation and path analysis studies conducted by Sasikumar *et al.,* (1992) revealed positive correlation between yield and characters like plant height, tiller and leaf number as well as length and breadth of leaf. Rout *et al.*, (1998) recommended leaf number as a parameter for selection for improving rhizome yield in ginger. Nybe, (2001) reported that morphological characters were not sufficient to classify ginger types, although some of them could be identified by rhizome characters. Length and girth of primary and secondary fingers were positively correlated with yield.

2.5.2 Biochemical markers

Lack of clear-cut morphological features coupled with absence of cultivar specific characters, make discrimination of cultivars rather difficult. Biochemical markers assume significance in this context. The use of biochemical markers in germplasm characterization has been demonstrated in banana by Bhat *et al.*, (1992) and in wild soyabean by Bult and Kiyangi (1992). Isozymes being multiple forms of protein are primary gene products, variation in their structure should give reliable information about the variability in the genome as they are less susceptible to environmental influence than secondary products of metabolism which are formed as a result of enzyme activity. This technique of isozyme electrophoresis is being widely used to study genotypic variation in living organisms. Isozymes are theoretically well suited to identify closely related individuals or clones, simply by comparision of phenotypic banding patterns.

Shamina *et al.*, (1997) studied twenty five accessions in ginger (*Zingiber officinale* Rosc.) germplasm for variations in total free amino acids, proteins, total phenols and isozymes. Considerable variations were observed for total free amino acids, proteins and total phenols. The variability for the isozyme loci in the population was generally low. Dendrograms were prepared based on the average similarity of the accessions with respect to the isozyme profiles and accessions collected from the same geographical area had a tendency to cluster together.

2.5.3 Molecular markers

With the advent of molecular biology techniques, DNA-based markers could very efficiently augment morphological, cytological and biochemical characters in germplasm characterization, varietal identification, clonal fidelity testing, assessment of genetic diversity, validation of genetic relationship, phylogenetic and evolutionary studies, marker assisted selection and gene tagging. The molecular approach for identification of plant genotypes seems to be more effective as it allows direct access to the hereditary material (Paterson *et al.*, 1991) unlike the morphological markers. Though RAPD markers are reported to be more suitable for genetic diversity analysis of clonal organisms (Bardakei, 2001), the ISSR markers are more reproducible than RAPD markers (Goulao *et al.*, 2001). Surprisingly very few reports are available in ginger with respect to utilization of molecular markers for germplasm characterization (Nayak *et al.*, 2005; Palai and Rout, 2007).

DNA fingerprinting has given new impetus to the biological sciences. Because of its versatility, it was rapidly adopted as a research tool in medicine (Nakamura *et al.*, 1987) and forensic science (Lewin, 1989). At present, DNA fingerprinting has also been applied in many aspects of crop biology, from analysis of genetic diversity within breeding populations in plants, to differentiate between cultivars, as well as to identify plants containing genes of interest.

DNA fingerprinting is a technique, which has been widely adapted to differentiate organisms at the species and subspecies levels (McClean *et al.*, 1994). The techniques used for cultivar identification are designed to detect the presence of specific DNA sequences or combination of sequences that uniquely identify the plant. Cultivar identification can be achieved more accurately using DNA fingerprinting data, especially in materials characterized by high genetic variation between cultivars. The most closely related cultivars are usually distinguished with the DNA fingerprinting methods (Beckmann and Soller, 1986). The application of DNA fingerprinting could be very valuable in the identification of cultivars and species and could help to create more efficient breeding programs through the detection of genetic linkages between DNA fingerprinting bands and agriculturally important quantitative trait loci (QTL). The high variability of DNA fingerprinting described in humans, animals and plants allows the identification of different individuals, genotypes and species (Lin *et al.*, 1993).

The advent of Polymerase Chain Reaction (PCR) ushered a revolutionary approach in producing genetic fingerprints, supplanting hybridization-based techniques. PCR-based methods can be accomplished using either arbitrary markers of unknown location in the genome or those markers that target specific genome sites. 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 on 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) while Caetano-Anolles *et al.*, (1991) reported on DNA Amplification Fingerprinting (DAF). The PCR reaction requires deoxynucleotides to provide both energy and nucleotides for synthesis of DNA polymerase primer, template and buffer containing magnesium (Taylor, 1991). Typical PCR

amplification utilizes oligonucleotide primers that hybridize to opposite 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 by DNA polymerase leading to amplification of the target sequence. The result is an exponential increase in the number of copies of the region amplified by the primer (Saiki *et al.*, 1988; Mullis, 1990). The technique can be applied to detect polymorphism in various plants, animals, bacterial species and fungi (Williams *et al.*, 1990).

The introduction of the PCR technique has revolutionized standard molecular techniques and has allowed for the proliferation of new tools to detect DNA polymorphism (Hu and Quiros, 1991). Polymerase chain reaction (PCR) based techniques make use of random or specific primers to amplify random or specific DNA fragments from the genome. The electrophoresis pattern of fragments generated by each primer for one isolate can be used as DNA fingerprints for assaying diversity (Tommercup *et al.*, 1998). Polymorphism between two individuals is generally scored as a presence or absence (non-amplification) of a particular DNA fragment. PCR is simple, fast, specific, sensitive and relatively low cost. The main advantage of this technique over other techniques is its inherent simplistic analysis (a single reaction can contain all reagents) and the ability to conduct PCR test with extremely, small quantities of tissue for DNA extraction (Welsch *et al.*, 1991). On the other hand PCR is limited in its usefulness because of the time and cost required to obtain the DNA sequence information required for primer design (Samec and Nasinec, 1995; Thottappilly *et al.*, 2000). They are simple to perform, easily amenable for automation and be used to assay a large number of samples. These include Randomly Amplified Polymorphic DNA (RAPD) (Welsh and McClelland, 1990; Williams *et al.*, 1990), Amplified Fragment Length of Polymorphisms (AFLP) (Vos *et al.*, 1995), Simple Sequences Repeats (SSR) (Tautz,1989), Inter Simple Sequences Repeats (ISSR), DNA Amplification Fingerprinting(DAF), Sequence Tagged Sites (STS)

Sequences Characterized Amplified Regions (SCAR) and Expression Sequences Tags (EST). Of these, RFLP and micro satellites are co-dominant markers, while RAPD and AFLP markers are largely dominant markers.

2.5.3.1 Randomly Amplified Polymorphic DNA (RAPD)

Random Amplified Polymorphic DNA is a PCR-based technique. RAPD markers are generated by the use of short (10-mer) synthetic oligonucleotides in single strand primer (Williams *et al.*, 1990). In this technique, a decamer primer of arbitrary sequence is allowed to anneal at a relatively low temperature priming the amplification of DNA fragments distributed at random in the genome (Williams *et al.*, 1990).

Amplification products are visualised by separation on agarose and stained with ethidium bromide. They usually result in DNA fragment patterns that are polymorphic between genotypes, there by detecting diversity within them (Tommercup *et al.*, 1998). However, a key requirement for reliable and reproducible RAPD results is a consistent approach to sample preparation and DNA isolation. There are several advantages of RAPDs compared to other DNA based techniques. It is simple, rapid and does not involve radioactivity and costs less (Varghese *et al.*, 1997). Another advantage of the RAPD method is that a universal set of random primers can be used for genomic analysis of any organism (Welsch and McClelland, 1990). Short random primers have been used to reproducibly amplify segments of genomic DNA from a wide variety of species including plants (Williams *et al.*, 1990; Quiros *et al.*, 1991; Fregene *et al.*, 1997). Polymorphisms detected by RAPDs are inherited in a Mendelian fashion as dominant markers (William *et al.*, 1990; Welsch *et al.*, 1991). This polymorphism has been proved to be useful for identifying variation at different levels. RAPD analysis enables differentiation between very closely related organisms due to high resolution of the technique (Tommerup *et al*,

1998). The polymorphic fragments generated by RAPDs are useful as genetic markers to identify organisms (Williams *et al.*, 1990) and the relative degree of similarity between individual populations and species (Yang and Quiros, 1993; Tonukari *et al.*, 1997).

Vierling and Nguyen (1992) pointed out that, the polymorphism detected between amplification products of different individuals using the short, random, single primers made RAPD marker studies good for genetic diversity, genetic relationships, genetic mapping, plant breeding, DNA fingerprinting and population genetics.

RAPD markers, in particular, have been successfully employed for determination of intra-species diversity in several plants, whereas fewer reports are available on determination of inter-species diversity (Goswami and Ranade, 1999).

Spice crops

The variation among 16 promising cultivars of ginger as observed by Nayak et al. (2005) through differential rhizome yield (181.9 to 477.3 g) was proved to have a genetic basis using cytological and Random Amplified Polymorphic DNA (RAPD). The analysis revealed a differential polymorphism of DNA showing a number of polymorphic bands ranging from 26 to 70 among 16 cultivars.

DNA finger printing of *Piper nigrum* L. and *Piper longum* L. cultivars using RAPD markers was reported by Keshavachandran *et al.* (2005). Fourteen land races and three advanced cultivars of *P. nigrum* and eleven land races and one advanced cultivar of *P.longum* were studied. Forty decamer primers were used for screening, among which ten primers were used for final analysis. These generated 119 amplification products. Cultivar specific single bands were reported for a few land races and accessions of both the *Piper* species. They observed the influence of random primers on the uniformity of RAPD fingerprints developed from different tissues of a particular variety.

Using phylogenetic analysis and metabolic profiling, Jiang *et al.*, (2006) investigated the diversity within and among Zingiber species and reported that *Zingiber officinale* from different geographical origins were indistinguishable.

Molecular characterization of selected superior somaclones in ginger was attempted by Paul, (2006) using RAPD markers and she could observe variation within somaclones and from the original source parent cultivar.

The identification and genetic variation within eight high yielding varieties of ginger was carried out through RAPD markers (Palai and Rout, 2007). A total of 55 distinct DNA fragments ranging from 0.5–2.4 Kb were amplified by using twelve selected primers. The cluster analysis indicated that the eight varieties formed two major clusters.

A comparison of *Curcuma spp.* in Yakushima Island, Japan, with *Curcuma aeruginosa* and *C. zedoaria* in Java, Indonesia was carried out using RAPD pattern by Kitamura *et al.* (2007). It was found that a good correlation was observed between *Curcuma sp.* in Yakushima and *C. aeruginosa* in Java, whereas *C. zedoaria* in Java gave different band patterns in all cases using six primers.

Panda *et al.* (2007) examined genetic stability of micropropagated clones of *Curcuma longa* using RAPD. The analysis revealed monomorphic bands in all the *invitro* grown plants thus confirming genetic uniformity among the clones.

The genetic stability of micropropagated clones of *Z. officinale* was evaluated by Mohanty *et al.*, (2008) at regular intervals of 6 months up to 24 months in culture using cytophotometric estimation of 4C nuclear DNA content and Random Amplified Polymorphic DNA (RAPD) analysis. Cytophotometric analysis revealed a unimodal distribution of the DNA content with a peak corresponding to the 4C value (23.1 pg), and RAPD analysis revealed monomorphic bands showing the absence of polymorphism in all fifty regenerants analyzed, thus confirming the genetic uniformity among *in vitro* grown somaclones.

DNA finger printing of eight varieties of *Zingiber officinale* Rosc. using RAPD markers was reported by Harisaranraj *et al.* (2009). A total of 55 distinct DNA fragments ranging from 0.5-2.4 Kb were amplified by using twelve selected primers. The cluster analysis indicated that the eight varieties formed two major clusters.

A preliminary characterization of mango ginger (*Curcuma amada*) was undertaken by Jatoi *et al.* (2010) to describe genetic structure acquired from farmers and *ex situ* genebank in Myanmar using rice SSR based RAPDs. The high polymorphism (> 91%) depicted has displayed existence of genetic variability in the germplasm investigated. Large number of source-specific alleles was amplified which revealed that non-coding regions of the mango ginger were more variable compared with the functional regions.

Genetic diversity analysis of a set of forty-nine ginger clones cultivated in North-East India was carried out using Random Amplified Polymorphic DNA (RAPD) markers by Sajeev *et al.* (2011). The set included clones of released varieties and clones collected from various parts of North East India. Jaccard's genetic similarity, cluster analysis and principal component analysis identified five clusters. Specific bands for these clones were also identified. Principal component analysis of the molecular data supported grouping of the clones into six hypothetical populations based on their source or location of collection.

Molecular genetic fingerprints of nine *Curcuma* species from Northeast India were developed using PCR based markers by Das *et al.* (2011). Twelve random amplified polymorphic DNA (RAPD), 19 Inter simple sequence repeats (ISSRs), and four amplified fragment length polymorphism (AFLP) primers produced 266 polymorphic fragments. ISSR confirmed maximum polymorphism of 98.55% whereas RAPD and AFLP showed 93.22 and 97.27%, respectively. Dendrograms based on three molecular data using unweighted pair group method with arithmetic

mean (UPGMA) was congruent and classified the *Curcuma* species into two major clusters.

DNA fingerprinting of seven KAU released varieties of black pepper (Panniyur 1 to Panniyur 7) was undertaken by Mogalayi, (2011) at Centre for Plant Biotechnology and Molecular Biology, Kerala Agricultural University. Bulked DNA from black pepper varieties were screened with 30 RAPD, 34 ISSR and 29 SSR primers for amplification. The genomic DNA from each variety was amplified with 10 each of selected RAPD and ISSR primers and 8 SSR primer pairs. The amplification pattern was scored and depicted to develop fingerprint for each variety.

Field crops

RAPD assay has been used for the identification of cultivars and genotypes and for genetic fingerprinting and related studies in a variety of crop spices. Detection of DNA sequence polymorphism among closely related lines of common wheat (*Triticum aestivum*) has been reported by (He *et al.*, 1992). A high level polymorphism was observed among a number of commercial varieties and breeding lines of wheat. Over 38 percent of the 65 primers, used for PCR amplification, produced readily detectable and reproducible DNA polymorphism.

Raychoudhary *et al.*, (2001) used 58 random decamer primers for identification and classification of aromatic rice based on DNA fingerprinting and found that 96.5 per cent of the primers detected polymorphism among the genotypes.

Fernández *et al.*, (2002) analyzed the phylogenetic relationships of 16 barley cultivars from different countries and all having a known pedigree, using 353 PCR markers (125 RAPDs and 228 ISSRs). The band profiles generated were reproducible in spite of the different DNA extractions, PCR techniques, electrophoretic methods and gel scorings used. The RAPD primer S10 and four ISSR primers (811, 820, 835

and 881) were both able to distinguish all cultivars. A strong and quite linear relationship was observed between Resolving Power (Rp) of a primer and its ability to distinguish genotypes. The dendrograms obtained using these two molecular markers are in agreement with their known origin, showing clusters that separate very well the spring/winter and six-rows/two-rows cultivars.

Random amplified polymorphic DNA markers generated by 30 random decamer primers were used to fingerprint 12 released cultivars and a breeding line of *Gossypium hirsutum* and 1 cultivar of *G. barbadense* presently under cultivation in Australia. Among a total of 453 developed markers, 69 (15.2%) were only present in the *G. barbadense* cultivar Pima S-7. Of the remaining markers, 128 (33.3%) were fixed in all 13 *G. hirsutum* cultivars. In pairwise comparisons of the degree of band sharing, nine closely-related cultivars showed 92.1 to 98.9 per cent genetic similarity. Cluster analysis of genetic distance estimates between each of the cultivars. Ten of the *G. hirsutum* cultivars can be characterized individually based upon cultivar-specific RAPD markers (Multani and Lyon, 2006).

Raina *et al.* (2001) illustrated the use of RAPD and ISSR fingerprints as the genetic marker analysis of genetic diversity, varietal identification and phylogenetic relationships in peanut cultivars and wild species.

Fruit crops

RAPD was used to estimate the genetic relationships among 20 selected banana cultivars from different regions of Kenya. Nineteen random primers were used. The polymorphisms in PCR amplification products were subjected to the UPGMA and plotted in a phenogram. The dendogram constructed from the similarity data showed that all the 20 cultivars analysed were related (Ongusoa *et al.*, 2002).

Thirty-six fruit characteristics of Iranian soft-seed pomegranate accessions were measured together with applying 29 random decamer primers already reported to be polymorphic on pomegranate (Sarkhosh *et al.*, 2009). Factor analysis on mean values of fruit characteristics determined ten main factors and applied for grouping of the accessions using Ward's method. Also 14 of the random primers showed good amplification and polymorphism on these samples, and a total of 43 RAPD markers were produced. This study showed that information based on fruit characteristics and RAPD markers are complementary for genetic discrimination in soft-seed pomegranate accessions.

Oil seeds

Deepamala *et al.*, (2005) reported fingerprinting of 14 sunflower cultivars with RAPD, ISSR and AFLP markers utilizing 361, 21 and four primer combinations, respectively. On an individual assay basis, AFLP was proven to be the best marker system as compared with the other two markers. To understand genetic relationships among these cultivars, Jaccards similarity coefficient and UPGMA clustering algorithm were applied to the three marker data sets. However, strong correlation was observed between RAPD and ISSR marker systems.

Genetic diversity evaluations among ten canola (*Brassica napus*) genotypes were determined using RAPD and ISSR markers (Abdelmigid, 2012). The RAPD and ISSR primers with the highest degree of polymorphism were selected. A total of 67 bands of polymorphic RAPD bands were detected out of 77 bands. The number of amplified fragments with RAPD primers ranged from eight to twenty one and the polymorphism ranged from 68.4 to 100.0, with an average of 87%. The five ISSR primers produced 94 bands across ten genotypes, of which 76 were polymorphic. The percentage of polymorphism using ISSR primers ranged from 25 to 100.0 with an average of 78.8%. Clustering of genotypes within the groups was not similar when

RAPD and ISSR derived dendrograms were compared, whereas, the pattern of clustering of the genotypes remained akin in ISSR and the combined data of RAPD and ISSR.

2.5.3.2 Inter Simple Sequence Repeats (ISSR)

ISSR is a PCR based technique which involves amplification of DNA segments in between two identical micro satellite repeat regions on the complementary strands. The inter simple sequence repeat (ISSR-PCR) (Zietkiewiez *et al.*, 1994) is a newly developed method which relies on one primer for PCR and holds promise for variety identification. It involves the amplification of regions between adjacent, inversely oriented microsatellites using a single simple sequence repeats (SSR) containing primer and can be undertaken for any spices that contains a sufficient number and distribution of SSR motifs and has the advantage that genomic sequence data is not required (Gupta *et al.*, 1994; Goodwin *et al.*, 1997). This technique amplifies large numbers of DNA fragments per reaction, representing multiple loci from across the genome; it is an ideal method for fingerprinting varieties and a useful alternative to single-locus or hybridization – based methods. This method was found useful in fingerprinting varieties of rice (Parsons *et al.*, 1997; Blair *et al.*, 1999).

This technique amplifies larger number of DNA fingerprints per reaction, representing multiple loci from across the genome. It is an ideal method for fingerprinting varieties and is useful in fingerprinting varieties of corn (Kantety *et al.*, 1995) and finger millet (Salimath *et al.*, 1995).

ISSRs have high reproducibility possible due to the use of longer primer (16-25 mers) as compared to RAPD primers (decamers) which permits the subsequent use of high annealing temperature (45°C-60°C) leading to higher stringency. The amplified products are usually 200-2000bp long and amenable to detection by both

agarose gel electrophoresis and polyacryamide gel electrophoresis. ISSR segregate mostly as dominant markers following simple Mendelian inheritance (Gupta *et al.*, 1994; Tsumura *et al.*, 1996; Ratnaparkhe *et al.*, 1998; Wang *et al.*, 1998).

ISSR markers are effective multilocus markers for application such as diversity analysis, fingerprinting and genome mapping, gene tagging and marker assisted selection. as no prior sequence knowledge is required, they are more rapidly applied than SSR markers, and they are more reliable and robust than RAPD markers, mainly due to the method of detection, and possibly also to the fact that primers are longer, and hence PCR condition are more stringent.

ISSRs have been successfully used to estimate the extent of genetic diversity at inter and intra specific level in a wide range of crop spices which include rice (Joshi *et al.*, 2000), wheat (Nagaoka and Ogihara, 1997), finger millet (Salimath *et al.*, 1995) vigna (Ajibade *et al.*, 2000), sweet potato (Huang and Sun, 2000) and plant ago (Wolff and Morganrichard, 1998)

ISSR markers linked to the traits of agronomic importance have been sequenced and used as STS markers in markers aided selection. An attractive possibility is thus the use of ISSR as probes for *in situ* hybridization for physical mapping of homologous chromosome sites (Pasakinsene *et al.*, 2000). Another advantage in the use of ISSR markers lies in their linkage to SSR loci. Although micro satellites themselves are probably non functional and selectively neutral, they are known to be linked to coding regions, so that ISSR are likely to be linked to coding region and to mark gene rich regions(Kojima *et al.*, 1998).

Spice crops

Syamkumar and Sasikumar (2007) developed molecular genetic fingerprints of 15 *Curcuma species* from India using Inter Simple Sequence Repeats (ISSR) and Random Amplified Polymorphic DNA (RAPD) markers to elucidate the genetic

diversity / relatedness among the species. Thirty-nine RAPD primers yielded 376 bands of which 352 were polymorphic and out of the 91 bands produced by the eight ISSR markers, 87 were polymorphic. Dendrograms were constructed based on the unweighted pair group method using arithmetic averages. Cluster analysis of data using UPGMA algorithm placed the 15 species into seven groups that are somewhat congruent with classification based on morphological characters proposed by the earlier works. However, the study also warrants the limitations of the conventional taxonomic tools for resolving the taxonomic confusion prevailing in the genus and suggests the need of molecular markers in conjunction with morpho-taxonomic and cytologic studies while revising the genus, which is currently in progress.

Molecular fingerprints of elite, exotic and primitive ginger genotypes were developed using RAPD and ISSR markers to characterize and protect the accessions (Prem *et al.*, 2008). Among the 30 molecular markers studied, 13 could easily discriminate the genotypes. Cluster analysis of data using UPGMA dendrogram placed the ginger genotypes into four separate groups. The grouping of elite genotype with the putative wild types in the dendrogram implies that there is some phylogenetic relationship between the putative wild types and modern cultivars. An exotic type from Japan, resembling the putative types in rhizome features, shared high similarity with the four indigenous putative types.

A global collection of ginger germplasm consisting of 46 accessions was characterized using two types of molecular markers, RAPD (Random Amplified Polymorphic DNA) and ISSR (Inter Simple Sequence Repeats) (Kizhakkayil and Sasikumar, 2010). UPGMA dendrograms constructed based on three similarity coefficients, i.e., Jaccard's, Sorensen–Dice and Simple Matching using the combined RAPD and ISSR markers placed the accessions in four similar clusters.

Taheri *et al.*, (2012) used PCR-based molecular markers (ISSRs) to assess genetic variation and relationships between five varieties of curcuma (*Curcuma*

alismatifolia) cultivated in Malaysia. Sixteen ISSR primers generated 139 amplified fragments, of which 77 per cent had high polymorphism among these varieties. These markers were used to estimate genetic similarity among the varieties using Jaccard's similarity coefficient.

Singh *et al.*, (2012) studied the genetic diversity among turmeric accessions from ten different agro-climatic regions comprising five cultivars and 55 accessions. Two DNA-based molecular marker techniques, viz., Random Amplified Polymorphism DNA (RAPD) and Inter Simple Sequence Repeat (ISSR) were used to assess the genetic diversity in turmeric genotypes. A total of 17 polymorphic primers (eleven RAPDs and six ISSRs) were used in this study. RAPD analysis of 60 genotypes yielded 94 fragments of which 75 were polymorphic with an average of 6.83 polymorphic fragments per primer. The polymorphism ranged from 45 to 100 with an average of 91 per cent. The six ISSR primers produced 66 bands across 60 genotypes of which 52 were polymorphic with an average of 8.6 polymorphic fragments per primer. The percentage of polymorphism using ISSR primers ranged from 83 to 100 with an average of 95.4 per cent. Nei's dendrogram for 60 samples using both RAPD and ISSR markers demonstrated an extent of 62 per cent correlation between the genetic similarity and geographical location.

Genetic variation among 42 cumin accessions, collected from different regions of Iran plus two accessions from Syria and Afghanistan were assessed based on three marker systems namely, ISSR, RAPD and morpho-agronomic traits (Ahmadvandi *et al.*, 2013). Specific grouping were carried out by each cluster analysis including ISSR, RAPD, ISSR+RAPD and morpho-agronomic markers based on their similarity matrix making eight, seven, six and three groups respectively. The results showed that grouping based on molecular markers and morpho-agronomic traits are different so these two systems could not discriminate accessions as a same way. It could be concluded that among three different molecular data sets, the RAPD

and RAPD+ISSR data have a significant and closer relationship to morpho-agronomic data.

Fruit crops

Goulao *et al.*, (2001) used two micro satellite-based methologies (SSR and ISSR) for fingerprinting and determination of similarity degree between 41 commercial cultivars of apple. A total of 13 SSR primer sets were used and 84 polymorphic alleles were amplified. Seven ISSR primer yielded a total of 252 bands, of which 176 (89.1%) were polymorphic. This study indicates that the results obtained based on the RAPD, AFLP, SSR and ISSR techniques are significantly correlated. The SSR and ISSR markers were found to be useful for cultivar identification and assessment of phenotypic relationship revealing advantages, due to higher reproducibility, over other commonly employed PCR-based methods, namely RAPD and RFLP.

Mansyah *et al.*, (2010) used Inter-simple sequence repeat (ISSR) markers to examine the level of genetic diversity in mangosteen. Twenty three accessions of the *Garcinia mangostana* collection from Sumatra region were screened for ISSR markers. Eleven random ISSR primers were chosen to differentiate the investigated accessions. The primers generated 72 bands of which 42 (58%) were polymorphic and 30 bands (42%) monomorphic. From the eleven primers tested, two primers were monomorphic. Seven of the nine polymorphic primers produced fingerprint profiles unique to the RT accession from Tembilahan (Riau Province). Cluster analysis divided the accessions into two major groups.

To identify different cultivars and monitor somaclonal variations of banana during rapid mass micro propagation, Inter simple sequence repeats (ISSR) marker study was done. DNA templates from 30 Banana cultivars were evaluated using 45 primers. Genetic diversity was analyzed. Total number of bands varied between the various cultivars from five to nine. The percentage of total polymorphism is about

85.1 per cent. Four large groups were obtained. Furthermore, a homogenous amplification profile was observed for all the micro propagated plants of 'Brazil' when compared to their mother plants; the developed profiles of different micro propagated clones were typical to that of the donor mother plants (Lu and Ying, 2011).

Beverage crops

Mondal, (2002) analyzed twenty-five diverse tea (*Camellia sinensis* (L.) O. Kuntze) cultivars using the Simple Sequence Repeat anchored Polymorearse Chain Reaction (SSR-anchored PCR) or Inter SSR-PCR (ISSR). Out of the 45 primers 12 were chosen for final study. Of the total amplified 130 bands out of which 108 (84 per cent) were polymorphic. A dendrogram was constructed using UPGMA method revealed three distinct clusters of Cambod, Assam and China type, which concur with the known taxonomical classification of tea. These results suggest that the ISSR-PCR method is potentially useful for genetic fingerprinting and molecular taxonomic classification of tea genotypes.

Inter-simple sequence repeat (ISSR) markers were used to evaluate genetic divergence among eight *Coffea* species and to identify the parentage of six interspecific hybrids by Ruas *et al.*, (2003). A total of 14 primers which contained different simple sequence repeats (SSR) were used as single primers or combined in pairs and tested for PCR amplifications. Two hundred and thirty highly reproducible fragments were amplified, which were then used to estimate the genetic similarity and to cluster the *Coffea* species and hybrids. High levels of interspecific genetic variation were revealed.

2.5.3.3 Simple Sequence Repeats (SSRs) or Microsatellites

The fingerprinting of accessions and analysis of genetic diversity in collections and natural populations are important aspects in the management and utilization of plant collections. Conventional characterization has relied on morphological and phenological characteristics. In recent years, SSRs have become the markers of choice to fingerprint accessions. An important advantage of SSRs is their ability to detect genetic diversity at a higher level of resolution than other methods. Furthermore, they are robust, information may be obtained from a small amount of plant material and at any stage of development, results can be obtained quickly, and the data are not affected by environmental conditions (Lanteri and Barcaccia, 2005). Also SSR primers and data can be shared among labs.

SSR markers are found in all eukaryotic genomes. They are short tandem repeat motifs usually consisting of one to six base pairs of nucleotides (Powell *et al.*, 1996). They were first referred to as microsatellites by Litt and Lutty, (1989) and later as Simple Sequence Repeats (SSRs) by Jacob *et al.*, (1991). Conserved regions flanking the repeats are suitable for designing PCR primer pairs to be used for amplifying the intervening repeat loci. These loci are highly variable on account of the number of repeat units found for each locus in any given population (Morgante and Oliveri, 1993). The high levels of heterozygosity, the co-dominant and PCR-based nature of these repeat loci have made SSRs the molecular markers of choice for genetic mapping and diversity studies (Wang *et al.*, 1994; Gupta *et al.*, 1996).

SSR markers are advantageous to applied plant breeding because they are co-dominant, easily assayed and detect high levels of polymorphism (Morgante and Olivieri, 1993) and for these reasons SSR markers have become highly valuable markers to breeders for the purposes of genome and QTL mapping. SSR markers have, thus, become the marker class of choice for the molecular mapping of many crop species (Roa *et al.*, 2000).

PCR-based methodologies provide an alternative method for isolation of microsatellite loci. Microsatellite loci have been developed using RAPD-PCR of genomic DNA (Ender *et al.*, 1996; Lunt *et al.*, 1999, Liu, 2008) and an AFLP-PCR approach called FIASCO (Fast Isolation by AFLP of Sequences Containing repeats) (Zane *et al.*, 2002; Sun *et al.*, 2008; Zang *et al.*, 2008).

Among the classes of repetitive DNA sequences that have proven amenable for PCR amplification, SSRs remain the best choice of markers (Jacob *et al.*, 1991). SSRs include a variety of di, tri, tetra and pentanucleotide tandem repeats (Hamada *et al.*, 1982; Tautz, 1989; Weber and May, 1989) that can detect high levels of polymorphism at multiple loci and which can serve as a major source of genetic variation thus aiding individual identification.

SSR loci can be amplified by PCR using primers, which are complimentary to the region flanking repeats. There is estimated to be a total of $5X10^3$ to $3X10^5$ microsatellites per plant genome (Condit and Hubbell, 1991). SSRs occur in many plant genomes such as maize (Shattuck-Eidens *et al.*, 1991; Senior and Heun, 1993), soybean (Akkaya *et al.*, 1992), Brassica (Poulsen *et al.*, 1994; Kresovch *et al.*, 1995), rice (Wu and Tanksley, 1993; Zhao and Kochert, 1993) and barley (Saghai-Maroof *et al.*, 1994).

The first amplification of microsatellites in plants has been in cultivar identification and they are the markers of choice in genotyping in cultivars (Weising *et al.*, 1991; Beyermann *et al.*, 1992.

Spice crops

Menezes *et al.*, (2009) reported nine microsatellite markers from an enriched library of *Piper nigrum L.* Twenty varieties regenerated from the Brazilian germplasm collection were analyzed and observed and expected heterozygosity values ranged over 0.11 to 1.00 and 0.47 to 0.87, respectively. The nine microsatellite

loci characterized would contribute to the studies of genetic diversity and conservation of *Piper nigrum.*

Field crops

Molecular genetic diversity analysis in rice using RAPD and SSR markers by Ravi *et al.*, (2003) revealed that the cluster produced based on RAPD and SSR markers were not conserved, since matrix correlation value was 0.582 as against the minimum required value of 0.800. Further, it was also noted that the two marker systems contrasted most notably in pair by pair comparison of relationship.

Guohao *et al.*, (2003) developed 56 SSR markers, out of which 19 could detect polymorphism among 24 cultivated peanut genotypes. The average number of alleles per locus was 4.25. They could identify 14 alleles at PM50 locus when 48 genotypes were surveyed. Using five such highly polymorphic markers they could differentiate 24 cultivated ground nut genotypes.

Vegetables

Alvarez *et al.*, (2001) used 17 microsatellite loci to study diversity among 31 tomato accessions comprising nine spices of the genus *lycopersicon.* The microsatellite polymorphisms were used to estimate the distribution of diversity throughout the genus to evaluate the efficiency of microsatellites for establishing species relationships in comparison with existing phylogeny reconstructions.

Pritesh *et al.*, (2010) reported twenty five determinate and indeterminate cultivars of tomato from different geographical locations of India were screened with twenty three SSR primers in order to determine genetic identities, genetic diversity and genetic relationships among these cultivars. 40 alleles were amplified using SSR primers with scorable fragment sizes ranging from approximately 150 to 1000 bp. UPGMA clustering grouped the cultivars into five groups with the USA cultivars

forming a distinct group. The genetic distance information obtained in this study might be useful to breeder for planning crosses among these cultivars.

Fruit crops

Viruel *et al.*, (2005) studied SSRs which allowed the unambiguous identification of all the mango genotypes. A total of 88 fragments were detected with the 16 simple sequence repeats (SSRs) with an average of 5.5 bands. Two primer pairs amplified more than a single locus. The mean expected and observed heterozygosities over the 14 single-locus SSRs averaged 0.65 and 0.69 respectively. The total value for the probability of identity was 2.74×10^{-9}. UPGMA cluster analysis and Principal coordinate's analysis group the genotypes according to their origin and their classification as monoembryonic or polyembryonic types reflecting the pedigree of the cultivars and the movement of mango germplasm.

Vouillamos *et al.*, (2006) genotyped 116 accessions of traditional grape cultivars from Armenia, Georgia and Turkey using 12 nuclear microsatellite markers, and found 17 identical genotypes and six cases of homonymy. They also stated that the origin of grape appears to be Georgia since four outgroup varieties from Western Europe was more closely related to Georgian cultivars than other germplasm.

Basaki *et al.*, (2013) conducted a study to evaluate the relationships between simple sequences repeat (SSR) markers and important traits using the multiple stepwise linear regressions in pomegranate. Initially, 30 SSR primers were used, seven of which produced polymorphic products, while the remaining was produced monomorphic.

2.5.3.4 Amplified Fragment Length Polymorphisms (AFLP)

Amplified Fragment Length Polymorphism (AFLP) technique combines principles of RFLP analysis with PCR technology (Vos *et al.*, 1995). Total genomic DNA is digested with two restriction enzymes. Adaptors of known sequence are then ligated to the DNA fragments. Primers complementary to the adaptors, with additional one to three selective nucleotides on the 3'end, are used to amplify the restriction fragments. The PCR amplified fragments can then be separated by gel electrophoresis and the banding patterns visualized. A range of enzymes and primers are available to manipulate the complexity of AFLP fingerprints to suit the application. The care is needed in the selection of primers with selective bases. AFLP profiles require no prior DNA sequence information and the number and nature of amplified fragments are altered by the choice of primer pair. The technique also has the advantage of sampling many loci simultaneously and, in addition, it is more robust than arbitrary priming techniques such as RAPD, because more stringent conditions are used in the PCR. AFLP has predominantly been applied in genetic mapping studies (Ballvora *et al.* 1995; Becker *et al.* 1995; Meksem *et al.* 1995; Van Eck *et al.* 1995). According to Van Eck *et al.* (1995) the technique is semi-quantitative because the intensity of AFLP bands can be used to determine zygosity. AFLP has been used to analyze varieties of various species, including cereals, potatoes, sunflowers, Brassicas, beans and lentils (Cooke and Reeves 1998; Law *et al.* 1998).

Spice crops

Nazeem *et al.*, (2005) studied the variability and relatedness among 49 varieties of black pepper (*Piper nigrum* L.) using molecular markers RAPD and AFLP. The similarity matrix was subjected to cluster analysis and dendrogram generated using the software NTSYS. The dendrogram revealed an average similarity of 63 per cent among accessions. The dissimilarity observed between the varieties

Panniyur 1 and Panniyur 3 the progenies of the same parentage, Uthirankotta and Cheriyakanyakadan was only 18 per cent.

Finger printing analysis with Amplified Fragment Length Polymorphic DNA (AFLP) was reported to be an ideal tool for cultivar identification and phylogenetic studies in black pepper (Joy *et al.*, 2007). The analysis was performed in 30 popular cultivars of black pepper and found considerable variability among cultivars of black pepper. It shows the high level of polymorphism and the unique characteristics of the major cultivars.

The genetic relationship among cassumunar gingers (*Zingiber cassumunar*) in Thailand was assessed by Amplified Fragment Length Polymorphism (AFLP) (Kladmook *et al.*, 2010). Twelve AFLP primer combinations generated a total of 309 fragments, of which, 242 bands were polymorphic with an average of 20.2 bands per primer pair. Genetic similarities were obtained using Jaccard similarity coefficients, and a phylogenetic tree was constructed using the UPGMA clustering method. High molecular variance (84%) was found within samples from the same region. The results implied dispersal of plant materials between collection regions. The genetic similarity assessed by AFLP showed that there are duplicate accessions in the germplasm collection.

Ghosh et *al.*, (2011) used Amplified Fragment Length Polymorphism (AFLP) to produce DNA fingerprints for three *Zingiber* species. Sixteen collections (six of *Z. officinale*, five of *Z. montanum*, and five of *Z. zerumbet*) were used in the study. Seven selective primer pairs were found to be useful for all the accessions. A total of 837 fragments were produced by these primer pairs. Species-specific markers were identified for all three *Zingiber* species (91 for *Z. officinale*, 82 for *Z. montanum*, and 55 for *Z. zerumbet*). The dendogram analysis generated from AFLP patterns showed that *Z. montanum* and *Z. zerumbet* are phylogenetically closer to each other than to *Z. officinale*.

Field crops

Amplified fragment length polymorphism (AFLP) technique was used to analyze the genealogical relationship of seven introgressed cotton varieties from the hybridization of upland (*Gossypium hirsutum* L.) and sea-island cottons (*Gossypium barbadense* L) (Liu *et al.*, 2010). Ten pairs of primer combinations with high polymorphism and resolution were selected from 64 primers. Ten primer combinations resulted in a total of 480 bands, of which 374 bands (77.9 per cent) were polymorphic and 51 bands (10.6 per cent) were specific. Cluster analyses showed that seven varieties of cottons were divided into two groups.

Fruit crop

Zhu *et al.* (2000) used four pairs of primers with high polymorphism and powerful distinctiveness has been selected from 68 pairs of primer after constructing the AFLP analysis system in apple varieties. Genetic diversity of P32M46 AFLP fingerprinting of 25 important apple varieties in China was analyzed, difference bands for each varieties were determined, and all apple varieties tested were identified based on the P32M46 AFLP fingerprinting.

Vegetable

De Riek, (2001) used AFLP based alternatives for the assessment of distinctness, uniformity and stability of sugar beet varieties. AFLP data were obtained for three consecutive seed deliveries of 15 sugar beet varieties. In total, 696 AFLP markers were scored on 1350 plants. A cluster analysis based on Nei's standard genetic distance between varieties was made.

The brief review of the literature indicated that the molecular markers either individually or combined could effectively be used in fingerprinting genotypes and determination of intraspecific or interspecific genetic diversity.

Materials and Methods

3. MATERIALS AND METHODS

The investigations on DNA fingerprinting of released varieties and selected superior somaclones of ginger (*Zingiber officinale* Rosc.) were carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Kerala Agricultural University, Thrissur during the period January 2012 to March 2013, with the objective of characterising two released varieties (Athira and Karthika), four selected superior somaclones (292R, 478R, 88R and B3) and source parent cultivars of ginger (Maran and Rio-de-Janeiro) using molecular markers and to develop a DNA fingerprint specific to each variety / somaclone. Two molecular marker systems viz., RAPD and ISSR were used in the investigation. The experimental materials used and methodologies adopted in the study are presented in this chapter.

3.1 MATERIALS

3.1.1 Plant materials

Two KAU released varieties of ginger (Athira and Karthika), four superior selected somaclones (292R, 478R, 88R and B3) and two source parent cultivars (Maran and Rio-de-Janeiro) maintained at CPBMB, College of Horticulture, Vellanikkara were used for the study. The released varieties and superior selected somaclones were developed through adventitious bud regeneration and field evaluation for eight years. Yield and quality attributes of the varieties and selected superior somaclones are furnished in Table 1. For recording morphological and rhizome characters, plants were raised in field at College of Horticulture, Vellanikkara (Plate 1). The crop was managed as per the package of practices recommendations of Kerala Agricultural University (KAU, 2011). For molecular analysis, plants maintained in pots in net house at CPBMB, College of Horticulture, Vellanikkara (Plate 3) were used.

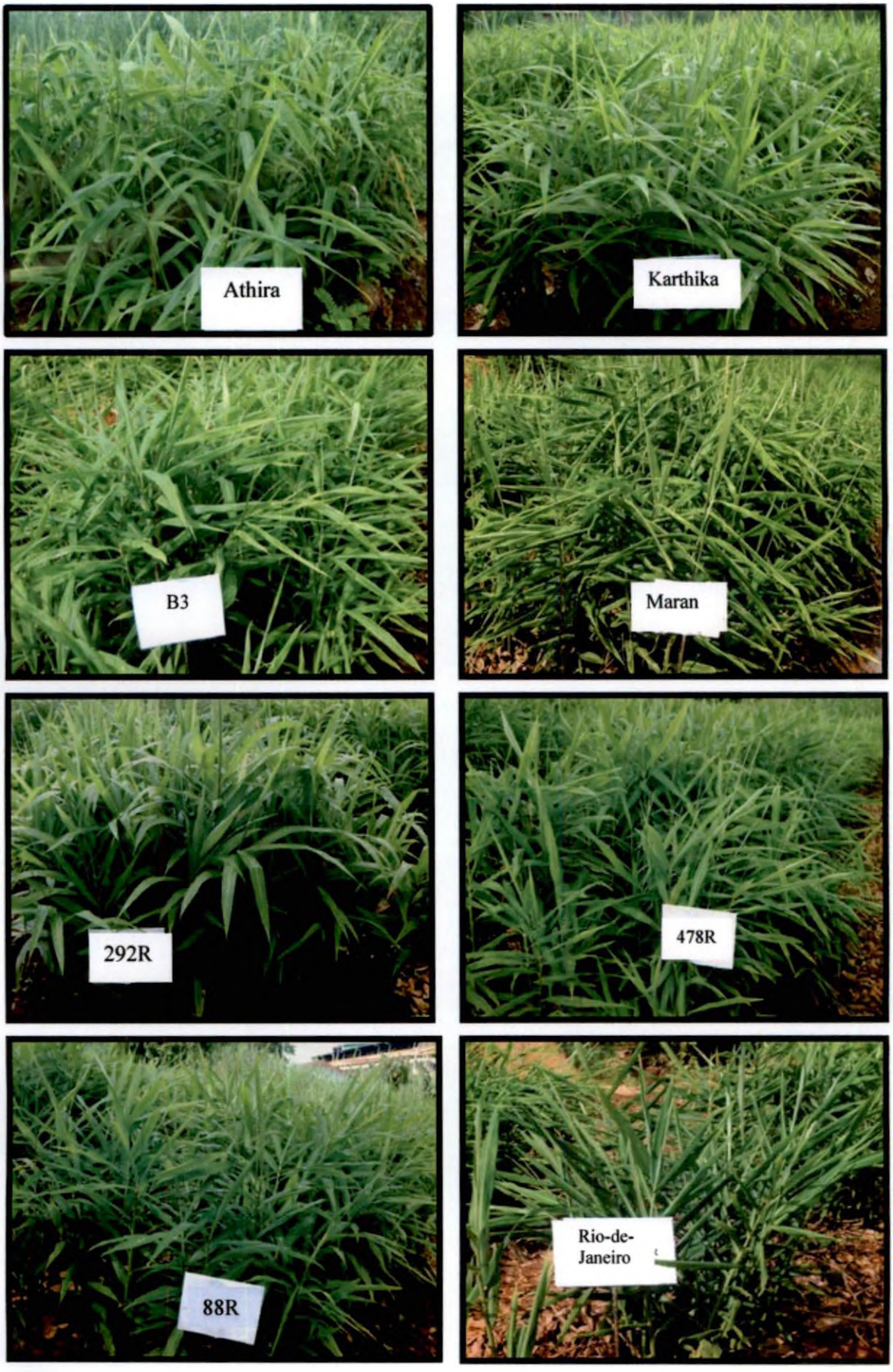


Plate 1 Growth habit of ginger varieties / somaclones

Table 1. Yield and quality attributes of released varieties and selected superior somaclones of ginger

Varieties / somaclones	Yield ($kg/3m^2$)	Yield t/ha	Driage (%)	Volatile oil (%)	Oleoresin (%)	Crude fibre (%)
Athira	10.28	21.00	22.60	3.10	6.80	3.40
Karthika	10.11	19.00	21.60	3.20	7.20	3.70
B3	11.19	22.40	23.00	3.00	6.70	3.60
Maran	6.92	13.80	21.80	2.60	5.70	3.90
292R	11.60	23.20	19.70	3.32	7.45	3.50
478R	11.88	23.76	23.00	3.00	6.99	3.85
88R	11.87	23.74	23.12	2.75	6.88	2.96
Rio-de-Janeiro	8.12	16.20	22.50	2.30	5.10	3.70

(Source: KAU, 2010)

3.1.2 Laboratory chemicals, glassware 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, dNTP, *Taq* buffer and molecular ladders (λDNA / *Hind*III+ *Eco*RI 1000bp) were supplied by Bangalore Genei Ltd and 100bp molecular ladder were supplied by Invitrogen™. All the plasticware used were obtained from Axygen and Tarson India Ltd. The decamer primers were obtained from Operon Technologies Inc. (Alamedda, Calif.) and Xcelris Genomics Primex and ISSR primers were obtained from Sigma Aldrich Chemicals Pvt. Ltd. and Xcelris Genomics Primex.

3.1.3 Equipment

The present research work was carried out using the facilities and equipment items available at Centre for Plant Biotechnology and Molecular Biology, College of Horticulture. Centrifugation was done with high speed refrigerated centrifuge (KUBOTA 6500, Japan). NanoDrop^{R} ND-1000 spectrophotometer was used for assessing the quality and quantity of DNA. The DNA amplification was carried out in Veriti^{R} Thermal cycler (Applied Biosystems). Agarose gel electrophoresis was done in horizontal Gel Electrophoresis Systems (BIO-RAD, USA) and gel documentation was done in Gel Documentation System (BIO-RAD, USA). Details of laboratory equipments used for the study are provided in Annexure I.

3.2 METHODS

3.2.1 Morphological Characterization

3.2.1.1 Vegetative Characters

Vegetative characters were recorded at four months after planting in plants raised in the field (Plate 1). Characters like growth habit, height, number of tillers,

orientation of top leaf, number of leaves on main tiller, leaf length, leaf width, intensity of leaf green colour, leaf petiole length, leaf orientation and tiller diameter were recorded. Data were recorded from nine plants per variety / clone, selected from three beds.

3.2.1.2 Rhizome Characters

Rhizome characters such as size, shape and arrangement of rhizomes, number of primary, secondary and tertiary fingers, length of primary and secondary fingers, girth of primary and secondary fingers, internodal length of primary and secondary fingers, thickness of flesh, thickness of inner core and colour of flesh and scales were recorded in the released varieties and selected superior somaclones after harvest (Plate 2).

3.2.2 Molecular Characterization

3.2.2.1 Genomic DNA isolation from ginger varieties / somaclones

Isolation of good quality genomic DNA is one of the most important pre-reqisites for doing RAPD and ISSR analyses. Young, tender, fully opened and pale green leaves were collected early in the morning on ice from individual plants grown in net house (Plate 3). The surface was cleaned by washing with sterile water and wiping with clean tissue paper. The fresh leaves were ground to fine powder using liquid nitrogen along with β-mercaptoethanol and PVP in ice-cold mortar and pestle in order to prevent browning due to phenol oxidase. DNA was extracted by following CTAB method reported by Rogers and Bendich (1994).

Reagents (Details of composition of reagents are provided in the Annexure II).

1. CTAB Buffer (2X)
2. 10 % CTAB Solution

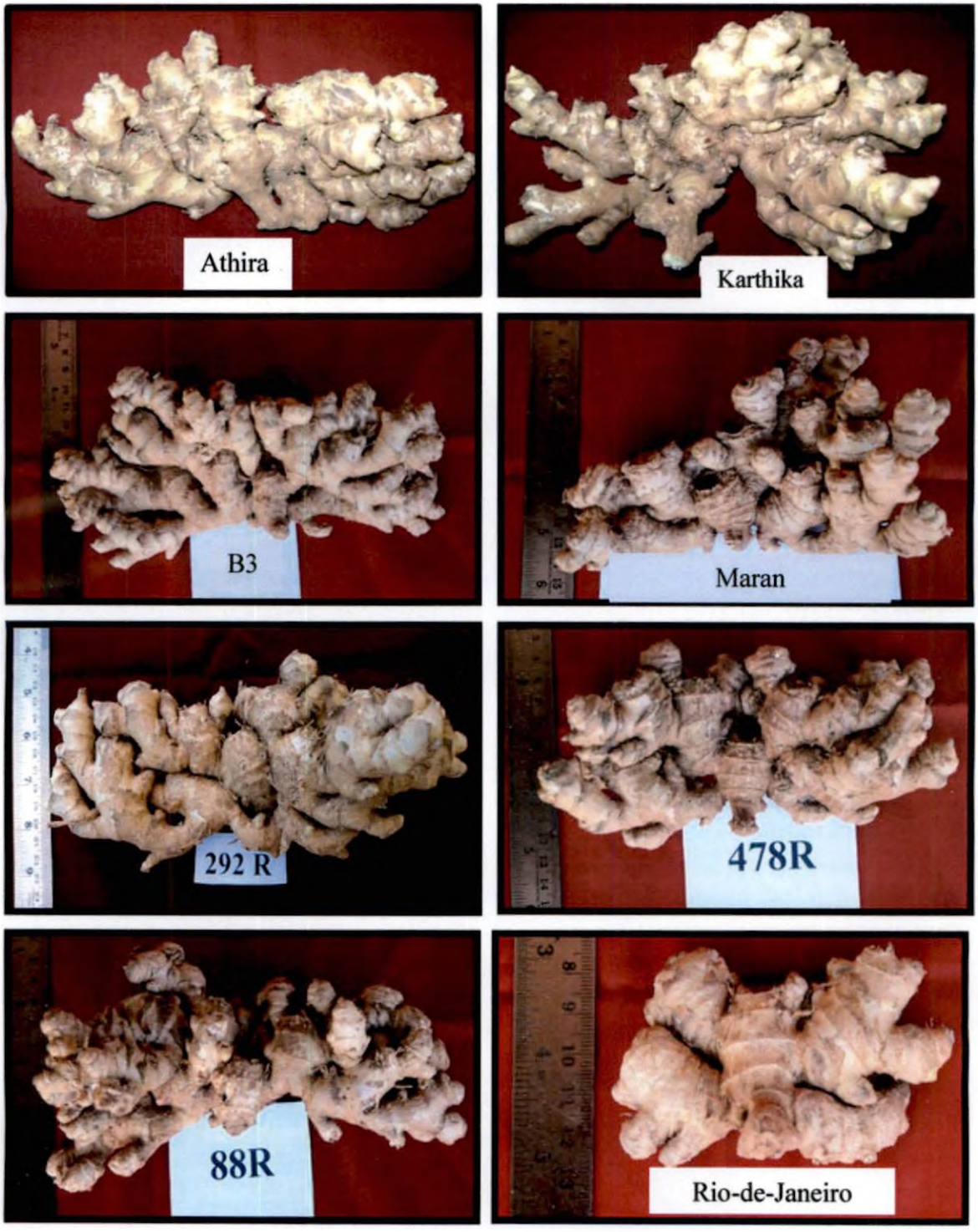


Plate 2 Rhizome characters of the varieties / somaclones

a. Ginger varieties / somaclones grown in net house

A – Folded, immature

B – Young, tender and pale green - ideal stage

C - Medium mature leaf

D - Mature leaf

b. Different maturity stages of leaves in ginger varieties / somaclones

Plate 3 Source plants for genomic DNA isolation

3. TE buffer
4. Chloroform: Isoamyl alcohol (24:1) v/v
5. Isopropanol
6. Ethanol 70 % and 100 %

Procedure

One gram of clean leaf tissue was ground in a pre-chilled mortar and pestle in the presence of liquid nitrogen and a pinch of PVP. Extraction buffer (2X) 4ml and 50µl of β-mercaptoethanol were added to it. The homogenized sample was transferred to an autoclaved 50ml centrifuge tube and added 3ml of pre-warmed extraction buffer (total 7ml). After mixing thoroughly, the mixture was incubated at 65°C for 20 to 30 minutes with occasional mixing by gentle inversion. Equal volume (7ml) of chloroform: isoamyl alcohol (24:1 v/v) was added and the mixture was mixed by inversion to emulsify. Then it was spun at 10,000rpm for 15 minutes at 4°C. After the centrifugation, the top aqueous layer was transferred to a clean centrifuge tube and $1/10^{th}$ volume of ten per cent CTAB was added. Equal volume of chloroform: isoamyl alcohol (24:1) was added and mixed by inversion and centrifuged at 10,000 rpm for 15 minutes at 4°C. The aqueous phase was transferred to a clean centrifuge tube and added 0.6 volume of chilled isopropanol and mixed by quick gentle inversion till the DNA was precipitated. The mixture was again centrifuged at 10,000 rpm for 15 minutes at 4°C and the supernatant was gently poured off. The DNA pellet was washed with 70 per cent ethanol followed by 100 per cent ethanol, spun for 5 minutes at 10,000 rpm and decanted the ethanol. The DNA pellet was air dried for 30 minutes and the pellet was dissolved in 50µl of TE buffer or sterilized water and stored at -20°C.

3.2.2.2 DNA Purification

The DNA isolated would contain RNA as contaminant and was hence purified by treatment with RNase and further precipitated (Sambrook *et al.*, 1989).

Reagents

1. Chilled isopropanol
2. 70 % ethanol
3. TE buffer
4. Chloroform: Isoamyl alcohol (24:1, v/v)
5. RNase (1 %)

The RNase-A from Sigma, USA was used to prepare RNase. One per cent solution was prepared by dissolving RNase-A in autoclaved distilled water. The solution was dispensed into aliquots and stored at -20°C.

Procedure

RNase solution (2 µl) was added to 100µl DNA sample, and incubated at 37°C in dry bath (GeNei, Thermocon) for one hour. The volume was made up to 250µl with distilled water and equal volume of chloroform: isoamyl alcohol (24:1) was added and mixed gently. This was then centrifuged at 10,000 rpm for 15 minutes at 4°C. The aqueous phase was collected into a fresh micro centrifuge tube and equal volume of chloroform: isoamyl alcohol (24:1) was added. Again it was centrifuged at 10,000 rpm for 15 minutes at 4°C. The aqueous phase was transferred into a fresh micro centrifuge tube and 0.6 volume of chilled isopropanol was added and mixed by gentle inversion till the DNA was precipitated. The mixture was incubated at -20°C for half an hour for complete precipitation and centrifuged at 10,000 rpm for 15

minutes at 4°C. The pellet of DNA obtained was washed with 70 per cent ethanol. The pellet was air dried and dissolved in 50µl of autoclaved distilled water.

3.2.2.3 Quantification of DNA

3.2.2.3.1 Assessment of quality and quantity of DNA by electrophoresis

Reagents and equipments

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

Materials used for agarose gel electrophoresis

1. Agarose

2. 50X TAE buffer (pH 8.0)

3. Tracking / loading dye (6X)

4. Ethidium bromide (stock 10 mg/ml; working concentration 0.5 µg/ml)

5. Electrophoresis unit, power pack, gel casting tray, comb

6. UV transilluminator

7. Gel documentation and analysis system

Chemical composition of reagents and dye are provided in Annexure III.

Procedure

1X TAE buffer was prepared from the 50X TAE stock solutions. Agarose (0.8 per cent) was weighed and dissolved in TAE buffer by boiling. Then ethidium bromide was added at a concentration of 0.5µg/ml and mixed well. The open end of

gel casting tray was sealed with a cellotape and kept on horizontal levelled surface. The comb was placed and the dissolved agarose was poured on to the tray. The gel was allowed to solidify for about 30 to 45 minutes at room temperature after which the comb and the tape were gently removed. The tray was placed in electrophoresis chamber and 1X TAE buffer was added to the tank. Then the DNA sample (3µl) along with tracking dye (1µl) was loaded into the wells using a micropipette carefully. The molecular marker used was λDNA / *Hind*III+ *Eco*RI double digest (1000bp). After closing the tank, the anode and cathode ends were connected to the power pack and the gel was run at a constant voltage (100 V) 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 electrophoresis unit and viewed under UV light in a transilluminator (Herolab^{R}). The DNA fluoresces under UV light due to ethidium bromide dye. The quality of DNA was judged by clarity of DNA band. The image was documented and saved in the Gel Documentation System.

3.2.2.3.2 Assessment of quality and quantity of DNA by NanoDrop method

The quality and quantity of genomic DNA was estimated using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., USA). Before taking sample readings, the instrumment was set to zero by taking 2µl of autoclaved distilled water as blank. Each sample was quantified by taking 1.5 µl and was measured in ng/µl. The absorbance of nucleic acid samples was recorded at a wavelength of 260 nm and 280 nm and OD_{260}/OD_{280} ratios were recorded to assess the purity of DNA. The ratio of 1.8 to 2.0 for OD_{260}/OD_{280} indicated that the DNA is pure and free from proteins. The quantity of DNA in the pure sample was calculated using the relation $1OD_{260}$ is equivalent to 50 µg double stranded DNA/ml sample.

1OD at 260 nm= 50 µg DNA/ml

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

3.2.2.4 Molecular markers used for the study

Two types of marker systems were used for the study which includes RAPD (Random Amplified Polymorphic DNA) and ISSR (Inter Simple Sequence Repeats) assays. Under each marker analysis system, eight samples were amplified separately with the selected primers with four replications per treatment. For both the marker systems only reproducible bands were scored for developing fingerprints.

3.2.2.4.1 RAPD (Random Amplified Polymorphic DNA) assay

Good quality genomic DNA (40 to 50ng/µl) isolated from eight samples of ginger by CTAB method were subjected to RAPD analysis as per the procedure reported by Williams *et al.*, (1990). Random decamer primers supplied by Operon Technologies Inc. (Alamedda, Calif.) and Xcelris Genomics Primex with good resolving power were used for amplification of DNA. After initial screening of primers, the decamer primers for RAPD assay were selected.

Screening of random primers for RAPD analysis

The basic RAPD procedure suggested by Venkatachalam *et al.*, (2007) was used for selection of primers. Thirty five primers (Operon Technologies Inc. and Xcelris Genomics Primex) in the series OPA, OPC, OPD, OPE, OPG, OPK, OPP, OPU, OPAH, RN, RY and S were screened with bulked DNA samples from the variety Athira and somaclones 292R and 478R. Genomic DNA at the concentration of 50ng/µl was subjected to amplification using selected random primers.

The amplification was carried out in a Veriti^{R} Thermal cycler (Applied Biosystems). The PCR reaction was performed using a 20µl reaction mixture.

Composition of the reaction mixture for PCR (20.0 µl)

Materials	Quantity (µl)
Genomic DNA (30 ng/µl)	2.0
10 X assay buffer (B)	2.0
$MgCl_2$	2.0
dNTPs (10mM/ml)	0.6
Taq DNA polymerase (3U/µl)	0.4
Decamer Primer	2.0
Autoclaved distilled water	11.0
Total	**20.0**

A master mix of all reagents for the required number of reactions was prepared first and aliquots were dispensed into PCR tubes followed by addition of template DNA in each tube, and one tube without template DNA was kept as blank.

The PCR tubes were kept in the thermal cycler and the following programme was run:

Step 1: 94 °C for 4 minutes – Initial denaturation

Step 2: 94 °C for 45 seconds – Denaturation

Step 3: 37 °C for 1 minute – Primer annealing

Step 4: 72 °C for 2 minutes – Extension

} 35 Cycles

Step 5: 72 °C for 8 minutes – Final extension

Step 6: 4 °C for 10 minutes – Hold

The amplified products were loaded on two per cent agarose gel using 50X TAE buffer stained with ethidium bromide along with marker (100bp Invitrogen).

Electrophoresis was performed at 70V for two hours. The profile was visualized under UV transilluminator and documented using gel documentation system (BIORAD, USA). The documented RAPD profiles were carefully examined for amplification of DNA as bands. The size of polymorphic bands in kb / bp of bases were recorded in comparison with marker and using the software Quantity One.

Out of 35 decamer primers screened for RAPD analysis, primers which gave good amplification products for each ginger variety / clone were selected for further assay (Table 2).

3.2.2.4.2 ISSR (Inter Simple Sequence Repeats) assay

Good quality DNA (25 to 30 ng/µl) isolated from eight samples of ginger was subjected to ISSR analysis as per the procedure reported by Zietkiewicz *et al.*, (1994). The primers for ISSR assay were supplied by Sigma Aldrich chemicals Pvt. Ltd. and Xcelris Genomics Primex. After an initial screening, primers were selected for ISSR assay. Primers with good resolving power were used for amplification of DNA.

Screening of ISSR primers for ISSR assay

The amplification was carried out in Veriti^{R} Thermal cycler (Applied Biosystems). Thirty ISSR primers (Sigma Aldrich chemicals Pvt. Ltd. and Xcelris Genomics Primex) in the series UBC, ISSR and SPS were screened with bulked DNA samples from the variety Athira and somaclones 292R and 478R. Genomic DNA at the concentration of 25 to 30 ng/µl was subjected to amplification using selected primers. The annealing temperature of ISSR primers used in the study ranged from 43°C to 55°C.

Composition of the reaction mixture for PCR (20.0 µl)

Materials	Quantity (µl)
Genomic DNA (30 ng/µl)	2.0
10 X assay buffer (B)	2.0
$MgCl_2$	2.0
dNTPs (10mM/ml)	0.6
Taq DNA polymerase (3U/ul)	0.4
Primer	2.0
Distilled water	11.0
Total	**20.0**

The PCR tubes were kept in the thermal cycler and were run in the following programme:

Step 1: 94 °C for 4 minutes – Initial denaturation

Step 2: 94 °C for 45 seconds – Denaturation

Step 3: 43 °C for 1 minute – Primer annealing

Step 4: 72 °C for 2 minutes – Extension

} 35 Cycles

Step 5: 72 °C for 8 minutes – Final extension

Step 6: 4 °C for 10 minutes – Hold

The amplified products were run on two per cent agarose gel using 50X TAE buffer stained with ethidium bromide along with 100bp marker. Electrophoresis was performed at 50V for three hours. The profile was visualized under UV transilluminator and documented using Gel Documentation System (BIORAD, USA). The documented ISSR profiles were carefully examined for amplification of

DNA as bands. The size of polymorphic bands in kb / bp of bases were recorded in comparison with marker and using the software Quantity One.

Out of 30 ISSR primers screened for ISSR analysis, primers which gave good amplification products for each ginger variety / clone were selected for further assay (Table 3).

3.2.2.5 Data Analysis

Morphological Characterization

The observations recorded on vegetative and rhizome characters were subjected to Analysis of Variance and the level of significance was compared between varieties / somaclones for different parameters recorded.

Mahalanobis D^2 analysis was carried out for assessing the genetic divergence between varieties / somaclones of ginger (Mahalanobis, 1936). The intra and inter cluster Mahalanobis distances were calculated as described by Singh and Choudhary (1997).

Molecular Characterization

Scoring of bands on agarose gel was done with the Quantity One software loaded in Gel Documentation System. λDNA marker (*Eco*RI+*Hind* III double digest, 1000bp) and 100bp ladder were used as molecular weight size marker for each gel along with DNA samples. The bands were scored as one and zero for the presence and absence respectively and their size recorded in relation to the molecular weight markers used and with the software Quantity One. The fingerprint results obtained from RAPD and ISSR were transformed into data matrix as discrete variables.

DNA fingerprint of each variety / somaclone was generated based on the presence of clear and distinct bands and size of the bands. Fingerprints were developed with the two marker systems used in the present investigations and also the combined marker system. Separate colour codes were given to highlight the presence of unique bands, bands shared with two varieties, three varieties etc. In fingerprints generated with RAPD and ISSR marker systems, the presence of unique band was represented in violet colour. Navy blue was used to highlight the bands shared with two varieties / somaclones, light blue for bands shared with three varieties / somaclones, pink for bands shared with four varieties / somaclones, yellow for bands shared with five varieties / somaclones, orange was for bands shared with six varieties / somaclones, red for bands shared with seven varieties / somaclones and green for bands shared with eight varieties / somaclones. In combined fingerprint generated with RAPD and ISSR markers, blue was used to indicate the bands produced by RAPD marker alone, yellow was used to indicate the bands produced by ISSR marker alone and green was used to indicate the bands produced by both RAPD and ISSR markers.

The analysis of variability among the varieties / somaclones and variability of the somaclones with the source parent cultivars were carried out using the primers that produced more polymorphism. Clearly resolved, unambiguous polymorphic bands were scored visually for their presence or absence. The scores were obtained in the form of a matrix with 'one' and 'zero', indicating the presence or absence of bands in each variety / somaclone respectively.

Jaccard's coefficient of similarity was measured and a dendrogram based on similarity coefficients was generated using Unweighted Pair Group Method with Arithmetic Mean (UPGMA). Only distinct and well resolved fragments were scored. The resulting data were analyzed using the software package Numerical Taxonomy and Multivariate Analysis System NTSYS (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 (Ib) is = 1- [2(0.5-p)], where p is the proportion of accessions containing the band. Resolving power of the primer is represented as: Rp=ΣIb.

Polymorphic Information Content (PIC) value of a primer is calculated as: PIC = $1-\Sigma pi^2$, where *pi* is the frequency of the i^{th} allele. Polymorphic Information Content was used to confirm the suitability of the primers selected for DNA fingerprinting (Nei, 1973).

Table 2. RAPD primers used for screening ginger varieties / somaclones

Sl. No.	Primers	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'

Table 3. ISSR primers used for screening ginger varieties / somaclones

Sl. No.	Primers	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 S 2	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'

Results

4. RESULTS

The results of the investigations on "DNA fingerprinting of released varieties and selected superior somaclones of ginger (*Zingiber officinale* Rosc.)" undertaken at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara from January 2012 to March 2013 are presented in this chapter.

The research works included mainly the morphological and molecular characterization of two released varieties of KAU (Athira and Karthika), four superior selected somaclones (292R, 478R, 88R and B3) and source parent cultivars (Maran and Rio-de-Janeiro) to develop DNA fingerprint specific to each variety / clone. The two marker systems viz. RAPD and ISSR were used in the investigations to develop DNA fingerprints of the varieties / somaclones.

4.1 Morphological Characterization

Morphological characterization of released varieties and selected superior somaclones was attempted.

a) Vegetative Characters

Vegetative characters like growth habit, height of plant, number of tillers, orientation of top leaf, number of leaves on main tiller, leaf length, leaf width, intensity of leaf green colour, leaf petiole length, leaf orientation and tiller diameter recorded at four months after planting of the varieties / somaclones are presented in Table 4.

Significant variation in plant height was noticed in the varieties and selected somaclones of ginger. Plant height recorded was highest (83.81 cm) for the variety Karthika and statistically it was on par with the variety Athira and somaclones 88R

and B3, while it was medium (71.5 cm) for somaclone 478R which was on par with the source parent cultivars Maran and Rio-de-Janeiro. The plant height was lowest for the somaclone 292R (54cm) which is a distinguishing feature for the clone.

There was significant variation in the number of tillers per plant in the ginger varieties and selected somaclones. The number of tillers per plant was more in the variety Karthika (17.6) which was on par with the somaclones B3, 292R and 88R while it was less for the variety Athira (13.0) which was on par with somaclone 478R and source parent cultivar Rio-de-Janeiro. Lowest number of tillers (9.2) was recorded for source parent cultivar Maran. The tiller girth recorded was highest (3.60 cm) for source parent cultivar Rio-de-Janeiro. All the other somaclone except B3 were on par with source parent cultivars with respect to tiller girth. The somaclone B3 recorded the lowest tiller girth of 3.20 cm.

Significant variation was noticed in number of leaves on main tiller in the varieties and somaclones of ginger studied. Highest number of leaves (21.1) was recorded for the somaclone 478R which is a distinguishing character for the clone. The number of leaves on main tiller in other varieties and somaclones ranged from 14.6 to 16.5.

The source parent cultivar Maran recorded the highest leaf length (30.4 cm), which can be considered as a remarkable character. Leaf lengths recorded for the other varieties / somaclones except 478R were on par. The somaclone 478R exhibited the lowest leaf length of 24 cm.

Data recorded on leaf width showed significant variation in the varieties and selected somaclones of ginger. Leaf width was more for the source parent cultivar Rio-de-Janeiro (3.16 cm), which was on par with the other source parent cultivar Maran and variety Athira. Leaf width was 2.78 cm in the somaclone B3 which was on par with 292R and Karthika. The leaf width recorded was less for the somaclones 478R and 88R.

Significant variation was also noticed in petiole length in the varieties and somaclones studied. The petiole length was more for the variety Karthika (0.55 cm) which was on par with all the other clones except 478R and B3.

Plant growth habit was semi erect in the varieties Athira, Karthika and somaclones B3, 478R and the source parent cultivars Maran and Rio-de-Janeiro while it was erect in somaclones 292R and 88R. Orientation of top leaf is erect in all the somaclones studied whereas, it was semi-erect in two source parent cultivars.

The colour of leaf was dark green in the somaclone 292R which can be used as an identifying feature. Leaf orientation observed was mostly semierect in the varieties Athira and Karthika, somaclones B3 and 292R and source cultivar Rio-de-Janeiro, whereas, it was spreading in the somaclones 478R, 88R and source cultivar Maran.

Quantitative clustering for vegetative characters was done in ginger varieties / somaclones as per Mahalanobis D^2 analysis and the clones investigated were grouped into three clusters. The different members of each cluster are presented in Table 5 and inter and intra cluster Mahalanobis distances are presented in Fig. 1a. The cluster wise vegetative parameters are presented in Table 6. The clones exhibiting higher values for vegetative parameters were grouped in cluster II. However, the distance between cluster II and cluster I was found low indicating better vegetative characters in cluster I also. The highest inter cluster distance was observed between cluster II and III indicating the higher variability in vegetative characters between the clones in the clusters.

Of the seven vegetative parameters analysed, plant height and number of tillers showed more divergence between the clusters. The single member in cluster III (Maran source parent cultivar) exhibited more length and width of leaves. However, the number of tillers, number of leaves on main tiller and plant height were found low indicating less leaf area and less photosynthetic efficiency of the cultivar as compared

to improved varieties. The improved varieties / somaclones were placed in distant clusters from the source parent cultivar Maran. Seventy five per cent of the improved somaclones were distantly clustered from the original source parent cultivar Maran. However, the cultivar Rio-de-Janeiro which exhibited better vegetative parameters was grouped along with the improved varieties / somaclones.

b) Rhizome Characters

Rhizome characters such as size, shape and arrangement of rhizomes, number of primary, secondary and tertiary fingers, length of primary and secondary fingers, girth of primary and secondary fingers, internodal length of primary and secondary fingers, thickness of flesh, thickness of inner core and colour of flesh and scales were recorded after harvest. The data recorded on rhizome characters are presented in Table 7.

Size of rhizome was bold in Athira, 292R, 478R and Rio-de-Janeiro, whereas, it is medium bold in Karthika, B3, Maran and 88R. The shape of rhizome is flat in Athira, Maran, 292R, 478R, 88R and Rio-de-Janeiro, whereas, it is round in Karthika and B3.

Significant variation was also noticed in the number of secondary and tertiary fingers of the varieties and selected somaclones in ginger.

Highest number of secondary and tertiary fingers was recorded in the variety Athira which can act as a distinguishing feature for the variety. Lowest number of secondary and tertiary fingers was recorded in the source cultivar Rio-de-Janeiro. All the other varieties / somaclones studied were found on par with respect to number of secondary and tertiary fingers.

Table 4. Vegetative characters of ginger varieties / somaclones

Varieties	Plant growth habit	Plant height (cm)	No. of tillers	No. of leaves on main tiller	Leaf length (cm)	Leaf width (cm)	Petiole length (cm)	Tiller girth (cm)	Intensity of leaf green colour	Leaf orientation	Orientation of top leaf
Athira	Semi erect	78.52	13.00	16.00	26.92	2.90	0.51	3.42	Green	Semi erect	Erect
Karthika	Semi erect	83.81	17.57	16.14	27.81	2.65	0.55	3.46	Green	Semi erect	Erect
B3	Semi erect	75.88	14.42	16.12	27.80	2.78	0.42	3.18	Green	Semi erect	Erect
Maran	Semi erect	63.46	9.16	14.60	30.38	2.93	0.48	3.35	Green	Spreading	Semi erect
292R	Erect	54.03	15.80	15.38	27.52	2.75	0.48	3.56	Dark green	Semi erect	Erect
478R	Semi erect	71.53	13.00	21.11	24.23	2.45	0.30	3.4	Green	Spreading	Erect
88R	Erect	76.68	16.14	16.55	25.81	2.45	0.50	3.32	Green	Spreading	Erect
Rio-de-Janeiro	Semi erect	69.36	10.67	15.00	25.80	3.16	0.50	3.60	Green	Semi erect	Semi erect
CD (0.05)	–	3.66	2.34	2.39	2.26	0.28	0.11	0.31	–	–	–

Table 5. Clustering of ginger varieties / somaclones based on Mahalanobis distances for vegetative characters

Cluster No.	Varieties / somaclones	No. of genotypes
I	Athira, 292R, 478R, Rio-de-Janeiro	4
II	Karthika, B3, 88R	3
III	Maran	1

Table 6. Vegetative characters in different clusters of ginger varieties / somaclones

Characters	Cluster I	Cluster II	Cluster III
Plant height (cm)	68.36	78.79	63.46
No. of tillers	10.86	16.04	9.16
No. of leaves on main tiller	16.87	16.27	14.60
Leaf length (cm)	26.11	27.14	30.38
Leaf width (cm)	2.815	2.62	2.93
Petiole length (cm)	0.45	0.49	0.48
Tiller girth (cm)	3.5	3.32	3.35

Significant variation was also observed in the varieties and somaclones in the girth of secondary fingers. The girth of secondary fingers was more in 292R (9.10 cm) which was on par with the variety Athira. In variety Karthika the girth was 6.4 cm which was found to be on par with the somaclones B3, 478R and 88R and source parent cultivar Maran. The lowest girth was observed in the source cultivar Rio-de-Janeiro (5.3 cm). Significant variation was seen in thickness of flesh in the ginger varieties and selected somaclones. Thickness of flesh recorded was more for the variety Athira (1.83 cm), which was on par with the variety Karthika and somaclones B3, 292R and 478R and source cultivar Rio-de-Janeiro. The thickness of flesh was found less in the somaclone 88R (1.36 cm) and the source parent cultivar Maran (1.33 cm).

There was significant variation in length of primary and secondary fingers in the varieties and somaclones studied. Length of primary and secondary fingers was more in the source cultivar Rio-de-Janeiro and somaclones studied and least for source cultivar Maran.

Significant variation was also observed in the girth of primary fingers with highest value recorded in the variety Athira (10.86 cm) which was on par with somaclones 292R, 478R and 88R and source parent cultivar Maran and Rio-de-Janeiro. Significant variation was noticed for the internodal length of primary fingers. The internodal length recorded was highest (1.46 cm) in the somaclone 88R and lowest in the parent cultivar Rio-de-Janeiro (0.53 cm).

No significant variation was noticed in the internodal length of secondary fingers. Significant variation was observed in the thickness of inner core. Thickness of inner core recorded was more for the somaclone 292R (12.6 cm) which was on par with the variety Athira and source cultivar Rio-de-Janeiro.

Colour of flesh was pale yellow in Athira, Maran, 292R and Rio-de-Janeiro, pale yellow with bluish tinge in B3 and bluish yellow in Karthika, 478R and 88R.

Colour of scale is brown in Athira, Karthika, Maran, B3, 478R and 88R whereas, it is dark brown in 292R and it is light brown in Rio-de-Janeiro.

Quantitative clustering for rhizome characters attempted as per Mahlanobis D^2 analysis could group the varieties / somaclones into three separate clusters. The variety / somaclone exhibiting bold rhizomes, more number of primary, secondary and tertiary fingers were clustered in cluster II. The members of each cluster are presented in Table 8 and the inter and intra cluster Mahalanobis distances are presented in Fig. 1b. The cluster wise rhizome characters are presented in Table 9.

The improved variety Athira and the somaclone 292R which exhibited better rhizome characters like bold rhizome, more number of primary, secondary and tertiary fingers, more number of layers of rhizome and more thickness of flesh and inner core were grouped into cluster II. The inter cluster distance between cluster II and III was found low as compared to cluster II and I indicating better rhizome characters for members in cluster III also. The improved variety Karthika exhibiting better rhizome characters was thus grouped in cluster III. The rhizome characters which exhibited more divergence were number of fingers, girth of primary and secondary fingers, and thickness of flesh and inner core of rhizome.

Table 7. Rhizome characters of ginger varieties / somaclones

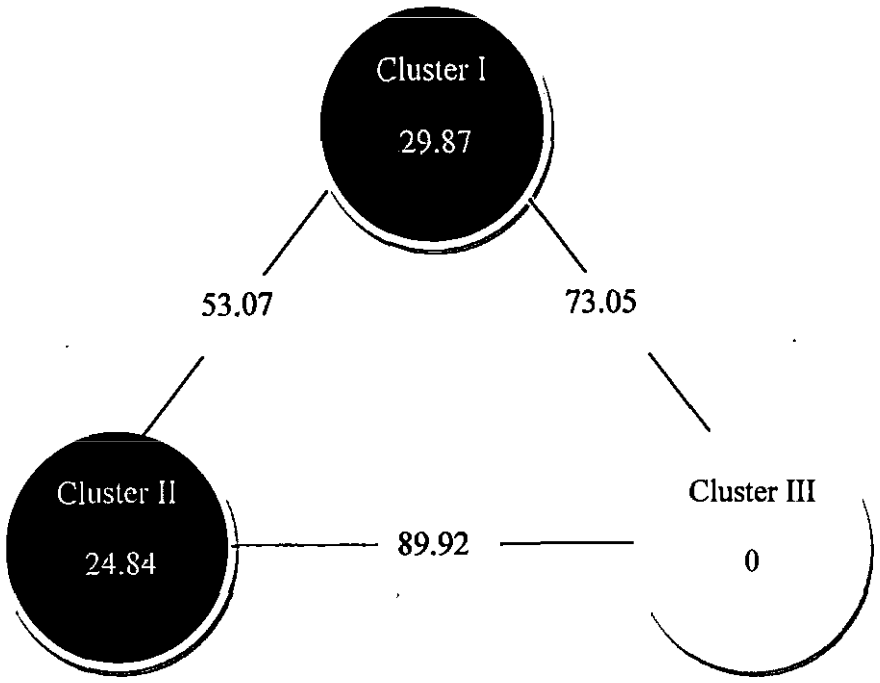
Varieties	Size	Shape	Arrangement of rhizomes (No. of layers)	No. of primary fingers	No. of secondary fingers	No. of tertiary fingers	Length of primary fingers (cm)	Length of secondary fingers (cm)	Girth of primary fingers (cm)	Girth of secondary fingers (cm)	Internodal length of primary finger (cm)	Internodal length of secondary finger (cm)	Thickness of flesh (cm)	Thickness of inner core (cm)	Colour of flesh	Colour of scale
Athira	Bold	Flat	3.66 (2.04)	4.33 (2.20)	14.66 (3.89)	28.00 (5.34)	3.96	3.33	10.86	8.33	0.97	0.67	1.83	1.16	Pale yellow	Brown
Karthika	Medium bold	Round	3.33 (1.93)	4.67 (2.27)	9.33 (3.12)	16.33 (4.10)	3.17	2.87	6.67	6.4	0.87	0.97	1.53	0.83	Bluish yellow	Brown
B3	Medium bold	Round	3.00 (1.86)	4.00 (2.12)	9.00 (3.08)	12.00 (3.53)	3.70	3.26	6.53	6.23	0.93	1.26	1.43	0.83	Pale yellow with bluish tinge	Brown
Maran	Medium bold	Flat	2.00 (1.58)	4.00 (2.12)	10.00 (3.24)	16.00 (4.06)	2.86	2.43	8.23	6.86	0.93	0.83	1.33	0.90	Pale yellow	Brown
292R	Bold	Flat	3.33 (1.95)	4.33 (2.20)	9.33 (3.13)	19.33 (4.44)	3.93	3.73	8.20	9.10	0.83	1.13	1.63	1.20	Pale yellow	Dark Brown
478R	Bold	Flat	3.00 (1.86)	4.33 (2.20)	10.00 (3.22)	11.66 (3.49)	3.40	3.40	7.10	6.60	0.87	1.10	1.70	0.80	Bluish yellow	Brown
88R	Medium bold	Flat	3.67 (2.04)	5.33 (2.41)	10.66 (3.32)	17.67 (4.24)	3.23	3.57	7.40	7.06	1.46	1.00	1.36	0.86	Bluish yellow	Brown
Rio-de-Janeiro	Bold	Flat	1.33 (1.34)	3.33 (1.20)	6.00 (2.54)	8.67 (3.03)	4.33	5.23	7.46	5.30	0.53	0.43	1.53	1.06	Pale yellow	Light Brown
CD (0.05)	–	–	0.36	0.18	0.48	0.41	0.58	0.45	1.14	1.27	0.14	0.93	0.39	1.43	–	–

Table 8. Clustering of ginger varieties / somaclones based on Mahalanobis distances for rhizome characters

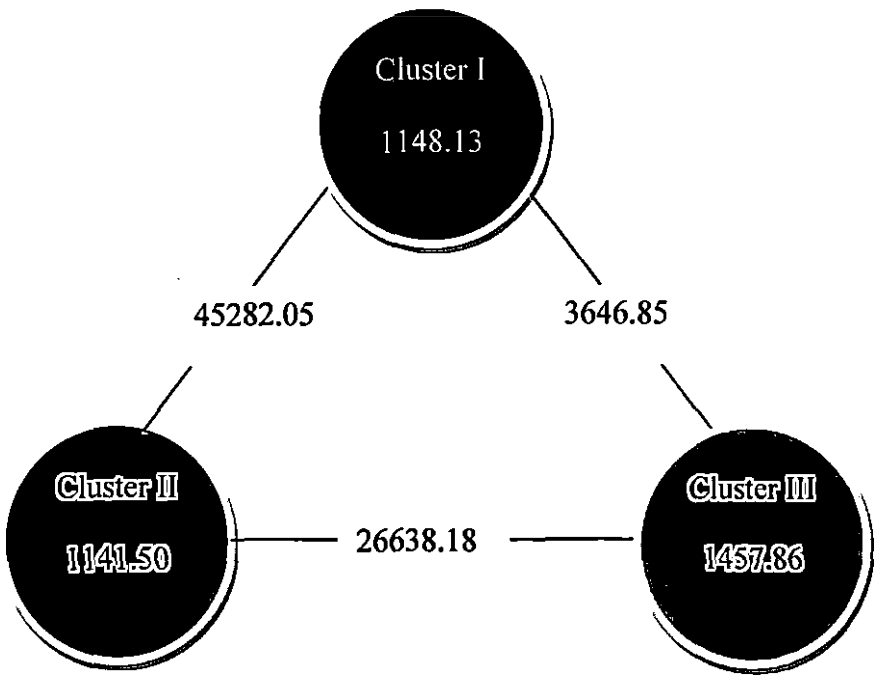
Cluster No.	Varieties / somaclones	No. of genotypes
I	B3, 478R, Rio-de-Janeiro	3
II	Athira, 292R	2
III	Maran, Karthika, 88R	3

Table 9. Rhizome characters in different clusters of ginger varieties / somaclones

Rhizome characters	Cluster I	Cluster II	Cluster III
No. of layers	2.44	3.49	3.00
No. of primary fingers	3.89	4.33	4.66
No. of secondary fingers	9.89	11.99	9.99
No. of tertiary fingers	10.77	23.67	16.67
Length of primary fingers (cm)	3.81	3.93	3.09
Length of secondary fingers (cm)	3.96	3.53	2.96
Girth of primary fingers (cm)	7.03	9.53	7.43
Girth of secondary fingers (cm)	6.04	8.72	6.77
Internodal length of primary finger (cm)	0.78	0.90	1.09
Internodal length of secondary finger (cm)	0.93	0.89	0.93
Thickness of flesh (cm)	1.55	1.73	1.40
Thickness of inner core (cm)	0.89	1.18	0.86



a. Vegetative characters



b. Rhizome characters

Fig. 1 Inter and intra cluster Mahalanobis distances for vegetative and rhizome characters in ginger varieties / somaclones

4.2 Genomic DNA isolation from ginger varieties / somaclones

4.2.1 Source of DNA

For isolation of genomic DNA, leaf samples were collected from potted plants maintained in net house of the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara. As reported in many other crops, young, immature, fully opened and pale green coloured leaves were found best for the recovery of good quality DNA in sufficient quantity from ginger (Plate 3b).

4.2.2 Isolation and purification of DNA

Genomic DNA isolated through the CTAB method reported by Roger and Bendich (1994) was not pure and had RNA contamination (Plate 4a). However, RNAse treatment after the DNA isolation resulted in good quality DNA (Plate 4b).

4.3 Quantification of DNA

The quality and quantity of isolated DNA was analysed using both electrophoresis and NanoDrop^{R} ND-1000 spectrophotometer. Intact clear bands indicated that DNA extracted was non-degraded and was of good quality. The ratio of absorbance for the DNA isolated ranged from 1.80 to 1.90, which indicated that the quality of DNA was good (Table 10). The DNA after appropriate dilutions was used as template for RAPD and ISSR analyses.

4.4 Molecular Characterization

Two marker systems viz., RAPD and ISSR were used for molecular characterization. After initial primer screening using bulked DNA from three clones,

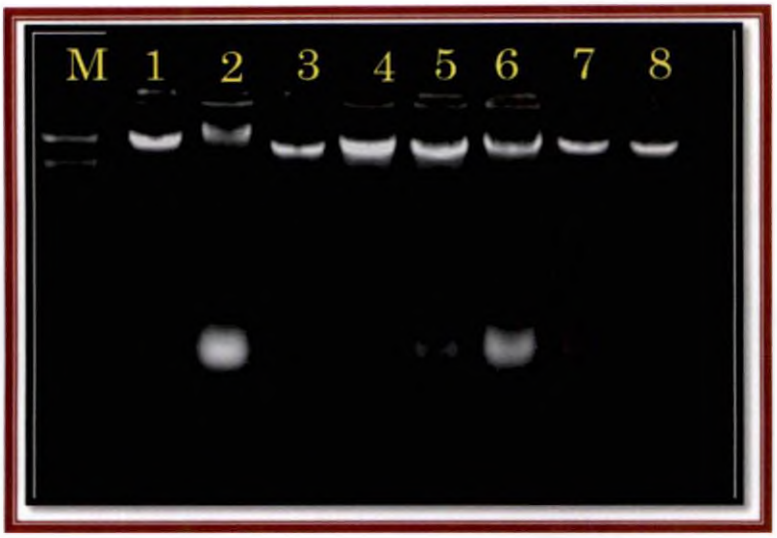
RAPD and ISSR analyses were done with selected primers using genomic DNA from eight ginger varieties / somaclones.

4.4.1 Random Amplified Polymorphic DNA (RAPD analysis:

The RAPD analysis with thermal settings described in the section 3.2.2.4.1 gave good amplification. List of RAPD primers used for screening is given in Table 2. The amplification pattern produced by thirty five primers belonging to different RAPD primer series viz., OPA, OPC, OPD, OPE, OPG, OPK, OPP, OPU, OPAH, RN, RY and S are provided in Table 11 and Plate 5. Based on the presence of clear and distinct bands, ten decamer primers were selected for RAPD assay of ginger varieties / somaclones which are OPA-04, OPA -12, OPA-27, OPA-28, OPD-15, OPD-20, OPP-16, OPU-03, RN-08 and S-11 (Table 12). Finally the analysis of RAPD profiles was carried out using NTSYS.

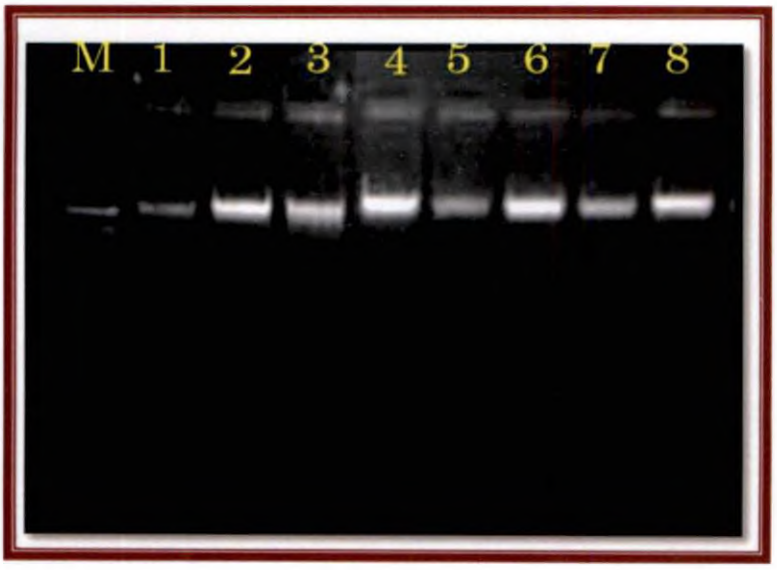
4.4.2 Inter Simple Sequence Repeat (ISSR) analysis:

The ISSR analysis with thermal settings described in the section 3.2.2.4.2 gave good amplification. List of ISSR primers used for screening is given in Table 3. The amplification pattern produced by thirty primers belonging to different ISSR primer series viz., UBC, ISSR and SPS are provided in Table 13 and Plate 6. Based on the presence of clear and distinct bands, eleven primers were selected for ISSR assay of ginger varieties / somaclones. The selected primers were UBC 834, UBC 835, UBC 840, UBC 844, UBC 866, SPS-03, ISSR-04, ISSR-05, ISSR-06, ISSR-08 and ISSR-10 (Table 14). Finally the analysis of ISSR profiles was carried out using NTSYS.



M: Marker Lambda DNA (*Eco* RI/ *Hind* III digest 1000bp)
1: Athira, 2: Karthika, 3: B3, 4: Maran,
5:292R, 6: 478R, 7: 88R, 8: Rio-de-Janeiro

a. Isolated DNA from ginger varieties / somaclones



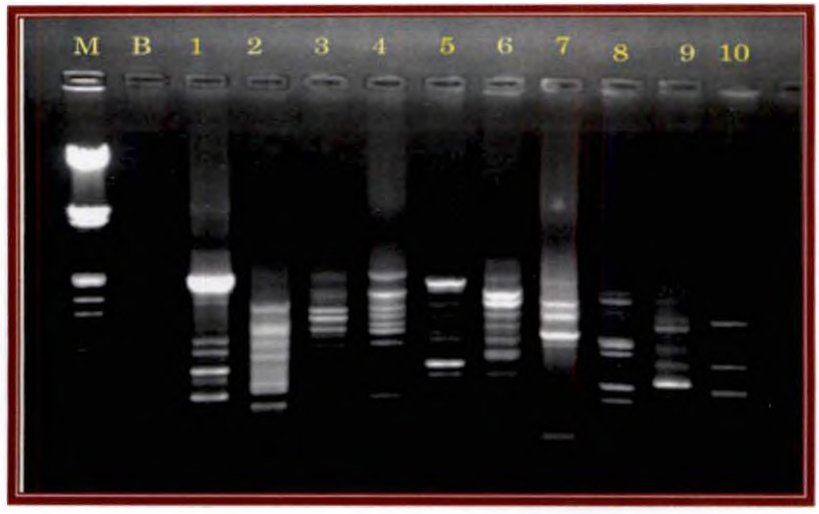
M: Marker Lambda DNA (*Eco* RI/ *Hind* III digest 1000bp)
1: Athira, 2: Karthika, 3: B3, 4: Maran,
5:292R, 6: 478R, 7: 88R, 8: Rio-de-Janeiro

b. Isolated DNA from ginger varieties / somaclones after RNase treatment

Plate 4 Isolated DNA from ginger varieties / somaclones

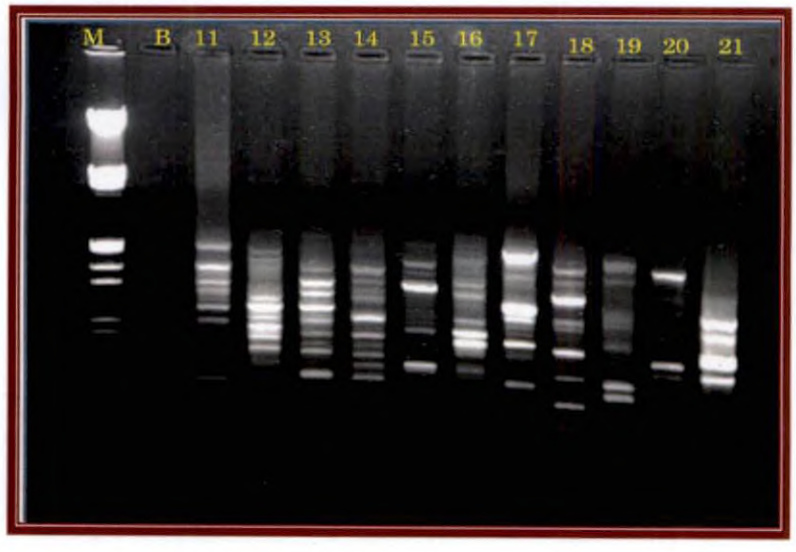
Table 10. Assessment of quality and quantity of DNA isolated from ginger varieties / somaclones

Sl. No.	Varieties / Somaclones	A260	A280	A260/A280	ng/ul
1	Athira	16.277	8.428	1.82	880.90
2	Karthika	6.897	3.342	1.87	1108.16
3	B_3	22.268	11.413	1.86	971.09
4	Maran	11.998	6.667	1.90	923.79
5	292R	14.063	7.401	1.87	1254.11
6	478R	20.567	10.552	1.80	1059.24
7	88R	23.396	12.467	1.88	1147.73
8	Rio-de-Janeiro	31.279	15.485	1.83	863.62



M: 100bp ladder, B: Blank
1: OPA O4, 2: OPC 02, 3: OPAH 3, 4: OPA 08, 5: OPA 12,
6: OPP 17, 7: OPA 02, 8: OPA 27, 9: OPAH 05, 10: OPP 16

a. Amplification with RAPD primers



M: 100bp ladder, B: Blank
11: OPA 28, 12: RY 08, 13: RN 08, 14: OPD 15, 15: OPA 27,
16: RN 07, 17: OPU 03, 18: S 11, 19: OPAH 09, 20: OPD 20, 21: OPE 07

b. Amplification with RAPD primers

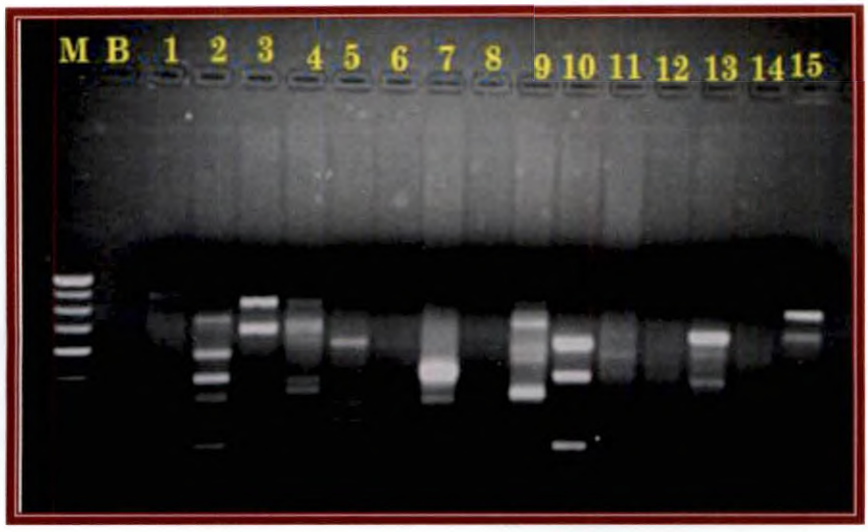
Plate 5 Screening of RAPD primers for amplification of genomic DNA from ginger varieties / somaclones

Table 11. Amplification pattern of RAPD primers in ginger varieties / somaclones

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

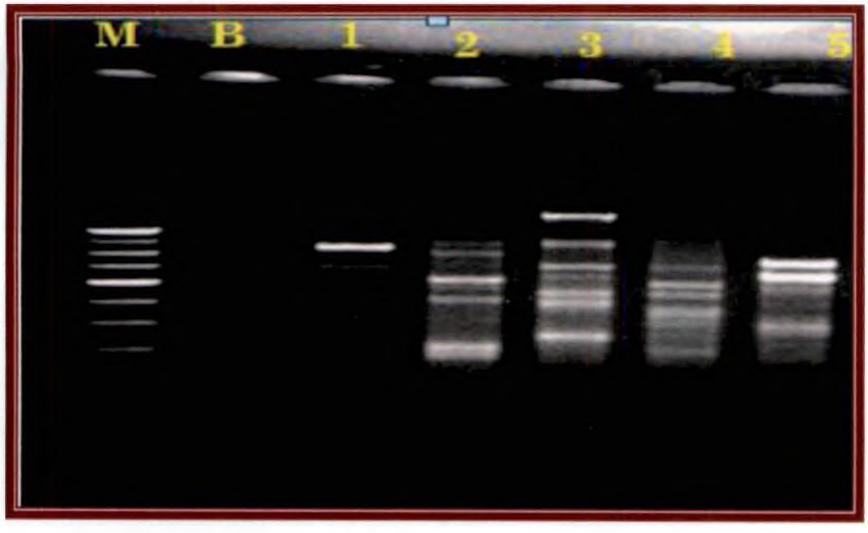
Table 12. Selected RAPD primers

Sl. No.	Name of Primer	Sequence
1	OPA-04	5'AATCGGGCTG3'
2	OPA -12	5' TCGGCGATAG3'
3	OPA-27	5'GAAACGGGTG3'
4	OPA-28	5'GTGACGTAGG3'
5	OPD-15	5'CATCCGTGCT3'
6	OPD-20	5'ACCCGGTAAC3'
7	OPP-16	5'CCAAGCTGCC3'
8	OPU-03	5'CTATGCCGAC3'
9	RN-08	5'ACCTCAGCTC3'
10	S-11	5'GTAGACCCGT3'



M: 100bp ladder, B: Blank
1: UBC 811, 2: UBC 840, 3: UBC 815, 4: UBC 835, 5: UBC 834,
6: UBC 890, 7: UBC 868, 8: UBC 354, 9: UBC 866, 10: UBC 844,
11: ISSR 07, 12: ISSR 15, 13: SPS 03, 14: SPS 08, 15: ISSR 05

a. Amplification with ISSR primers



M: 100bp DNA ladder B: Blank
1: ISSR 09, 2: ISSR 10, 3: ISSR 06, 4: ISSR 04, 5: ISSR 08.

b. Amplification with ISSR primers

Plate 6 Screening of ISSR primers for amplification of genomic DNA from ginger varieties / somaclones

Table 13. Amplification pattern of ISSR primers in ginger varieties / somaclones

Sl. No.	Primers	Amplification Pattern			
		No. of bands	Types of bands		Remarks
			Distinct	Faint	
1.	UBC 840	5	3	2	Selected
2	UBC 844	3	3	0	Selected
3	UBC 890	0			------
4	UBC 811	3	0	3	------
5	UBC813	0			------
6	UBC 815	2	2	0	------
7	UBC354	0			------
8	UBC S 2	1	1	0	------
9	UBC 866	7	2	5	Selected
10	UBC 826	0			------
11	UBC 848	1	0	1	------
12	UBC 845	0			------
13	UBC 868	2	1	1	------
14	UBC 834	5	2	3	Selected
15	UBC 835	6	2	4	Selected
16	UBC 836	4	0	4	------
17	UBC 807	0			------
18	UBC 817	1	0	1	------
19	UBC 818	1	0	1	------
20	UBC 820	1	0	1	------
21	ISSR 04	7	2	5	Selected
22	ISSR 05	3	3	0	Selected
23	ISSR 06	6	1	5	Selected
24	ISSR 07	2	0	2	------
25	ISSR 08	4	2	2	Selected
26	ISSR 09	2	1	1	------
27	ISSR 10	6	2	4	Selected
28	ISSR 15	2	0	2	------
29	SPS 03	5	1	4	Selected
30	SPS 08	3	0	3	------

Table 14. Selected ISSR primers

Sl. No.	Name of Primer	Sequence	Annealing temperature (°C)
1	UBC 834	5'AGAGAGAGAGAGAGAGYT3'	45
2	UBC 835	5'AGAGAGAGAGAGAGAGY3'	43
3	UBC 840	5'GAGAGAGAGAGAGAGAYT3'	45
4	UBC 844	5'CTCTCTCTCTCTCTCTRC3'	47
5	UBC 866	5'CTCCTCCTCCTCCTCCTC3'	55
6	SPS-03	5'GACAGACAGACAGACA3'	43
7	ISSR-04	5'ACACACACACACACACC3'	47
8	ISSR-05	5'CTCTCTCTCTCTCTTG3'	43
9	ISSR-06	5'GAGAGAGAGAGAGAGAC3'	47
10	ISSR-08	5'GAGAGAGAGAGAGAGAT3'	45
11	ISSR-10	5'ACACACACACACACACG3'	47

4.5 DNA fingerprinting of released varieties and selected somaclones of ginger

1. Variety Athira

A. RAPD profile

The amplification pattern observed for the genomic DNA of ginger variety Athira with ten selected RAPD primers is presented in Plate 7a and Table 15.

The number of clear and distinct bands produced by ten RAPD primers was twenty eight. The size of amplicons ranged from 400bp to 1300bp (Table 15). Primers OPA 04, OPA 27, OPP 16 and S 11 gave three distinct bands, while it was only one for OPA 28 and OPU 03. The primers OPD 20 and RN 08 gave five distinct and clear bands. Primers OPA 12 and OPD 15 gave two distinct and clear bands. Fingerprint developed based on presence of clear and distinct bands and size of bands produced with ten RAPD primers is presented in Fig. 2a.

In the fingerprint generated with RAPD profile, no unique band was found for the variety Athira. The variety Athira shared 3.03 per cent of bands with three varieties / somaclones and another 3.03 per cent with four somaclones and 91 per cent with all the eight varieties / somaclones studied in the present investigations (Table 31).

B. ISSR profile

The amplification pattern observed for the genomic DNA of ginger variety Athira with eleven selected ISSR primers is presented in Plate 7b and Table 16.

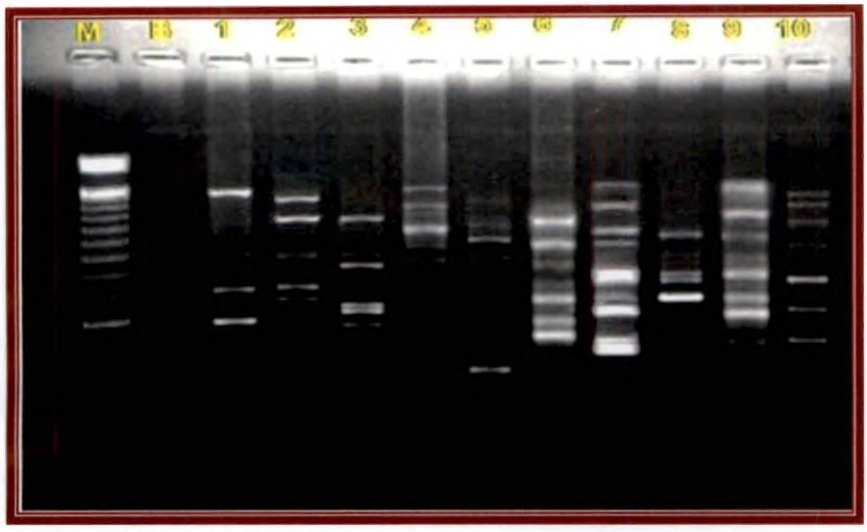
Twenty nine clear and distinct loci were generated with eleven ISSR primers. The amplicons ranged in size from 200bp to 1300bp (Table 16). Most of the primers amplified two and four distinct bands for the variety Athira whereas primers UBC

835 and SPS 03 amplified only one distinct band and primer ISSR 06 amplified five distinct bands. Fingerprint developed based on presence of clear and distinct bands and size of bands produced with eleven ISSR primers is presented in Fig. 2b.

In ISSR fingerprint, the salient feature observed for Athira was the unique band developed by the primer ISSR 06 (900bp) which is highlighted as violet in the fingerprint (Fig. 1b). Sharing of bands was found alike (1.53 per cent) with two, four five and six somaclones. The variety Athira shared 16.9 per cent bands with seven varieties / somaclones and 75 per cent with all the other ginger varieties / somaclones studied in the present investigations (Table 32).

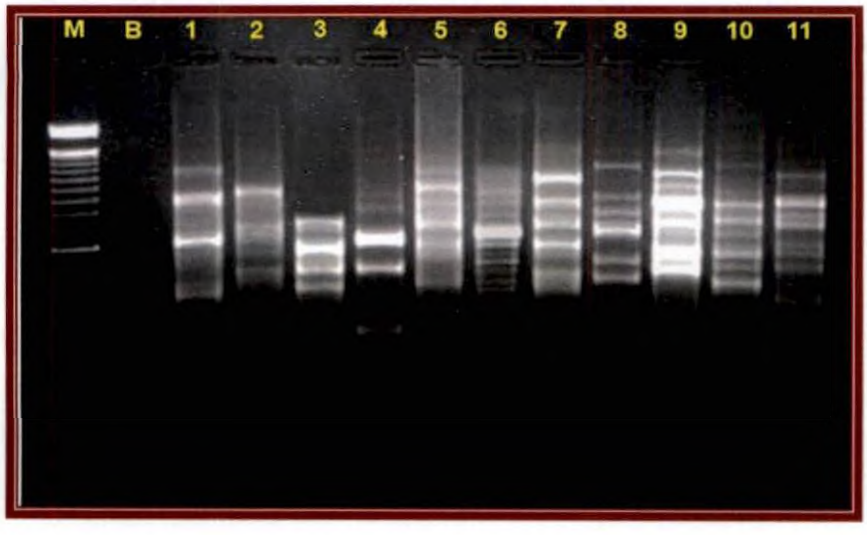
C. Combined RAPD and ISSR profiles

In combined fingerprint generated utilizing RAPD and ISSR profiles, the variety Athira produced a unique fingerprint pattern which showed that 31 per cent of the bands were produced by RAPD primers, 29 per cent by ISSR primers and 40 per cent of the bands were produced by both RAPD and ISSR primers (Fig 10a and Table 33).



M: 100bp ladder, B: Blank
1: OPA 04, 2: OPA 12, 3: OPA 27, 4: OPA 28, 5: OPD 15,
6: OPD 20, 7: OPP 16, 8: OPU 03, 9: RN O8, 10: S 11

a. Amplification with RAPD primers



M: 100bp ladder, B: Blank
1: UBC 834, 2: UBC 835, 3: UBC 840, 4: UBC 844, 5: UBC 866,
6: SPS 03, 7: ISSR 04, 8: ISSR 05, 9: ISSR 06, 10: ISSR 08,
11: ISSR 10

b. Amplification with ISSR primers

Plate 7 Amplification patterns of ginger variety Athira with the selected RAPD and ISSR primers

Table 15. Amplification pattern depicted for the ginger variety Athira with selected RAPD primers

Primers	1	2	3	4	5	6	7	8	9	10
	OPA 04	**OPA 12**	**OPA 27**	**OPA 28**	**OPD 15**	**OPD 20**	**OPP 16**	**OPU 03**	**RN 08**	**S 11**
Total Amplicons	5	8	5	7	7	7	7	3	8	7
Distinct Amplicons	3	2	3	1	2	6	3	1	5	3
Distribution (Mol. Size/bp)										
1400										
1300	–	–		–	–	–	–			–
1200		–	–	–			–		–	
1100				–	–	–			–	–
1000				–	–			–	–	–
900	–	–	–	–	–	–	–		–	–
800	–	–	–			–	–	–	–	
700	–	–	–		–	–	–	–	–	–
600	–	–	–	–	–	–	–		–	–
500		–	–			–	–		–	–
400		–		–	–					
300										
200										
100										

Table 16. Amplification pattern depicted for the ginger variety Athira with selected ISSR primers

Primers	1	2	3	4	5	6	7	8	9	10	11
	UBC 834	**UBC 835**	**UBC 840**	**UBC 844**	**UBC 866**	**SPS 03**	**ISSR 04**	**ISSR 05**	**ISSR 06**	**ISSR 08**	**ISSR 10**
Total Amplicons	4	3	6	5	7	7	6	6	7	7	7
Distinct Amplicons	2	1	4	2	2	1	4	2	5	4	2
Distribution (Mol. Size/bp)											
1400											
1300								–		–	
1200											
1100	–				–		–		–		
1000		–			–	–		–	–		–
900	–			–		–	–		–	–	
800			–	–	–	–		–	–	–	–
700		–			–	–	–	–		–	–
600	–		–	–	–	–	–		–	–	–
500		–	–	–	–	–	–	–	–	–	–
400	–		–		–	–	–	–	–	–	–
300			–	–							–
200			–								
100											

Primers	1	2	3	4	5	6	7	8	9	10
Mol. Size (bp)										
1400										
1300										
1200										
1100										
1000										
900										
800										
700										
600										
500										
400										
300										
200										

a. RAPD Fingerprint

Primers	1	2	3	4	5	6	7	8	9	10	11
Mol. Size (bp)											
1400											
1300											
1200											
1100											
1000											
900											
800											
700											
600											
500											
400											
300											
200											

b. ISSR Fingerprint

Colour code for sharing of bands among varieties / somaclones

Unique band
Shared by two
Shared by three
Shared by four
Shared by five
Shared by six
Shared by seven
Shared by eight

Fig. 2 Fingerprint of the variety Athira generated through RAPD and ISSR analyses

2. Variety Karthika

A. RAPD profile

The amplification pattern observed for the genomic DNA of ginger variety Karthika with ten selected RAPD primers is presented in Plate 8a and Table 17.

Twenty nine clear distinct loci were observed with ten RAPD primers. The amplicons ranged in size 400bp to 2000bp (Table 17). Primers OPA 12 and OPA 27 gave two and four clear and distinct bands respectively. Primer OPA 04, OPP 16 and S 11 gave three distinct and clear bands, whereas, primers OPA 27 and OPD 20 gave four distinct and clear bands. Only one distinct band was produced by primers OPA 28 and OPU 03. Primer OPD 15 gave two distinct bands and RN 08 gave five distinct bands. Fingerprint developed based on clear and distinct bands and size of bands produced with ten RAPD primers is presented in Fig. 3a.

The fingerprint developed with RAPD profile revealed that a unique band was developed for the variety Karthika with the random primer OPA 12 of size 2000bp. Other salient features of the fingerprint are: 2.94 per cent of the bands produced were shared with three, four and seven varieties / somaclones and 88 per cent of the bands were shared with all the eight varieties / somaclones studied in the present investigations (Table 31).

B. ISSR profile

The amplification pattern observed for the genomic DNA of ginger variety Karthika with eleven selected ISSR primers is presented in Plate 8b and Table 18.

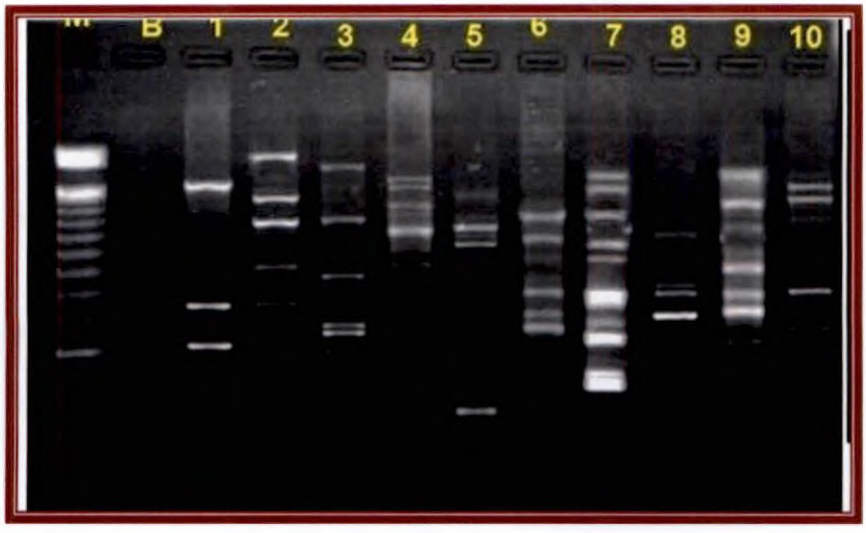
Twenty six clear and distinct loci were produced by eleven ISSR primers. The amplicons ranged in size from 200bp to 1300bp (Table 18). Most of the primers

amplified two distinct bands for the variety Karthika, while it was only one for the primers UBC 835and SPS 03. The primer UBC 840 gave four, ISSR 05 gave three and ISSR 08 gave five distinct and clear bands. Fingerprint developed based on clear and distinct bands and size of bands produced with ten RAPD primers is presented in Fig. 3b.

The fingerprint developed by ISSR profile showed that 1.61 per cent of the bands were shared with four varieties / somaclones, 3.26 per cent of the bands with six varieties / somaclones, 16.13 per cent of the bands with seven varieties / somaclones and 79 per cent of the bands with all the eight ginger varieties / somaclones studied in the present investigations (Table 32).

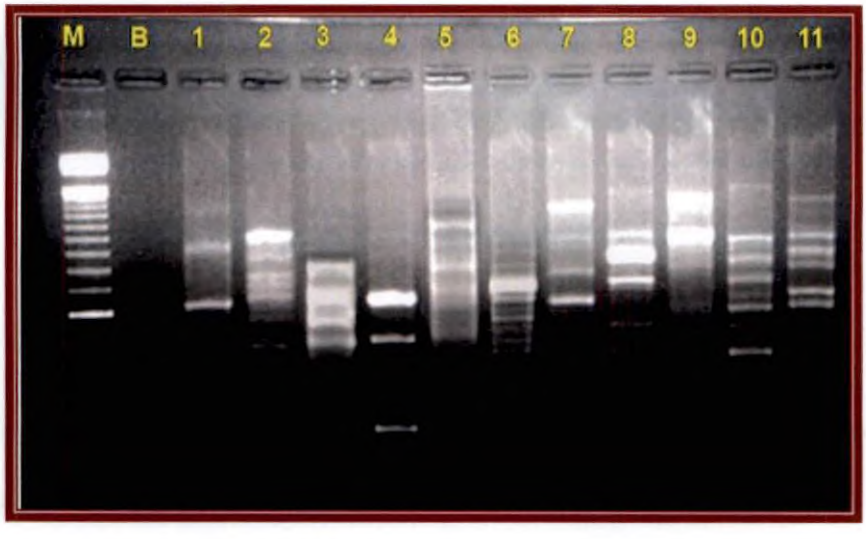
C. Combined RAPD and ISSR profiles

The combined fingerprint generated by the RAPD and ISSR profiles showed that 34 per cent of the bands were produced by RAPD primers, 27 per cent of the bands were produced by ISSR primers and 39 per cent of the bands by both RAPD and ISSR primers (Fig 10b and Table 33).



M: 100bp ladder, B: Blank
1: OPA 04, 2: OPA 12, 3: OPA 27, 4: OPA 28, 5: OPD 15,
6: OPD 20, 7: OPP 16, 8: OPU 03, 9: RN O8, 10: S 11

a. **Amplification with RAPD primers**



M: 100bp ladder, B: Blank
1: UBC 834, 2: UBC 835, 3: UBC 840, 4: UBC 844, 5: UBC 866,
6: SPS 03, 7: ISSR 04, 8: ISSR 05, 9: ISSR 06, 10: ISSR 08,
11: ISSR 10

b. Amplification with ISSR primers

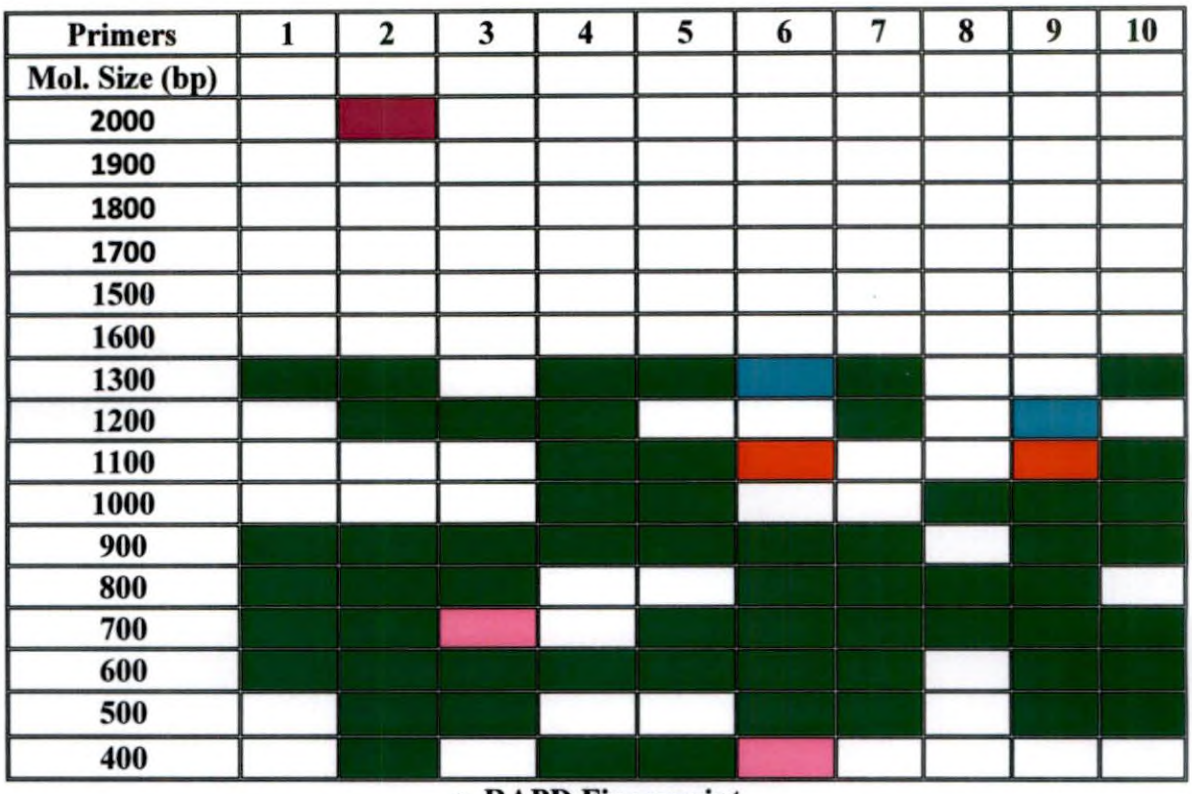
Plate 8 Amplification patterns of ginger variety Karthika with the selected RAPD and ISSR primers

Table 17. Amplification pattern depicted for the ginger variety Karthika with selected RAPD primers

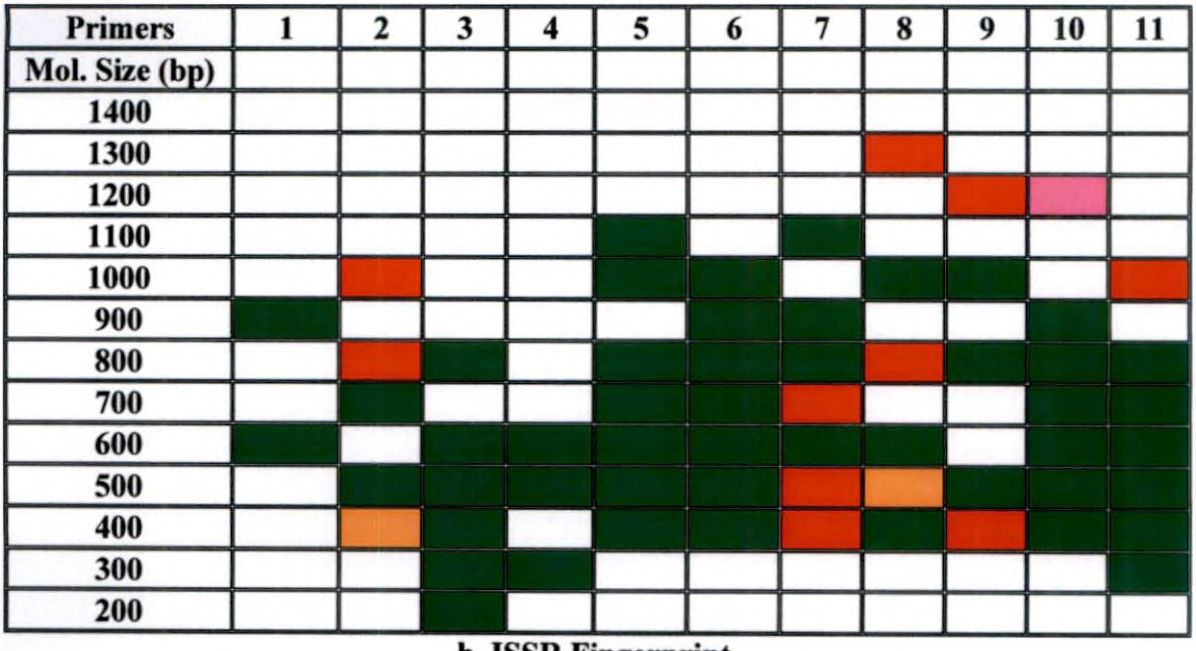
Primers	1	2	3	4	5	6	7	8	9	10
	OPA 04	**OPA 12**	**OPA 27**	**OPA 28**	**OPD 15**	**OPD 20**	**OPP 16**	**OPU 03**	**RN 08**	**S 11**
Total Amplicons	5	8	6	7	7	7	7	3	8	7
Distinct Amplicons	3	2	4	1	2	4	3	1	5	3
Distribution (Mol. Size/bp)										
2000		—								
1900										
1800										
1700										
1600										
1500										
1400										
1300	—	—		—	—	—	—			—
1200		—	—	—			—		—	
1100				—	—	—			—	—
1000				—	—			—	—	—
900	—	—	—	—	—	—	—		—	—
800	—	—	—			—	—	—	—	
700	—	—	—		—	—	—	—	—	—
600	—	—	—	—	—	—	—		—	—
500		—	—			—	—		—	—
400		—		—	—					

Table 18. Amplification pattern depicted for the ginger variety Karthika with selected RAPD primers

Primers	1	2	3	4	5	6	7	8	9	10	11
	UBC 834	**UBC 835**	**UBC 840**	**UBC 844**	**UBC 866**	**SPS 03**	**ISSR 04**	**ISSR 05**	**ISSR 06**	**ISSR 08**	**ISSR 10**
Total Amplicons	2	5	6	3	7	7	6	6	5	7	7
Distinct Amplicons	2	1	4	2	2	1	2	3	2	5	2
Distribution (Mol. Size/bp)											
1400											
1300								–		–	
1200									–		
1100					–		–				
1000		–			–	–		–	–		–
900	–					–	–			–	
800		–	–		–	–	–	–	–	–	–
700		–			–	–				–	–
600	–		–	–	–	–	–	–		–	–
500		–	–	–	–	–	–	–	–	–	–
400		–	–		–	–	–	–	–	–	–
300			–	–							–
200			–								
100											



a. RAPD Fingerprint



b. ISSR Fingerprint

Colour code for sharing of bands among varieties / somaclones

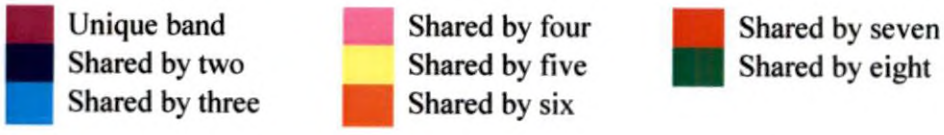


Fig. 3 Fingerprint of the variety Karthika generated through RAPD and ISSR analyses

3. Somaclone B3

A. RAPD profile

The amplification pattern observed for the genomic DNA of ginger somaclone B3 with ten selected RAPD primers is presented in Plate 9a and Table 19.

Ten RAPD primers produced twenty six clear and distinct loci. The amplicons ranged in size 400bp to 1300bp (Table 19). The primers OPA 04, OPA 12 and OPD 15 gave two distinct and clear bands whereas primers OPA 27, OPP 16 and S 11 gave three distinct and clear bands. Primers OPA 28 and OPU 03 gave only one distinct band. Primer OPD 20 gave four distinct bands and primer RN 08 gave five distinct bands. Fingerprint was developed based on clear and distinct bands and size of the bands produced by ten RAPD primers and is presented in Fig. 4a.

The fingerprint generated with RAPD profile for somaclone B3 showed a unique band of size 1400bp with the random primer OPA 12. Banding pattern further showed that 2.98 per cent of the bands produced were shared with three, four and seven varieties / somaclones and 88.24 per cent of the bands with all the eight ginger varieties / somaclones studied in the present investigations (Table 31).

B. ISSR profile

The amplification pattern observed for the genomic DNA of ginger somaclone B3 with eleven selected ISSR primers is presented in Plate 9b and Table 20.

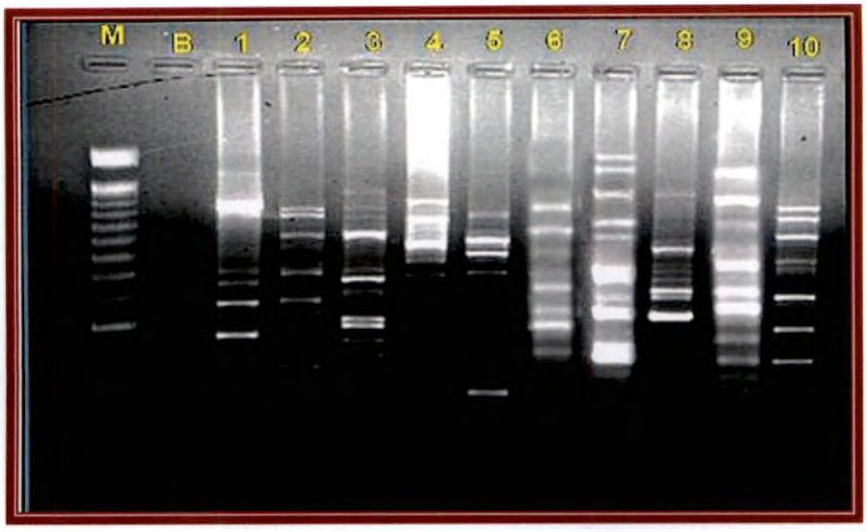
The number of clear and distinct loci produced with eleven ISSR primers was twenty seven. The amplicons ranged in size from 200bp to 1300bp (Table 20). Primers UBC 834, UBC 844, UBC 866, ISSR 05 and ISSR 10 gave two distinct and

clear bands whereas primers UBC 835 and SPS03 gave only one distinct band. Primers ISSR 04 and ISSR 06 gave three clear and distinct bands. Primer UBC 840 gave four and ISSR 08 gave five distinct bands. Fingerprint was developed based on clear and distinct bands (Fig. 4b).

Fingerprint developed for somaclone B3 with eleven ISSR primers is presented in Fig. 5b. The fingerprint revealed that 1.6 per cent of the bands were shared with two and five varieties / somaclones, 3.17 per cent with six varieties / somaclones, 15.87 per cent with two varieties / somaclones and 77.78 per cent with all the ginger varieties / somaclones studied in the present investigations (Table 32).

C. Combined RAPD and ISSR profiles

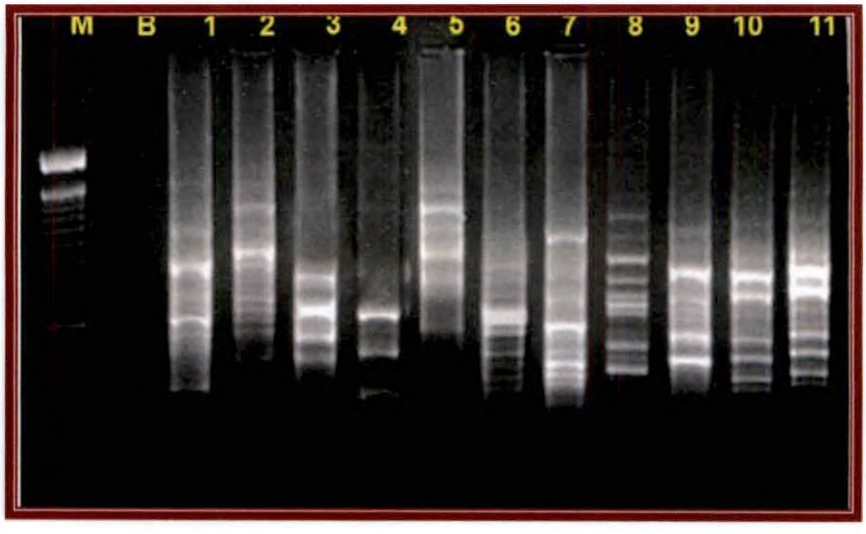
The combined fingerprint generated by the RAPD and ISSR profiles showed that 34.83 per cent of the bands were produced by RAPD primers, 25 per cent of the bands by ISSR primers and 40.45 per cent of the bands by both RAPD and ISSR primers (Fig 10c and Table 33).



M: 100bp ladder, B: Blank
1: OPA 04, 2: OPA 12, 3: OPA 27, 4: OPA 28, 5: OPD 15,

6: OPD 20, 7: OPP 16, 8: OPU 03, 9: RN O8, 10: S 11

a. Amplification with RAPD primers



M: 100bp ladder, B: Blank
1: UBC 834, 2: UBC 835, 3: UBC 840, 4: UBC 844, 5: UBC 866,
6: SPS 03, 7: ISSR 04, 8: ISSR 05, 9: ISSR 06, 10: ISSR 08,
11: ISSR 10

b. Amplification with ISSR primers

Plate 9 Amplification patterns of ginger somaclone B3 with the selected RAPD and ISSR primers

Table 19. Amplification pattern depicted for the ginger somaclone B3 with selected RAPD primers

Primers	1	2	3	4	5	6	7	8	9	10
	OPA 04	**OPA 12**	**OPA 27**	**OPA 28**	**OPD 15**	**OPD 20**	**OPP 16**	**OPU 03**	**RN 08**	**S 11**
Total Amplicons	5	8	5	7	7	7	7	3	8	7
Distinct Amplicons	2	2	3	1	2	4	3	1	5	3
Distribution (Mol. Size/bp)										
1400										
1300	–			–	–	–	–			–
1200		–	–	–			–		–	
1100				–	–	–			–	–
1000				–	–			–	–	–
900	–	–	–	–	–	–	–		–	–
800	–	–	–			–	–	–	–	
700	–	–	–		–	–	–	–	–	–
600	–	–	–	–	–	–	–		–	–
500		–	–			–	–		–	–
400				–	–					
300										
200										
100										

Table 20. Amplification pattern depicted for the ginger somaclone B3 with selected ISSR primers

Primers	1	2	3	4	5	6	7	8	9	10	11
	UBC 834	**UBC 835**	**UBC 840**	**UBC 844**	**UBC 866**	**SPS 03**	**ISSR 04**	**ISSR 05**	**ISSR 06**	**ISSR 08**	**ISSR 10**
Total Amplicons	4	5	6	3	7	7	6	5	5	7	7
Distinct Amplicons	2	1	4	2	2	1	3	2	3	5	2
Distribution (Mol. Size/bp)											
1400											
1300								–			
1200		–									
1100	–				–		–				
1000		–			–	–		–	–	–	–
900	–					–	–			–	
800		–	–		–	–	–		–	–	–
700		–			–	–				–	–
600	–		–	–	–	–	–	–	–	–	–
500		–	–	–	–	–	–	–	–	–	–
400	–		–		–	–	–	–	–	–	–
300			–	–							–
200			–								
100											

Primers	1	2	3	4	5	6	7	8	9	10
Mol. Size (bp)										
1400										
1300										
1200										
1100										
1000										
900										
800										
700										
600										
500										
400										
300										
200										

a. RAPD Fingerprint

Primers	1	2	3	4	5	6	7	8	9	10	11
Mol. Size (bp)											
1400											
1300											
1200											
1100											
1000											
900											
800											
700											
600											
500											
400											
300											
200											

b. ISSR Fingerprint

Colour code for sharing of bands among varieties / somaclones

Unique band
Shared by two
Shared by three
Shared by four
Shared by five
Shared by six
Shared by seven
Shared by eight

Fig. 4 Fingerprint of the somaclone B3 generated through RAPD and ISSR analyses

4. Cultivar Maran

A. RAPD profile

The amplification pattern observed for the genomic DNA of ginger source parent cultivar Maran with ten selected RAPD primers is presented in Plate 10a and Table 21.

Twenty six clear distinct loci were observed with the ten RAPD primers. The amplicons ranged in size 400bp to 1300bp (Table 21). Primers OPA 04, OPD 20, OPP 16 and S11 gave three distinct and clear bands, whereas, primers OPA 28 and OPU 03 gave only one clear and distinct band. Primers OPA 12 and OPD 15 gave two bands and primers OPA 27 and RN 08 gave four clear and distinct bands. Fingerprint was developed based on clear and distinct bands produced with the ten selected RAPD primers (Fig. 5a).

In the fingerprint generated with RAPD profile, all the bands were shared with the ginger varieties / somaclones studied in the present investigations (Table 31).

B. ISSR profile

The amplification pattern observed for the genomic DNA of ginger source parent cultivar Maran with eleven selected ISSR primers is presented in Plate 10b and Table 22.

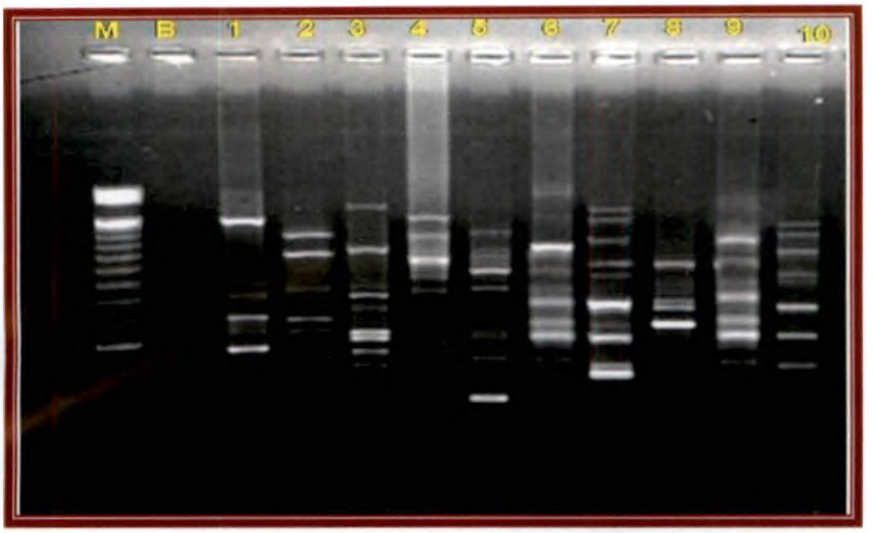
The selected eleven ISSR primers produced thirty one clear and distinct loci. The amplicons ranged in size from 200bp to 1300bp (Table 22). The primers UBC 840, ISSR 04, ISSR 05 and ISSR 06 gave four clear and distinct bands, whereas, primers UBC 834, UBC 844, UBC 866 and ISSR 10 gave two distinct bands. Primers UBC 835 and SPS 03 gave only one clear band and primer ISSR 08 gave five distinct and

clear bands. Fingerprint was developed based on clear and distinct bands produced by the selected ISSR primers (Fig. 5b).

In the fingerprint generated using the selected ISSR primers, it was seen that 5.8 per cent of the bands produced by the cultivar Maran was shared with two varieties / somaclones, 1.5 per cent of the bands with three and five varieties / somaclones, and 4.35 per cent of the bands were shared with six varieties / somaclones. The percentage of bands shared with seven varieties / somaclones was 16 and with all other ginger varieties / somaclones was 71 (Table 32).

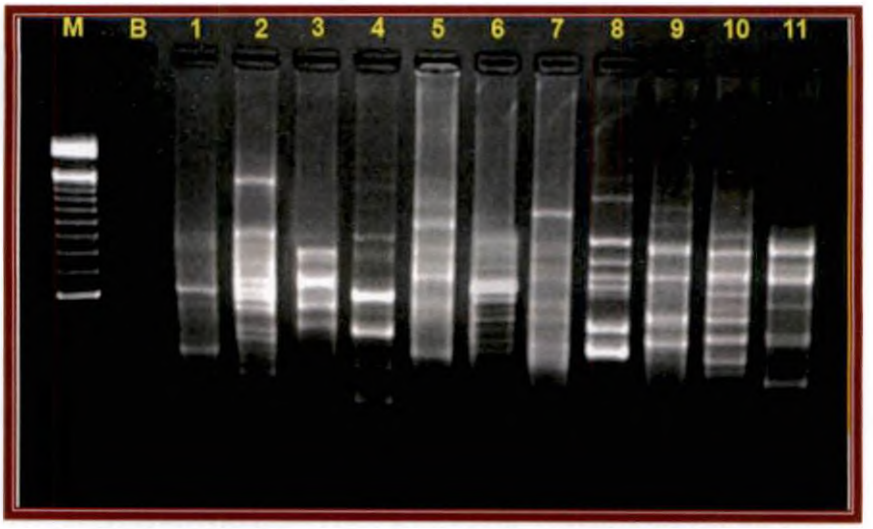
C. Combined RAPD and ISSR profiles

Combined RAPD and ISSR fingerprint produced by the selected primers in cultivar Maran revealed that 30 per cent of the bands were produced by RAPD primers, 30 per cent of the bands by ISSR primers and 40 per cent of the bands by both RAPD and ISSR primers (Fig 10d and Table 33).



M: 100bp ladder, B: Blank
1: OPA 04, 2: OPA 12, 3: OPA 27, 4: OPA 28, 5: OPD 15,
6: OPD 20, 7: OPP 16, 8: OPU 03, 9: RN O8, 10: S 11

a. Amplification with RAPD primers



M: 100bp ladder, B: Blank
1: UBC 834, 2: UBC 835, 3: UBC 840, 4: UBC 844, 5: UBC 866,
6: SPS 03, 7: ISSR 04, 8: ISSR 05, 9: ISSR 06, 10: ISSR 08,
11: ISSR 10

b. Amplification with ISSR primers

Plate 10 Amplification patterns of ginger cultivar Maran with the selected RAPD and ISSR primers

Table 21. Amplification pattern depicted for the ginger cultivar Maran with selected RAPD primers

Primers	1	2	3	4	5	6	7	8	9	10
	OPA 04	**OPA 12**	**OPA 27**	**OPA 28**	**OPD 15**	**OPD 20**	**OPP 16**	**OPU 03**	**RN 08**	**S 11**
Total Amplicons	5	7	6	8	7	7	7	3	7	7
Distinct Amplicons	3	2	4	1	2	3	3	1	4	3
Distribution (Mol. Size/bp)										
1600			–							
1500										
1400										
1300	–	–		–	–		–			–
1200		–	–	–			–			
1100				–	–	–			–	–
1000				–	–			–	–	–
900	–	–	–	–	–	–	–		–	–
800	–	–	–			–	–	–	–	
700	–	–	–	–	–	–	–	–	–	–
600	–	–	–	–	–	–	–		–	–
500		–	–			–	–		–	–
400				–	–					
300										
200										
100										

Table 22. Amplification pattern depicted for the ginger cultivar Maran with selected ISSR primers

Primers	1	2	3	4	5	6	7	8	9	10	11
	UBC 834	**UBC 835**	**UBC 840**	**UBC 844**	**UBC 866**	**SPS 03**	**ISSR 04**	**ISSR 05**	**ISSR 06**	**ISSR 08**	**ISSR 10**
Total Amplicons	3	6	6	3	7	7	5	6	6	7	6
Distinct Amplicons	2	1	4	2	2	1	4	4	4	5	2
Distribution (Mol. Size/bp)											
1400											
1300								–			
1200		–					–		–		
1100					–						
1000		–			–	–		–	–	–	
900	–					–	–			–	
800		–	–		–	–	–	–	–	–	–
700		–			–	–				–	–
600	–		–	–	–	–	–	–	–	–	–
500		–	–	–	–	–	–	–	–	–	–
400	–	–	–		–	–		–	–	–	–
300			–	–							–
200			–								
100											

Primers	1	2	3	4	5	6	7	8	9	10
Mol. Size (bp)										
1400										
1300										
1200										
1100										
1000										
900										
800										
700										
600										
500										
400										
300										
200										

a. RAPD Fingerprint

Primers	1	2	3	4	5	6	7	8	9	10	11
Mol. Size (bp)											
1400											
1300											
1200											
1100											
1000											
900											
800											
700											
600											
500											
400											
300											
200											

b. ISSR Fingerprint

Colour code for sharing of bands among varieties / somaclones

Unique band
Shared by two
Shared by three
Shared by four
Shared by five
Shared by six
Shared by seven
Shared by eight

Fig.5 Fingerprint of the cultivar Maran generated through RAPD and ISSR analyses

5. Somaclone 292R

A. RAPD profile

The amplification pattern produced for the genomic DNA of ginger somaclone 292R with ten selected RAPD primers is presented in Plate 11a and Table 23.

The ten RAPD primers generated twenty six clear distinct loci. The amplicons ranged in size 400bp to 1300bp (Table 23). Primers OPA 12, OPD 15 and OPD 20 gave two clear and distinct bands, whereas, primers OPA 28 and OPU 03 produced only one clear and distinct band. Primers OPA 04 and OPA 27 produced three distinct and clear bands and primers OPP 16, S 11 and RN 08 gave four distinct and clear bands. Fingerprint developed based on clear and distinct bands produced with selected RAPD primers is presented in Fig 6a.

The fingerprint generated by the somaclone 292R with RAPD profile revealed that a unique band of 400bp was produced by random primer OPA 04 for the somaclone 292R. It was further found that three per cent of the bands were shared with four and seven varieties / somaclones and 92 per cent of the bands with all the ginger varieties / somaclones studied in the present investigations (Table 31).

B. ISSR profile

The amplification pattern observed for the genomic DNA of ginger somaclone 292R with eleven selected ISSR primers is presented in Plate 11b and Table 24.

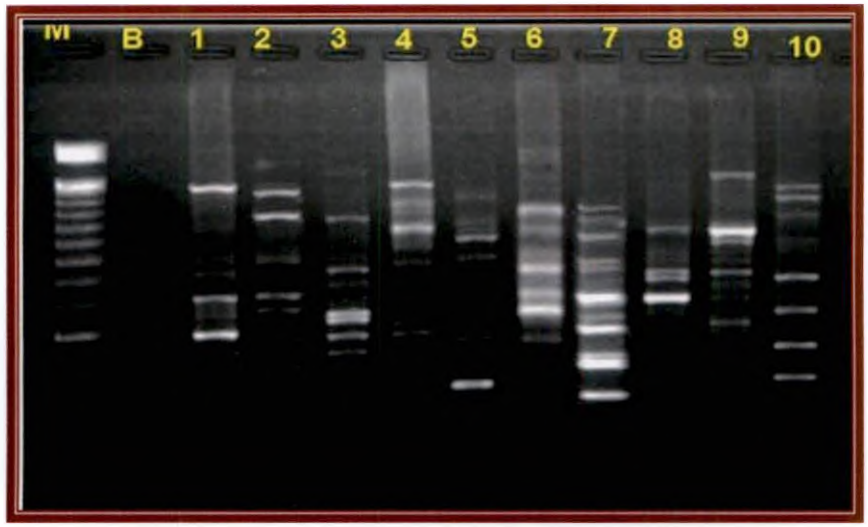
Twenty six clear and distinct loci were observed with eleven selected ISSR primers. The amplicons ranged in size from 200bp to 1200bp (Table 24). Primers UBC 834, UBC 844, UBC 866 and ISSR 10 produced two clear and distinct bands, whereas, primers UBC 840, ISSR 05, ISSR 06 and ISSR 10 produced three clear and distinct bands. Primers UBC 835 and UBC SPS 03 gave only one clear and distinct

band and primer ISSR 04 gave four clear and distinct bands. Fingerprint was developed based on clear and distinct bands produced by eleven selected ISSR primers (Fig 6b).

The fingerprint generated using ISSR prifile showed that 1.4 per cent of the bands were shared with two and three varieties / somaclones. Ten per cent of the bands were shared with four varieties / somaclones and four per cent of the bands were shared with six varieties / somaclones. The percentage of bands shared with seven varieties / somaclones was 14 and with all the ginger varieties / somaclones was 69 (Table 32).

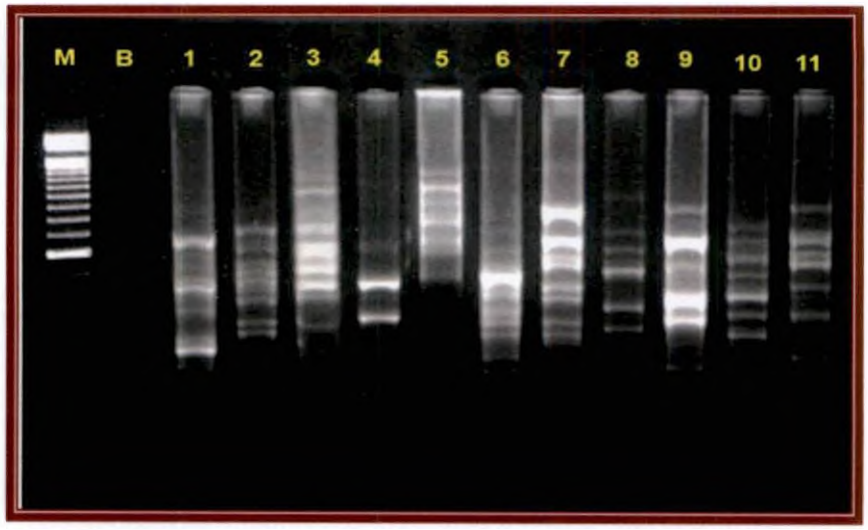
C. Combined RAPD and ISSR profiles

Combined RAPD and ISSR fingerprint generated in 292R revealed that 23 per cent of the bands were produced by RAPD primers, 31 per cent by ISSR primers and 46 per cent of the bands by both RAPD and ISSR primers (Fig 10e and Table 33).



M: 100bp ladder, B: Blank
1: OPA 04, 2: OPA 12, 3: OPA 27, 4: OPA 28, 5: OPD 15,
6: OPD 20, 7: OPP 16, 8: OPU 03, 9: RN O8, 10: S 11

a. Amplification with RAPD primers



M: 100bp ladder, B: Blank
1: UBC 834, 2: UBC 835, 3: UBC 840, 4: UBC 844, 5: UBC 866,
6: SPS 03, 7: ISSR 04, 8: ISSR 05, 9: ISSR 06, 10: ISSR 08,
11: ISSR 10

b. Amplification with ISSR primers

Plate 11 Amplification patterns of ginger somaclone 292R with the selected RAPD and ISSR primers

Table 23. Amplification pattern depicted for the ginger somaclone 292R with selected RAPD primers

Primers	1	2	3	4	5	6	7	8	9	10
	OPA 04	**OPA 12**	**OPA 27**	**OPA 28**	**OPD 15**	**OPD 20**	**OPP 16**	**OPU 03**	**RN 08**	**S 11**
Total Amplicons	6	8	5	7	7	6	8	3	7	8
Distinct Amplicons	3	2	3	1	2	2	4	1	4	4
Distribution (Mol. Size/bp)										
1400										
1300	—	—		—	—		—			—
1200		—	—	—			—			
1100				—	—	—		—	—	—
1000				—	—				—	—
900	—	—	—	—	—	—	—	—	—	—
800	—	—	—			—	—	—	—	
700	—	—			—	—	—		—	—
600	—	—	—	—	—	—	—		—	—
500		—	—			—	—		—	—
400	—	—		—	—		—			—
300										
200										
100										

Table 24. Amplification pattern depicted for the ginger somaclone 292R with selected ISSR primers

Primers	1	2	3	4	5	6	7	8	9	10	11
	UBC 834	**UBC 835**	**UBC 840**	**UBC 844**	**UBC 866**	**SPS 03**	**ISSR 04**	**ISSR 05**	**ISSR 06**	**ISSR 08**	**ISSR 10**
Total Amplicons	4	5	10	3	8	9	6	6	6	7	7
Distinct Amplicons	2	1	3	2	2	1	4	3	3	3	2
Distribution (Mol. Size/bp)											
1400											
1300				–							
1200			–	–				–	–		
1100			–	–		–	–				
1000				–		–		–	–	–	–
900	–		–	–		–	–			–	
800		–	–			–	–	–	–	–	–
700		–	–			–				–	–
600	–		–	–	–	–	–	–	–	–	–
500		–	–		–	–	–	–	–	–	–
400	–	–	–	–		–	–	–	–	–	–
300			–		–	–					–
200	–		–								
100											

Primers	1	2	3	4	5	6	7	8	9	10
Mol. Size (bp)										
1400										
1300										
1200										
1100										
1000										
900										
800										
700										
600										
500										
400										
300										
200										

a. RAPD Fingerprint

Primers	1	2	3	4	5	6	7	8	9	10	11
Mol. Size (bp)											
1400											
1300											
1200											
1100											
1000											
900											
800											
700											
600											
500											
400											
300											
200											

b. ISSR Fingerprint

Colour code for sharing of bands among varieties / somaclones

Unique band
Shared by two
Shared by three
Shared by four
Shared by five
Shared by six
Shared by seven
Shared by eight

Fig. 6 Fingerprint of the somaclone 292R generated through RAPD and ISSR analyses

6. Somaclone 478R

A. RAPD profile

The amplification pattern observed for the genomic DNA of ginger somaclone 478R with ten selected RAPD primers is presented in Plate 12a and Table 25.

The total number of distinct loci observed with ten selected RAPD primers was twenty six. The amplicons ranged in size 400bp to 1300bp (Table 25). Primers OPA 12, OPD 15 and OPD 20 gave two clear and distinct bands, whereas, primers OPA 28 and OPU 03 produced only one clear and distinct band. Primers OPA 04 and OPA 27 produced three distinct and clear bands and primers OPP 16, S 11 and RN 08 gave four distinct and clear bands. Fingerprint was developed based on clear and distinct bands produced by ten selected RAPD primers (Fig. 7a).

The salient feature of the fingerprint developed by RAPD profile in somaclone 478R was the unique band of size 300b with the primer OPA 28. Three per cent of the bands in the fingerprint were shared with four and seven varieties / somaclones and 92 per cent of the bands with all the eight varieties / somaclones studied in the present investigations (Table 31).

B. **ISSR profile**

The amplification pattern observed for the genomic DNA of ginger somaclone 478R with eleven selected ISSR primers is presented in Plate 12b and Table 26.

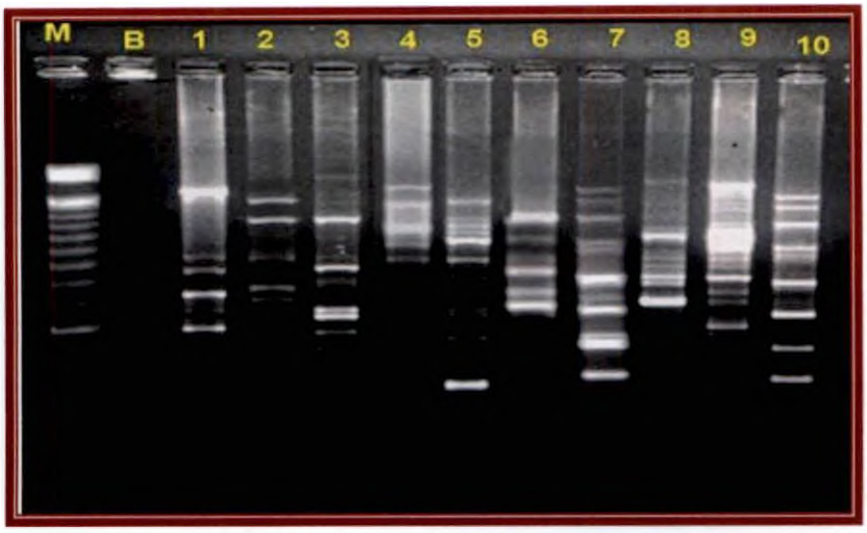
Twenty four clear and distinct loci were observed with eleven ISSR primers. The amplicons ranged in size from 200bp to 1300bp (Table 26). Primers UBC 834, UBC 840, UBC 844, UBC 866, ISSR 05 and ISSR 10 gave two clear and distinct bands, whereas, ISSR 06 and ISSR 08 gave three clear and distinct bands. Primers UBC 835 and SPS 03 gave only one clear and distinct band and primer ISSR 04 gave

four clear and distinct bands. Clear and distinct bands produced by eleven selected ISSR primers were used to develop the fingerprint (Fig. 7b).

In the fingerprint generated by ISSR profile, 1.4 per cent of the bands were shared with two varieties/somaclones, 12 per cent with four varieties / somaclones, 1.4 per cent with five varieties / somaclones and three per cent with four varieties / somaclones. About 14 per cent of the bands were shared with seven varieties / somaclones and 49 per cent of the bands with the eight varieties / somaclones studied in the present investigations (Table 32).

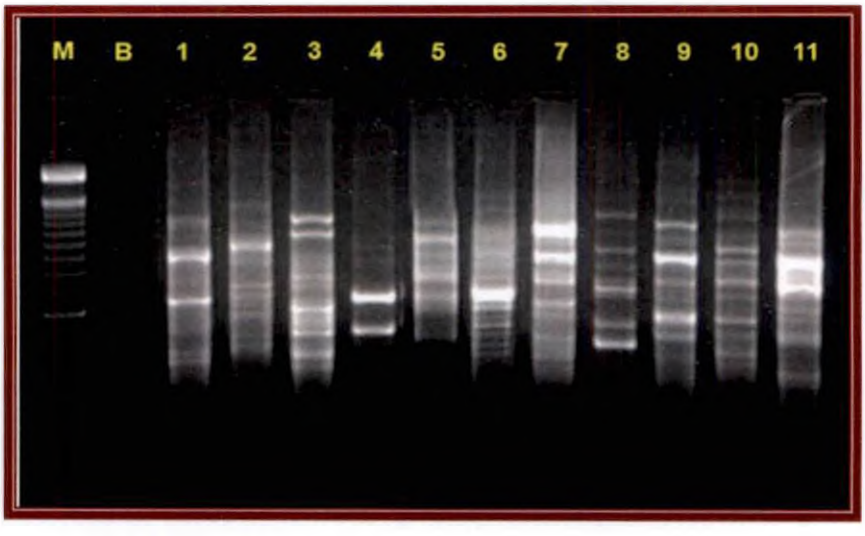
C. Combined RAPD and ISSR profiles

The combined RAPD and ISSR fingerprint generated revealed that 25 per cent of the bands were produced by RAPD primers, 30 per cent of the bands produced by ISSR primers and 45 per cent by both RAPD and ISSR primers (Fig 10f and Table 33).



M: 100bp ladder, B: Blank
1: OPA 04, 2: OPA 12, 3: OPA 27, 4: OPA 28, 5: OPD 15,
6: OPD 20, 7: OPP 16, 8: OPU 03, 9: RN O8, 10: S 11

a. **Amplification with RAPD primers**



M: 100bp ladder, B: Blank
1: UBC 834, 2: UBC 835, 3: UBC 840, 4: UBC 844, 5: UBC 866,
6: SPS 03, 7: ISSR 04, 8: ISSR 05, 9: ISSR 06, 10: ISSR 08,
11: ISSR 10

b. Amplification with ISSR primers

Plate 12 Amplification patterns of ginger somaclone 478R with the selected RAPD and ISSR primers

Table 25. Amplification pattern depicted for the ginger somaclone 478R with selected RAPD primers

Primers	1	2	3	4	5	6	7	8	9	10
	OPA 04	**OPA 12**	**OPA 27**	**OPA 28**	**OPD 15**	**OPD 20**	**OPP 16**	**OPU 03**	**RN 08**	**S 11**
Total Amplicons	5	8	5	8	7	6	8	3	7	8
Distinct Amplicons	3	2	3	1	2	2	4	1	4	4
Distribution (Mol. Size/bp)										
1400										
1300				–	–		–			–
1200	–	–	–	–			–			
1100		–		–	–	–		–	–	–
1000				–	–				–	–
900	–	–	–	–	–	–	–	–	–	–
800	–	–	–			–	–	–	–	
700	–	–			–	–	–		–	–
600	–	–	–	–	–	–	–		–	–
500		–	–			–	–		–	–
400		–		–	–		–			–
300				–						
200										
100										

Table 26. Amplification pattern depicted for the ginger somaclone 478R with selected ISSR primers

Primers	1	2	3	4	5	6	7	8	9	10	11
	UBC 834	**UBC 835**	**UBC 840**	**UBC 844**	**UBC 866**	**SPS 03**	**ISSR 04**	**ISSR 05**	**ISSR 06**	**ISSR 08**	**ISSR 10**
Total Amplicons	4	5	9	3	8	10	5	5	5	8	7
Distinct Amplicons	2	1	2	2	2	1	4	2	3	3	2
Distribution (Mol. Size/bp)											
1400											
1300								–			
1200			–							–	
1100	–		–		–	–	–				
1000		–			–	–		–	–	–	–
900	–		–		–	–	–			–	
800		–	–		–	–	–	–	–	–	–
700		–			–	–				–	–
600	–		–	–	–	–	–	–	–	–	–
500		–	–	–	–	–			–	–	–
400	–	–	–		–	–	–	–		–	–
300			–	–		–					–
200			–			–					
100											

Primers	1	2	3	4	5	6	7	8	9	10
Mol. Size (bp)										
1400										
1300										
1200										
1100										
1000										
900										
800										
700										
600										
500										
400										
300										
200										

a. RAPD Fingerprint

Primers	1	2	3	4	5	6	7	8	9	10	11
Mol. Size (bp)											
1400											
1300											
1200											
1100											
1000											
900											
800											
700											
600											
500											
400											
300											
200											

b. ISSR Fingerprint

Colour code for sharing of bands among varieties / somaclones

Unique band
Shared by two
Shared by three
Shared by four
Shared by five
Shared by six
Shared by seven
Shared by eight

Fig. 7 Fingerprint of the somaclone 478R generated through RAPD and ISSR analyses

7. Somaclone 88R

A. RAPD profile

The amplification pattern observed for the genomic DNA of ginger somaclone 88R with ten selected RAPD primers is presented in Plate 13a and Table 27.

A total of twenty six clear distinct loci were observed with the ten RAPD primers. The amplicons ranged in size 400bp to 1300bp (Table 27). Primers OPA 12, OPD 15 and OPD 20 gave two clear and distinct bands, whereas, primers OPA 28 and OPU 03 produced only one clear and distinct band. Primers OPA 04 and OPA 27 produced three distinct and clear bands and primers OPP 16, S 11 and RN 08 gave four distinct and clear bands. Fingerprint was developed based on clear and distinct bands produced by ten selected RAPD primers (Fig. 8a).

The fingerprint generated by RAPD profile showed that three per cent of the bands were shared with four and seven varieties / somaclones and 94 per cent of the bands with the eight varieties / somaclones studied in the present investigations (Table 31).

B .ISSR profile

The amplification pattern observed for the genomic DNA of ginger somaclone 88R with 11 selected ISSR analysis with the thermal settings standardized in section 3.2.4.2 is presented in Plate 13b and Table 28.

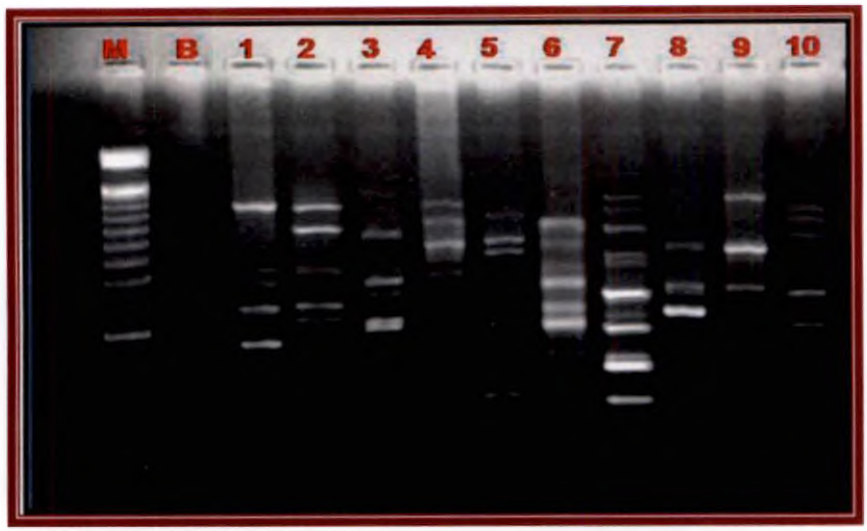
Twenty six clear and distinct loci were observed with eleven ISSR primers. The amplicons ranged in size from 200bp to 1300bp (Table 28). Primers UBC 840, ISSR 05, ISSR 06 and ISSR 08 gave three clear and distinct bands, whereas, primers UBC 834, UBC 844, UBC 866 and ISSR 10 gave two clear and distinct bands. Primers UBC 835 and SPS 03 gave one clear and distinct band and primer ISSR 04

gave four clear and distinct band. Fingerprint was developed based on clear and distinct bands (Fig. 8b).

The fingerprint developed by ISSR profile in the somaclone 88R showed that 1.3 per cent of the bands were shared with three varieties / somaclones, 11 per cent with four varieties / somaclones. 1.3 per cent with five varieties / somaclones and three per cent with six varieties / somaclones. The percentage of bands shared with seven varieties / somaclones was 16 and with the eight ginger varieties / somaclones studied in the present investigation was 49 (Table 32).

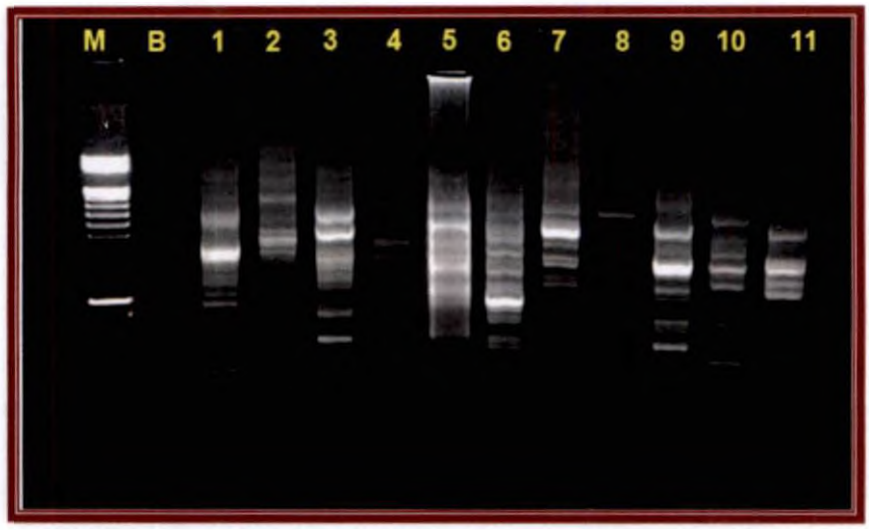
C. Combined RAPD and ISSR profiles

Combined fingerprint data produced by RAPD and ISSR profile revealed that 23 per cent of the bands were produced by RAPD primers, 33 per cent of the bands by ISSR primers and 44 per cent of the bands by both RAPD and ISSR primers (Fig 10g and Table 33).



M: 100bp ladder, B: Blank
1: OPA 04, 2: OPA 12, 3: OPA 27, 4: OPA 28, 5: OPD 15,
6: OPD 20, 7: OPP 16, 8: OPU 03, 9: RN O8, 10: S 11

a. **Amplification with RAPD primers**



M: 100bp ladder, B: Blank
1: UBC 834, 2: UBC 835, 3: UBC 840, 4: UBC 844, 5: UBC 866,
6: SPS 03, 7: ISSR 04, 8: ISSR 05, 9: ISSR 06, 10: ISSR 08,
11: ISSR 10

b. Amplification with ISSR primers

Plate 13 Amplification patterns of ginger somaclone 88R with the selected RAPD and ISSR primers

Table 27. Amplification pattern depicted for the ginger somaclone 88R with selected RAPD primers

Primers	1	2	3	4	5	6	7	8	9	10
	OPA 04	**OPA 12**	**OPA 27**	**OPA 28**	**OPD 15**	**OPD 20**	**OPP 16**	**OPU 03**	**RN 08**	**S 11**
Total Amplicons	5	8	5	7	7	6	8	3	7	8
Distinct Amplicons	3	2	3	1	2	2	4	1	4	4
Distribution (Mol. Size/bp)										
1400										
1300	–	–		–	–					–
1200		–	–	–			–			
1100				–	–	–	–	–	–	–
1000				–	–				–	–
900	–	–	–	–	–	–	–	–	–	–
800	–	–	–			–	–	–	–	
700	–	–			–	–	–		–	–
600	–	–	–	–	–	–	–		–	–
500		–	–			–	–		–	–
400		–		–	–		–			–
300										
200										
100										

Table 28. Amplification pattern depicted for the ginger somaclone 88R with selected ISSR primers

Primers	1	2	3	4	5	6	7	8	9	10	11
	UBC 834	**UBC 835**	**UBC 840**	**UBC 844**	**UBC 866**	**SPS 03**	**ISSR 04**	**ISSR 05**	**ISSR 06**	**ISSR 08**	**ISSR 10**
Total Amplicons	5	5	9	3	8	9	6	5	7	8	7
Distinct Amplicons	2	1	3	2	2	1	4	3	3	3	2
Distribution (Mol. Size/bp)											
1400											
1300								–			
1200			–						–	–	
1100	–		–		–	–	–				
1000		–			–	–		–	–	–	–
900	–		–		–	–	–			–	
800		–	–		–	–	–	–	–	–	–
700		–			–	–				–	–
600	–		–	–	–	–	–	–	–	–	–
500		–	–	–	–	–	–		–	–	–
400	–	–	–		–	–	–	–	–	–	–
300			–	–		–			–		–
200	–		–								
100											

Primers	1	2	3	4	5	6	7	8	9	10
Mol. Size (bp)										
1400										
1300										
1200										
1100										
1000										
900										
800										
700										
600										
500										
400										
300										
200										

a. RAPD Fingerprint

Primers	1	2	3	4	5	6	7	8	9	10	11
Mol. Size (bp)											
1400											
1300											
1200											
1100											
1000											
900											
800											
700											
600											
500											
400											
300											
200											

b. ISSR Fingerprint

Colour code for sharing of bands among varieties / somaclones

Unique band
Shared by two
Shared by three
Shared by four
Shared by five
Shared by six
Shared by seven
Shared by eight

Fig. 8 Fingerprint of the somaclone 88R generated through RAPD and ISSR analyses

8. Cultivar Rio-de-Janeiro

A. RAPD profile

The amplification pattern observed for the genomic DNA of ginger source parent cultivar Rio-de-Janeiro with ten selected RAPD primers is presented in Plate 14a and Table 29.

The ten RAPD primers produced twenty five clear distinct loci. The amplicons ranged in size 400bp to 1300bp (Table 29). Primers OPA 04, OPA 27 and RN 08 gave three clear and distinct bands, whereas, primers OPA 12, OPD 15 and OPD 20 gave two clear and distinct bands. Primers OPP 16 and S11 gave four clear and distinct bands, whereas, primers OPA 28 and OPU 03 gave only one clear and distinct band. Fingerprint was developed based on clear and distinct bands generated by ten selected RAPD primers (Fig. 9a).

The fingerprint generated with RAPD profile in the cultivar Rio-de-Janeiro revealed that 3.33 per cent of the bands was shared with four varieties / somaclones and 97 per cent of the bands with all the eight ginger varieties / somaclones studied in the present investigation (Table 31).

B. ISSR profile

The amplification pattern observed for the genomic DNA of ginger source parent cultivar Rio-de-Janeiro with 11 selected ISSR analysis with the thermal settings standardized in section 3.2.4.2 is presented in Plate 8a and Table 29.

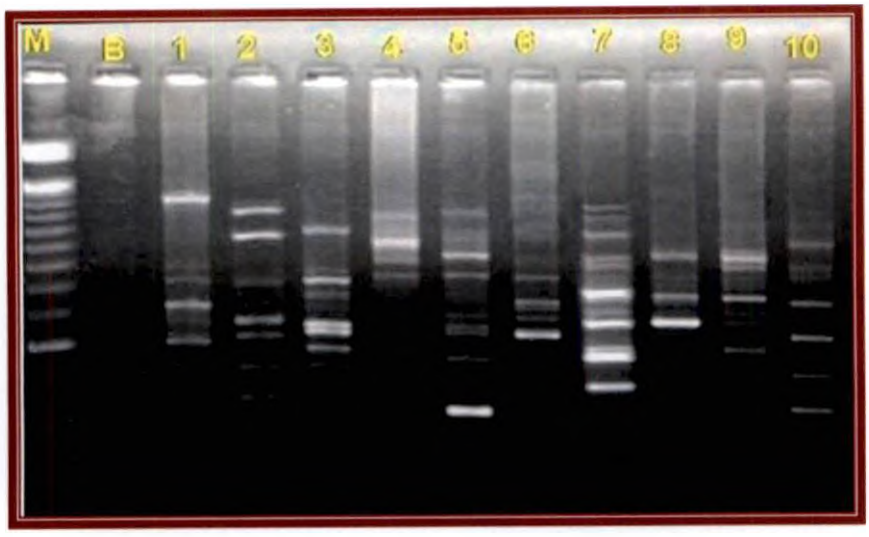
Twenty eight clear and distinct loci were observed with 11 ISSR primers. The amplicons ranged in size from 200bp to 1300bp (Table 29). Primers UBC 834, UBC 844, UBC 866 and ISSR 10 gave two clear and distinct bands, whereas primers UBC 840, ISSR 06 and ISSR 08 gave three clear and distinct bands. Primers UBC 835 and

SPS 03 gave one clear and distinct band and primer ISSR 04 gave four clear and distinct band. Fingerprint was developed based on clear and distinct bands (Fig 9b).

The fingerprint generated with ISSR profile in the cultivar Rio-de-Janeiro showed that four per cent of the bands were shared with two varieties / somaclones, 1.3 per cent of the bands with three varieties / somaclones, nine per cent with four varieties / somaclones, one percent with five, four per cent with six and 15 with seven varieties / somaclones. The cultivar Rio-de-Janeiro shared 65 per cent of the bands with all the eight ginger varieties/somaclones studied in the present investigations (Table 32).

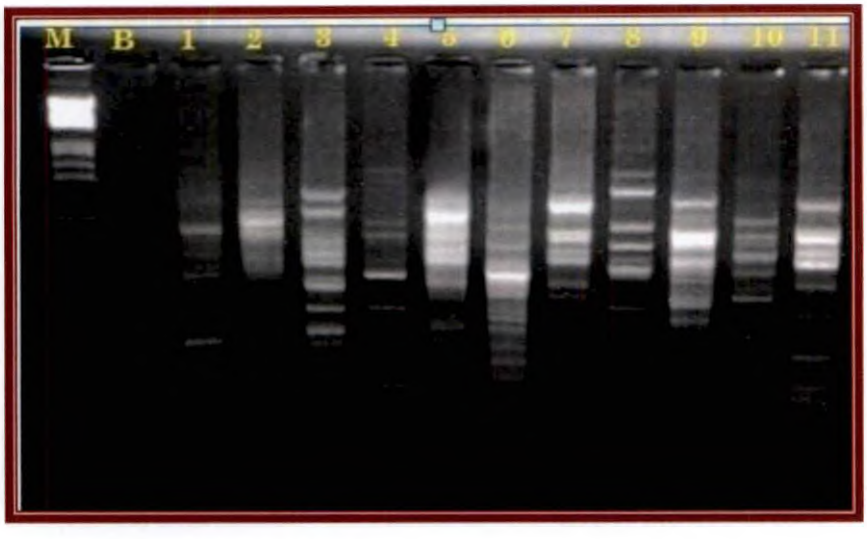
C. Combined RAPD and ISSR profiles

Combined fingerprint data generated by RAPD and ISSR profiles showed that 20 per cent of the bands were produced by RAPD primers, 36 per cent of the bands by ISSR primers and 44 per cent by both RAPD and ISSR primers (Fig 10h and Table 33).



M: 100bp ladder, B: Blank
1: OPA 04, 2: OPA 12, 3: OPA 27, 4: OPA 28, 5: OPD 15,
6: OPD 20, 7: OPP 16, 8: OPU 03, 9: RN O8, 10: S 11

a. Amplification with RAPD primers



M: 100bp ladder, B: Blank
1: UBC 834, 2: UBC 835, 3: UBC 840, 4: UBC 844, 5: UBC 866,
6: SPS 03, 7: ISSR 04, 8: ISSR 05, 9: ISSR 06, 10: ISSR 08,
11: ISSR 10

b. Amplification with ISSR primers

Plate 14 Amplification patterns of ginger cultivar Rio-de-Janeiro with the selected RAPD and ISSR primers

Table 29. Amplification pattern depicted for the ginger cultivar Rio-de-Janeiro with selected RAPD primers

Primers	1	2	3	4	5	6	7	8	9
	OPA 04	**OPA 12**	**OPA 27**	**OPA 28**	**OPD 15**	**OPD 20**	**OPP 16**	**OPU 03**	**RN 08**
Total Amplicons	5	8	5	7	7	5	8	3	6
Distinct Amplicons	3	2	3	1	2	2	4	1	3
Distribution (Mol. Size/bp)									
1400									
1300	—	—		—	—		—		
1200		—	—	—			—		
1100				—	—			—	
1000				—	—				—
900	—	—	—	—	—	—	—	—	—
800	—	—	—			—	—	—	—
700	—	—			—	—	—		—
600	—	—	—	—	—	—	—		—
500		—	—			—	—		—
400		—		—	—		—		
300									
200									
100									

Table 30. Amplification pattern depicted for the ginger cultivar Rio-de-Janeiro with selected ISSR primers

Primers	1	2	3	4	5	6	7	8	9	10	11
	UBC 834	**UBC 835**	**UBC 840**	**UBC 844**	**UBC 866**	**SPS 03**	**ISSR 04**	**ISSR 05**	**ISSR 06**	**ISSR 08**	**ISSR 10**
Total Amplicons	5	5	9	3	8	9	5	8	6	7	7
Distinct Amplicons	2	1	3	2	2	1	4	5	3	3	2
Distribution (Mol. Size/bp)											
1400											
1300								–			
1200			–					–			
1100	–		–		–	–	–				
1000		–			–	–		–	–	–	–
900	–		–		–	–	–			–	
800		–	–		–	–	–	–	–	–	–
700		–			–	–		–		–	–
600	–		–	–	–	–	–	–	–	–	–
500		–	–	–	–	–	–	–	–	–	–
400	–	–	–		–	–		–	–	–	–
300			–	–		–			–		–
200	–		–								
100											

Primers	1	2	3	4	5	6	7	8	9	10
Mol. Size (bp)										
1400										
1300										
1200										
1100										
1000										
900										
800										
700										
600										
500										
400										
300										
200										

a. RAPD Fingerprint

Primers	1	2	3	4	5	6	7	8	9	10	11
Mol. Size (bp)											
1400											
1300											
1200											
1100											
1000											
900											
800											
700											
600											
500											
400											
300											
200											

b. ISSR Fingerprint

Colour code for sharing of bands among varieties / somaclones

Unique band
Shared by two
Shared by three
Shared by four
Shared by five
Shared by six
Shared by seven
Shared by eight

Fig. 9: Fingerprint of the cultivar Rio-de-Janeiro generated through RAPD and ISSR analyses

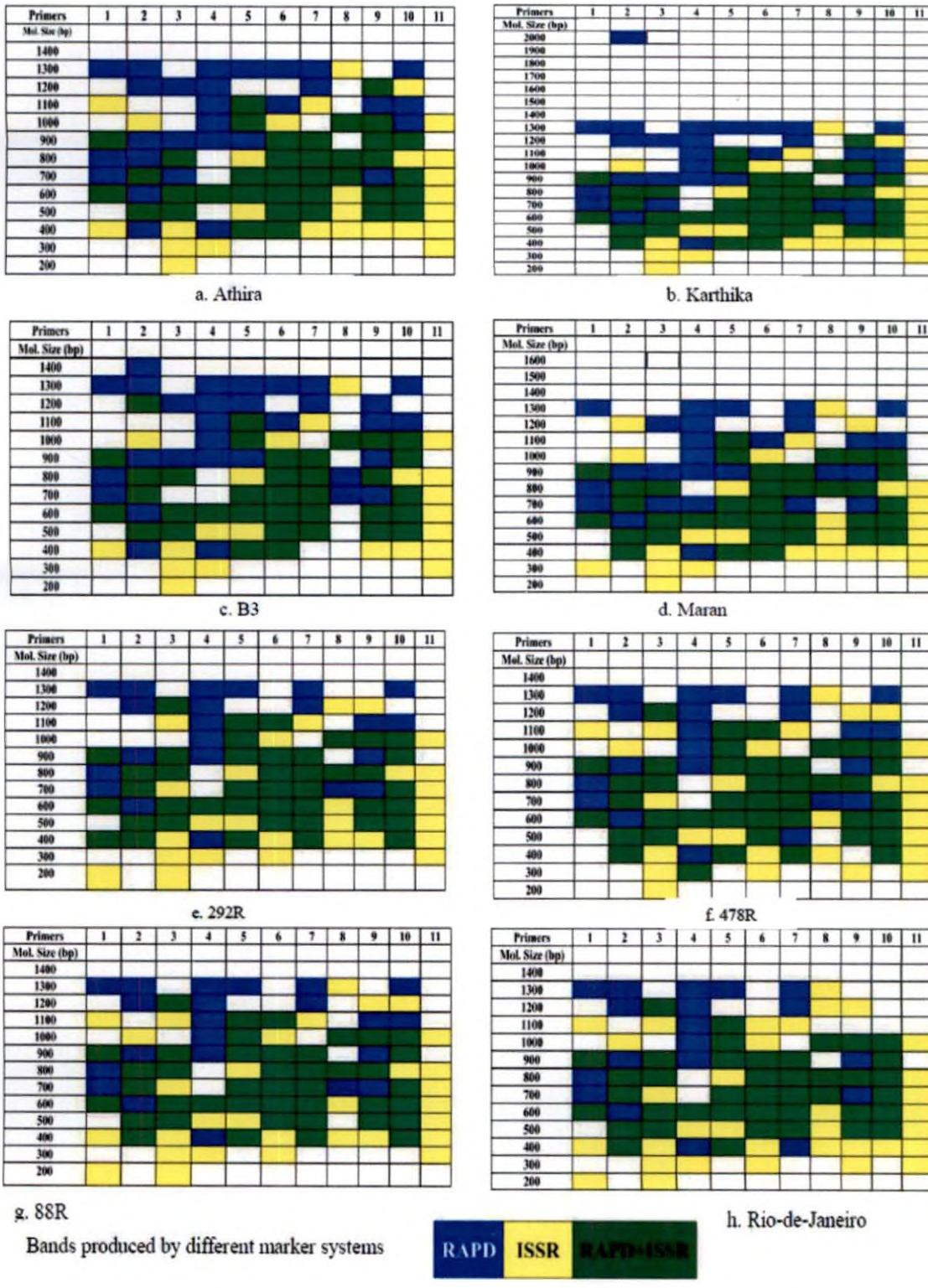


Fig. 10 Combined fingerprints of eight ginger varieties / somaclones

Table 31. Unique band / sharing of bands in fingerprints generated in ginger varieties / somaclones using RAPD profiles

Colour code	Unique band / sharing of bands	Number and percentage of bands shared							
		Athira	Karthika	B3	Maran	292R	478R	88R	Rio-de-Janeiro
Violet	Unique band	0	1 (1.53*)	1 (1.49*)	0	1 (1.53*)	1 (1.53*)	0	0
Navy blue	Shared by two	0	0	0	0	0	0	0	0
Light blue	Shared by three	2 (3.03*)	2 (2.94*)	2 (2.98*)	0	0	0	0	0
Pink	Shared by four	2 (3.03*)	2 (2.94*)	2 (2.98*)	0	2 (3.07*)	2 (3.07*)	2 (3.13*)	2 (3.33*)
Yellow	Shared by five	0	0	0	0	0	0	0	0
Orange	Shared by six	0	0	0	0	0	0	0	0
Red	Shared by seven	2 (3.03*)	2 (2.94*)	2 (2.98*)	0	2 (3.07*)	2 (3.07*)	2 (3.13*)	0
Green	Shared by eight	60 (90.9*)	60 (88.2*)	60 (89.5*)	60 (100*)	60 (92.3*)	60 (92.3*)	60 (93.8*)	60 (96.8*)
Total number of bands with selected primers		66	67	67	60	65	65	64	62

* (Percentage of bands shared)

Table 32. Unique band / sharing of bands in fingerprints generated in ginger varieties / somaclones using ISSR profiles

Colour code	Unique band / sharing of bands	Number and percentage of bands shared							
		Athira	Karthika	B3	Maran	292R	478R	88R	Rio-de-Janeiro
Violet	Unique band	1 (1.53*)	0	0	0	0	0	0	0
Navy blue	Bands shared by two varieties/somaclones	1 (1.53*)	0	1 (1.59*)	4 (5.80*)	1 (1.41*)	1 (1.41*)	0	3 (4.0*)
Light blue	Bands shared by three varieties/somaclones	0	0	0	1 (1.45*)	1 (1.41*)	0	1 (1.37*)	1 (1.33*)
Pink	Bands shared by four varieties/somaclones	1 (1.53*)	1 (1.61*)	0	0	7 (9.86*)	8 (11.3*)	8 (10.9*)	7 (9.33*)
Yellow	Bands shared by five varieties/somaclones	1 (1.53*)	0	1 (1.59*)	1 (1.45*)	0	1 (1.41*)	1 (1.37*)	1 (1.33*)
Orange	Bands shared by six varieties/somaclones	1 (1.53*)	2 (3.26*)	2 (3.17*)	3 (4.35*)	3 (4.23*)	2 (2.82*)	2 (2.74*)	3 (4.0*)
Red	Bands shared by seven varieties/somaclones	11 (16.92*)	10 (16.13*)	10 (15.9*)	11 (15.9*)	10 (14.0*)	10 (14.1*)	12 (16.4*)	11 (14.6*)
Green	Bands shared by eight varieties/somaclones	49 (75.38*)	49 (79.03*)	49 (77.8*)	49 (71.0*)	49 (69.0*)	49 (69.0*)	49 (67.1*)	49 (65.3*)
Total number of bands with selected primers		65	62	63	69	71	71	73	75

* (Percentage of bands shared)

Table 33. Analysis of fingerprints generated in ginger varieties / somaclones with RAPD and ISSR profiles

Colour code	Bands produced by	Number and percentage of bands produced							
		Athira	Karthika	B3	Maran	292R	478R	88R	Rio-de-Janeiro
Blue	RAPD	29 (31.18*)	32 (34.04*)	31 (34.83*)	28 (30.8*)	22 (23.4*)	23 (24.7*)	22 (23.2*)	19 (20.2*)
Yellow	ISSR	27 (29.03*)	25 (26.6*)	22 (24.72*)	27 (29.7*)	29 (30.9*)	28 (30.1*)	31 (32.63*)	34 (36.17*)
Green	RAPD+ISSR	37 (39.78*)	37 (39.3*)	36 (40.45*)	36 (39.5*)	43 (45.7*)	42 (45.2*)	42 (44.21*)	41 (43.61*)
Total number of bands with selected primers		93	94	89	91	94	93	95	94

* (Percentage of bands produced by each marker system)

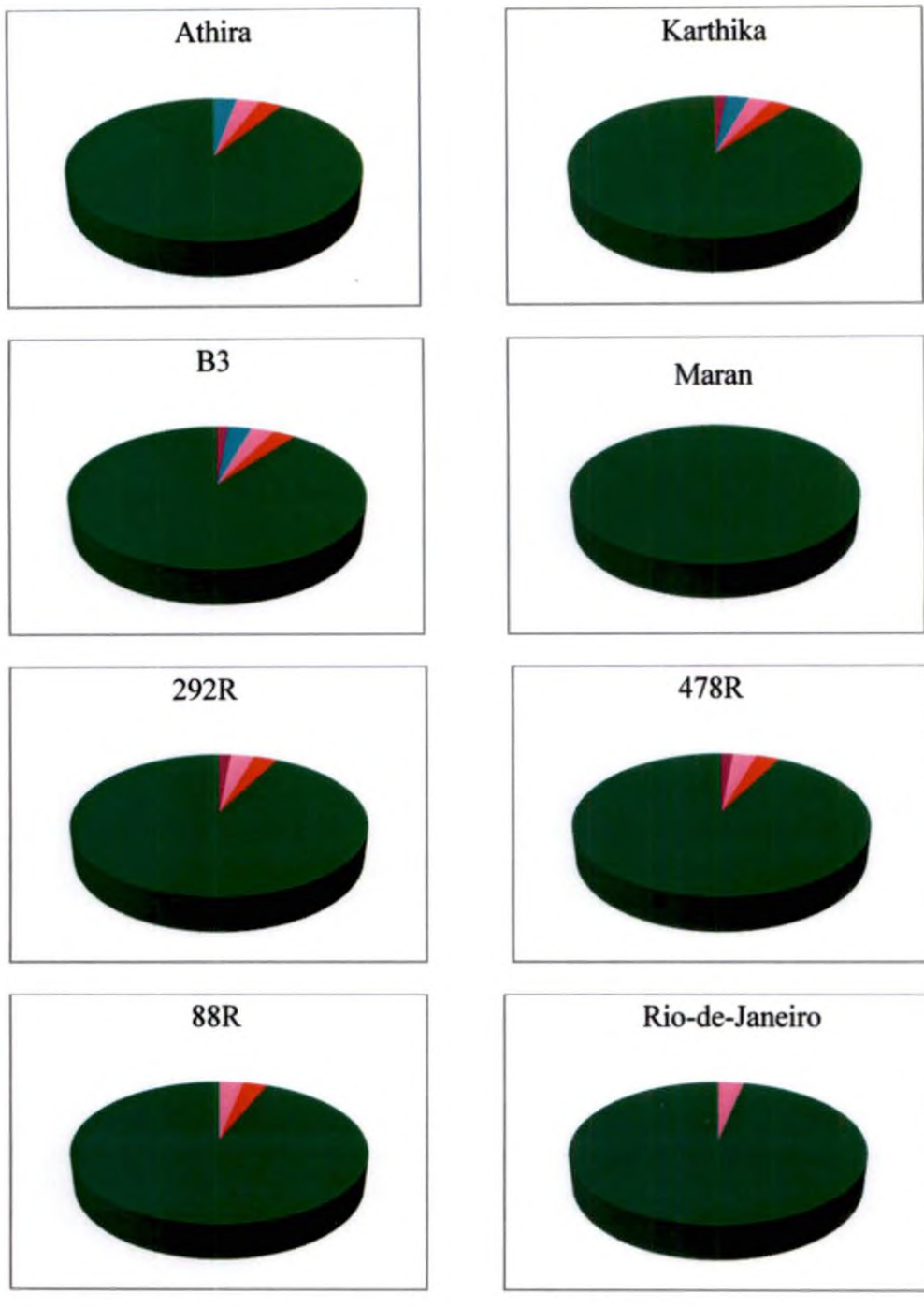


Fig. 11 Unique band / sharing of bands in fingerprints generated in ginger varieties / somaclones using RAPD profiles

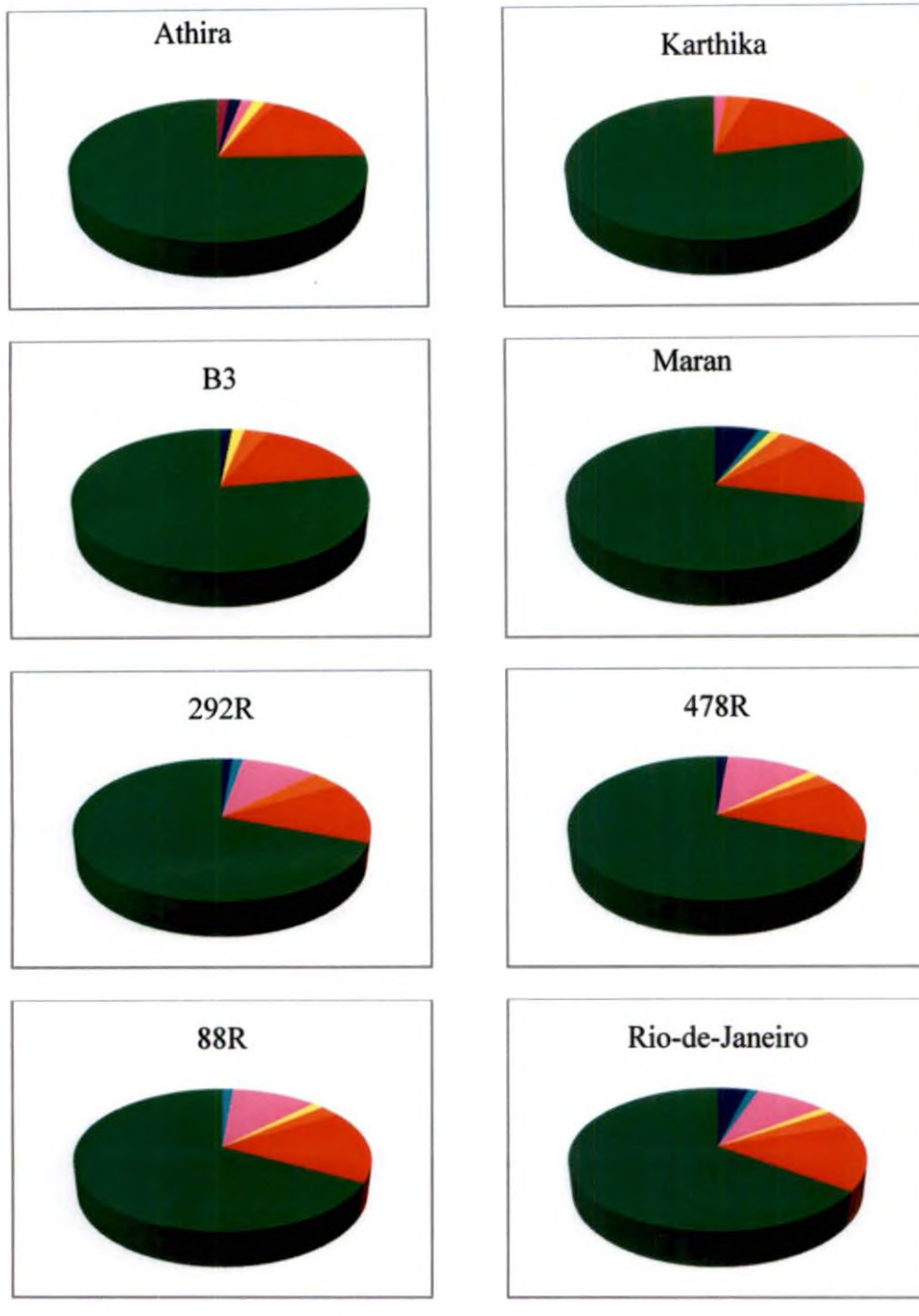


Fig. 12 Unique band / sharing of bands in fingerprints generated in ginger varieties / somaclones using ISSR profiles

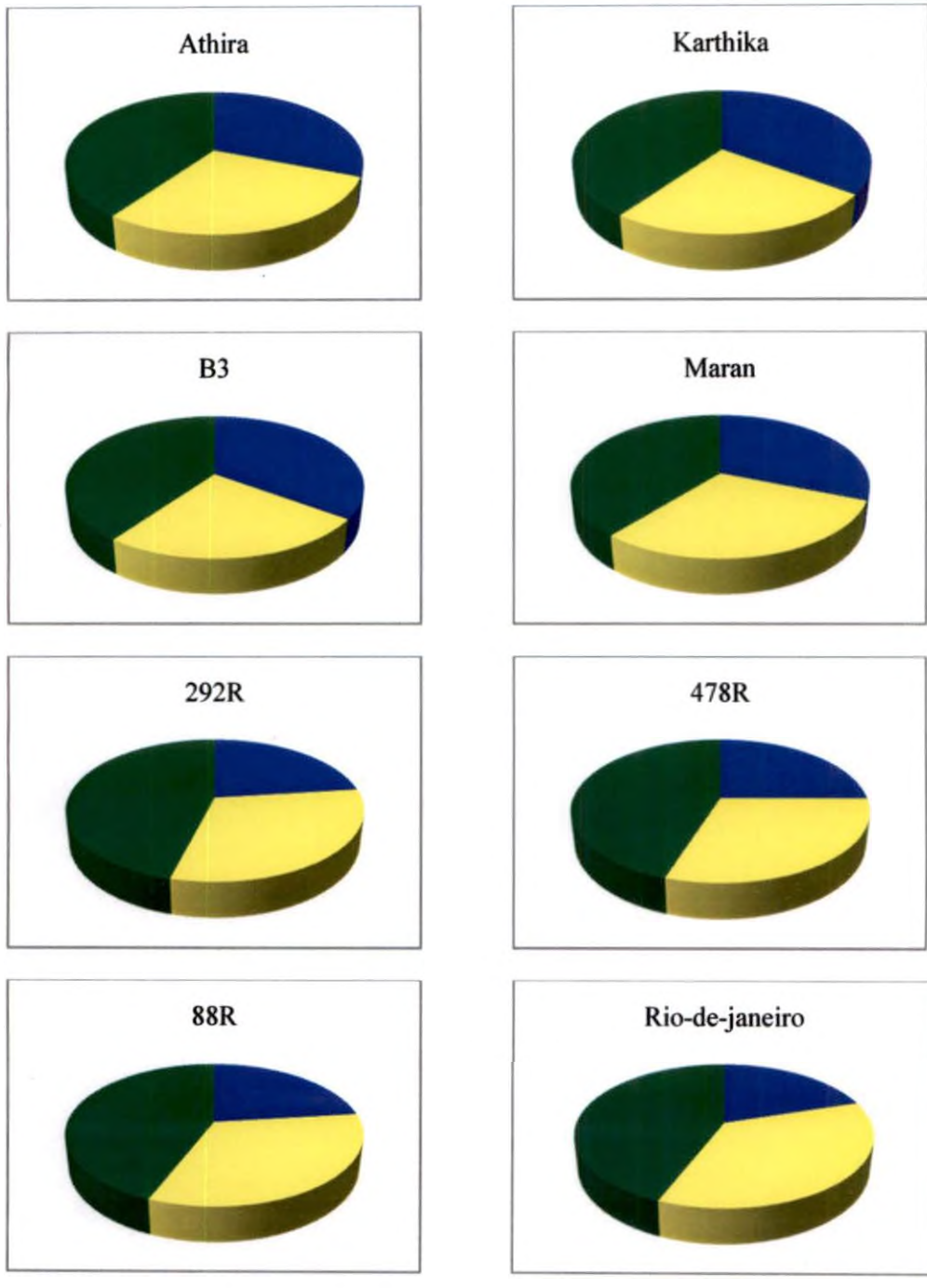


Fig. 13 Bands produced by different marker systems in fingerprints of ginger varieties / somaclones

4.6 Data analysis

Reproducible and well resolved fragments were scored manually and assigned one (1) for the presence of band and zero (0) for the absence of band. The data analysis was done using the software NTSYS pc.

4.6.1 Cluster analysis of the ginger varieties / somaclones

4.6.1.1 Cluster analysis using the RAPD profile

The RAPD data was analysed using NTSYS pc and dendrogram was generated by UPGMA analysis. The similarity coefficient ranged from 0.8356 to 1.0000 indicating 17.5 per cent variability in the varieties / somaclones. In Maran group, the selected superior somaclones were found variable from the original source parent cultivar by five to eight per cent. In Rio-de-Janeiro group, the selected superior somaclones were found variable from the original source parent cultivar by five to six per cent. When the variability from source parent cultivar Rio-de-Janeiro was compared, the somaclone 292R exhibited more variability (six per cent) as compared to other somaclones which showed five per cent. The genetic similarity matrix of ginger varieties / somaclones analysed by Jaccard's coefficient method of the NTSYS program using RAPD data is presented in Table 34.

Dendrogram (Fig. 14) separated the eight ginger varieties / somaclones into two major clusters with a Jaccard's similarity coefficient of 0.89 to 1.00.The first cluster included the clones derived from the cultivar Maran (Athira, Karthika and B3) and the source parent cultivar. The dendrogram revealed that the variety Athira was most diverse and Karthika was most closely related to the source parent cultivar Maran. The second cluster included the clones derived from the cultivar Rio-de-Janeiro (292R, 478R and 88R) and the source parent cultivar. It is evident from the dendrogram that the somaclone 292R was most diverse and somaclones 478R and

88R were very closely related to the source parent cultivar Rio-dr-Janeiro. The somaclones 478R and 88R showed highest similarity (98 per cent).

4.6.1.2 Cluster analysis using the ISSR profile

The ISSR data was analysed using NTSYS pc and dendrogram was generated by UPGMA analysis. The similarity coefficient ranged from 0.7733 to 1.0000 indicating 23 per cent variability in the varieties / somaclones studied. When the variability from source parent cultivar Maran was compared, the variety Athira exhibited more variability (15 per cent) as compared to other varieties / somaclones which ranged from ten to eleven per cent. In Rio-de-Janeiro group, the selected superior somaclones were found variable from the original source parent cultivar by eight to twelve per cent. The genetic similarity matrix of ginger varieties / somaclones analysed by Jaccard's coefficient method of the NTSYS program using ISSR data is presented in Table 35.

Dendrogram (Fig. 15) separated the eight ginger varieties / somaclones into two major clusters with a Jaccard's similarity coefficient of 0.80 to 1.00.The first cluster included the clones derived from the cultivar Maran (Athira, Karthika and B3) along with the source parent cultivar. The dendrogram showed that the variety Athira was most diverse and B3 most closely related to the source parent cultivar Maran. The second cluster included the clones derived from the cultivar Rio-de-Janeiro (292R, 478R and 88R) and the source parent cultivar. It is evident from the dendrogram that the somaclone 292R was most diverse and somaclones 478R and 88R were very closely related to the source parent cultivar Rio-de-Janeiro. The somaclones 478R and 88R showed highest similarity (96 per cent).

4.6.1.3 Cluster analysis using the combined RAPD and ISSR profiles

The RAPD and ISSR data were combined and the NTSYS pc was used for UPGMA analysis. The similarity coefficient ranged from 0.8041 to 1.0000 indicating 20 per cent variability in the varieties / somaclones studied. When the variability from source parent cultivar Maran was compared, the variety Athira exhibited more variability (eleven per cent) as compared to other varieties / somaclones which ranged from eight to nine per cent. In Rio-de-Janeiro group, the selected superior somaclones were found variable from the original source parent cultivar by seven to nine per cent. The genetic similarity matrix of ginger varieties / somaclones analysed by Jaccard's coefficient method of the NTSYS program using RAPD and ISSR combined data is presented in Table 36.

Dendrogram (Fig. 16) separated the eight ginger varieties / clones into two major clusters with a Jaccard's similarity coefficient of 0.84 to 1.00. The first cluster included the clones derived from the cultivar Maran (Athira, Karthika and B3) and the source parent cultivar. The combined dendrogram revealed that the variety Athira was most diverse and the variety Karthika was most closely related to the source parent cultivar Maran. The second cluster included the clones derived from the cultivar Rio-de-Janeiro (292R, 478R and 88R) and the source parent cultivar. The somaclone 292R was most diverse (0.9149) and the somaclones 478R and 88R were closely related to the source parent cultivar Rio-de-Janeiro. Highest similarity 97.60 per cent was observed between the somaclones 478R and 88R. The Maran somaclones exhibited more variation from the source parent compared to the Rio-de-Janeiro somaclones.

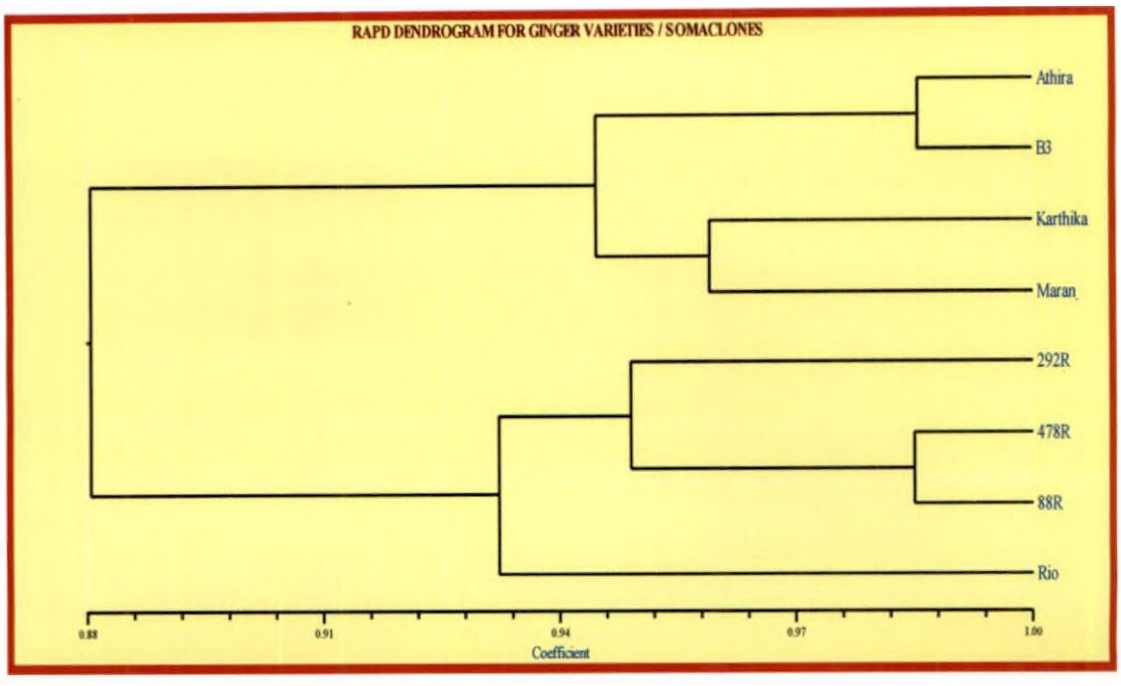


Fig. 14 Dendrogram generated with RAPD profile in ginger varieties / somaclones

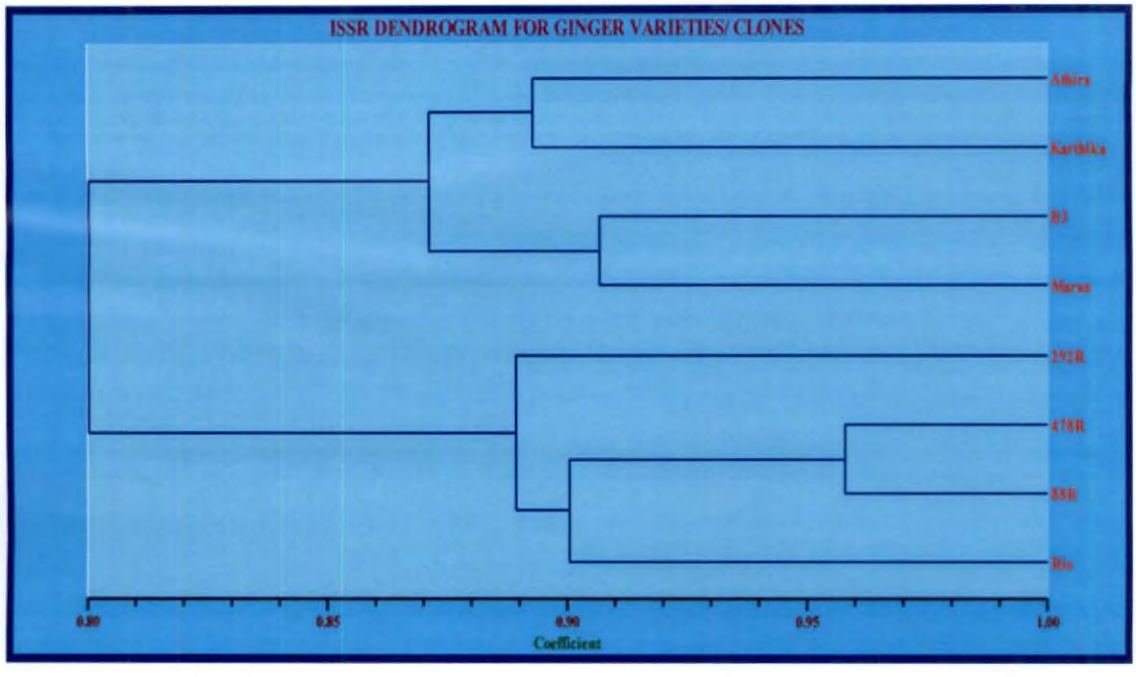


Fig.15 Dendrogram generated with ISSR profile in ginger varieties / somaclones

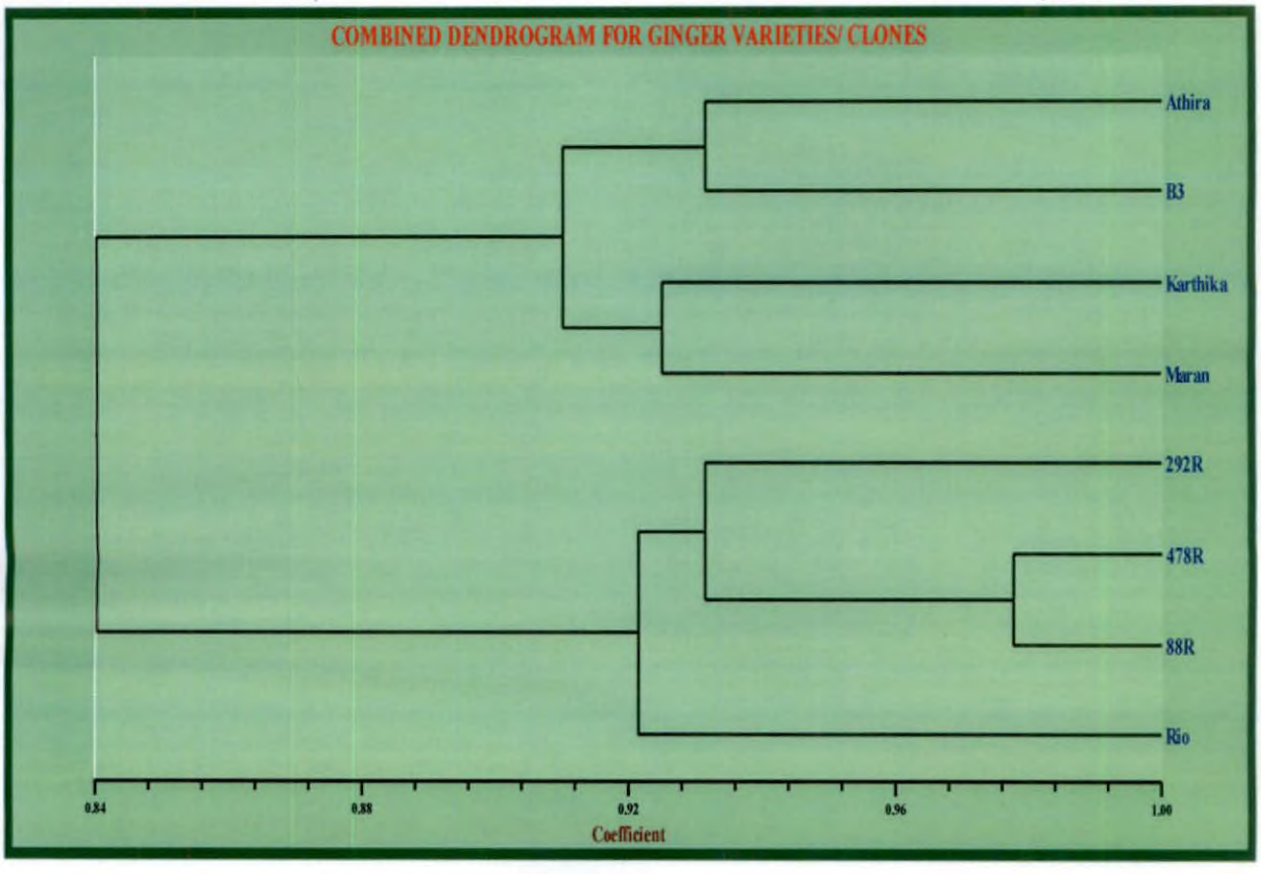


Fig.16 Dendrogram generated with RAPD and ISSR profiles in ginger varieties / somaclones

Table 34. Genetic similarity matrix of ginger varieties / somaclones analysed by Jaccard's coefficient method of the NTSYS program using RAPD data

	Athira	Karthika	B3	Maran	292R	478R	88R	Rio-de-Janeiro
Athira	1.0000							
Karthika	0.9571	1.0000						
B3	0.9851	0.9429	1.0000					
Maran	0.9429	0.9577	0.928	1.0000				
292R	0.9000	0.8630	0.9130	0.8750	1.0000			
478R	0.9130	0.8870	0.9345	0.8887	0.9848	1.0000		
88R	0.9122	0.8850	0.9265	0.8873	0.9765	0.9938	1.0000	
Rio-de-Janeiro	0.8714	0.8356	0.8841	0.8472	0.9403	0.9545	0.9545	1.0000

Table 35. Genetic similarity matrix of ginger varieties / somaclones analysed by Jaccard's coefficient method of the NTSYS program using ISSR data

	Athira	Karthika	B3	Maran	292R	478R	88R	Rio-de-Janeiro
Athira	1.0000							
Karthika	0.8923	1.0000						
B3	0.8788	0.8615	1.0000					
Maran	0.8507	0.8906	0.9063	1.0000				
292R	0.7632	0.7945	0.8082	0.8333	1.0000			
478R	0.7945	0.8028	0.7945	0.8169	0.9690	1.0000		
88R	0.7867	0.7945	0.8028	0.8082	0.8933	0.9577	1.0000	
Rio-de-Janeiro	0.7895	0.7733	0.7867	0.8108	0.8947	0.8800	0.9200	1.0000

Table 36. Genetic similarity matrix of ginger varieties / somaclones analysed by Jaccard's coefficient method of the NTSYS program using RAPD and ISSR combined data

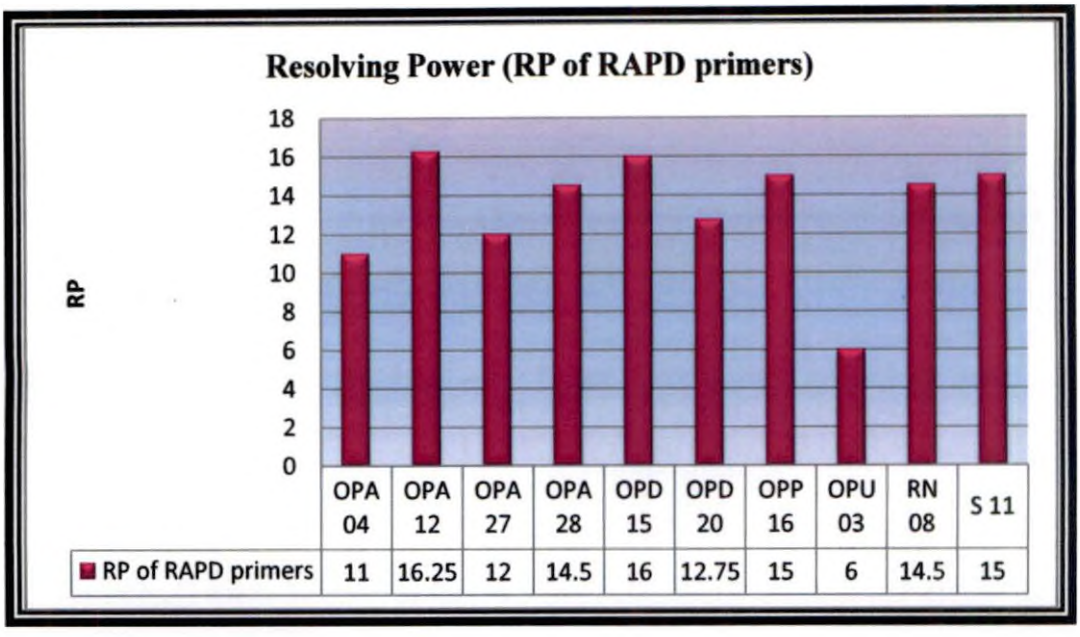
	Athira	Karthika	B3	Maran	292R	478R	88R	Rio-de-Janeiro
Athira	1.0000							
Karthika	0.9323	1.0000						
B3	0.9259	0.9037	1.0000					
Maran	0.8978	0.9179	0.9259	1.0000				
292R	0.8521	0.8705	0.8392	0.8521	1.0000			
478R	0.8288	0.8592	0.8288	0.8542	0.9286	1.0000		
88R	0.8472	0.8652	0.8345	0.8472	0.9779	0.9362	1.0000	
Rio-de-Janeiro	0.8288	0.8333	0.8041	0.8288	0.9149	0.9161	0.9362	1.0000

4.6.2 Resolving power (Rp) of selected RAPD and ISSR primers

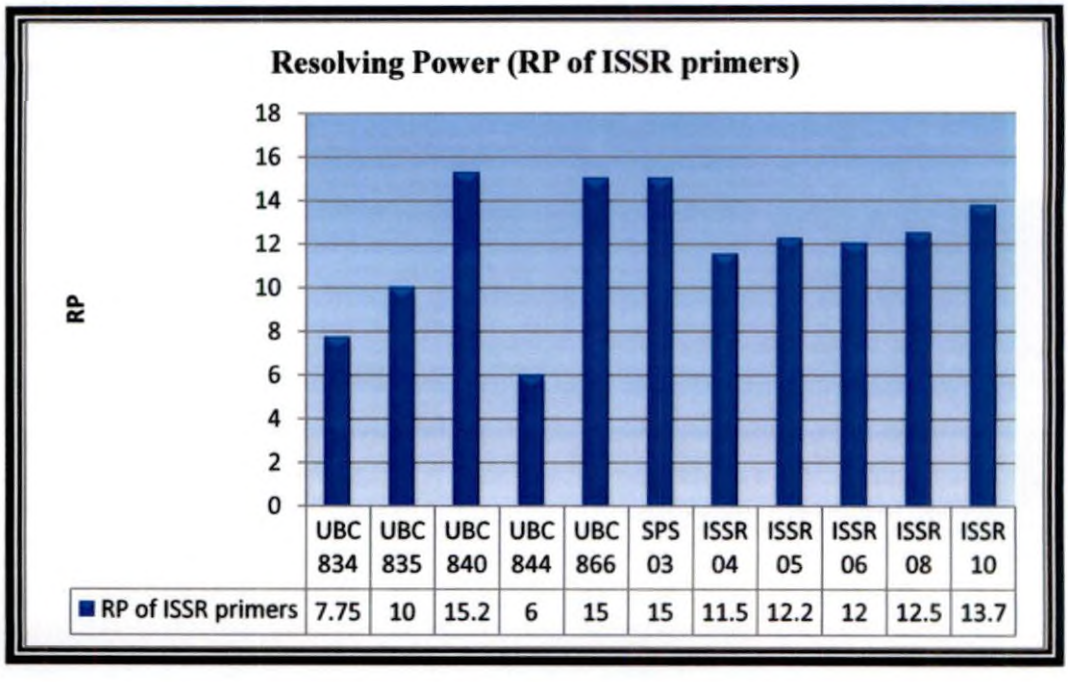
The Resolving power (Rp) calculated for the random primers is presented in Fig. 17a. It ranged between 6.00 (OPU 03) and 16.25 (OPA 12) with an average of 13.30 for RAPD primers. Highest Resolving Power was found in case of RAPD primer OPA 12 (16.25) and lowest in OPU 03 (6.00). So, the primer OPA 12 with highest Rp value can distinguish between the varieties / somaclones more efficiently. ISSR primers (Fig. 17b) recorded values ranging between 6.00 (UBC 844) and 15.25 (UBC 840) with an average of 11.90. Highest Resolving Power was found in case of ISSR primer UBC 840 (15.20) and hence it can distinguish betweent the varieties / somaclones more efficiently. Lowest Resolving Power was observed in UBC 844 (6.00).

4.6.3 Polymorphic Information Content (PIC) value of selected RAPD and ISSR primers

The Polymorphic Information Content (PIC) value calculated for the ten selected RAPD primers (Fig. 18a) varied from 0.67 (OPU 03) to 0.88 (OPA 12 and OPD 15) with a mean of 0.85. Highest PIC value was found in case of RAPD primers OPA 12, OPD 15, OPP 16 and S11 (0.87), hence, they were more suitable for DNA fingerprinting .The lowest PIC value was found in RADP primer OPU 03 (0.66). The eleven selected ISSR primers (Fig. 18b) recorded values ranging from 0.67 (UBC 844) to 0.88 (UBC 840 and SPS 03) with an average of 0.83. Highest PIC value was found in the two ISSR primers UBC 840 and SPS 03 (0.88) and lowest in the primer UBC 844 (0.66). The primers with more PIC value (UBC 840 and SPS 03) are found to more suitable for DNA fingerprinting.

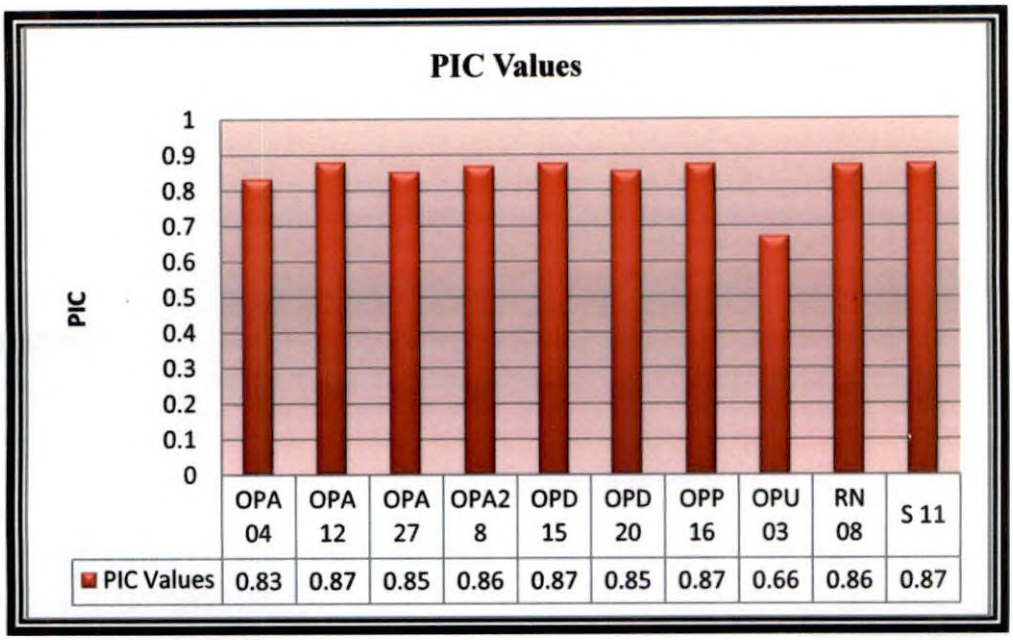


a. Resolving power of selected RAPD primers

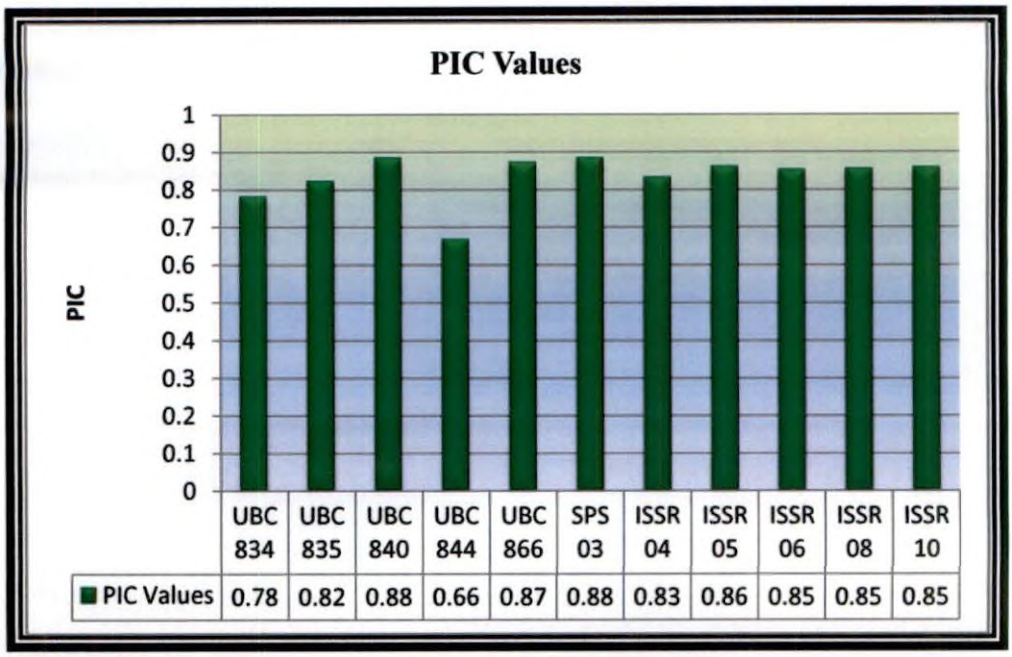


b. Resolving power of selected ISSR primers

Fig. 17 Resolving power (Rp) of selected RAPD and ISSR primers



a. Polymorphic Information Content of selected RAPD primers



b. Polymorphic Information Content of selected ISSR primers

Fig. 18 Polymorphic Information Content (PIC) of selected RAPD and ISSR primers

DNA fingerprinting could be done for the varieties and selected somaclones using two molecular marker systems viz., RAPD and ISSR. The investigations could bring out certain unique bands for genotype identification. The studies also helped to assess variability in the selected varieties / somaclones and to assess the extent of variability from parent cultivar. The dendrogram generated could also group Maran somaclones and Rio-de-Janeiro somaclones in two separate clusters. In cluster analyses it was found that the variety Athira was most diverse from the source parent cultivar Maran and the somaclone 292R was most diverse from the source parent cultivar Rio-de-Janeiro. The Maran somaclones exhibited more variation from the source parent compared to the Rio-de-Janeiro somaclone. RAPD, ISSR and combined fingerprints developed specific for the ginger varieties / somaclones could be utilized for registration and documentation of varieties and for settling IPR issues.

Discussion

5. DISCUSSION

Ginger (*Zingiber officinale* Rosc.), one of the most valued spice crops of the world, is well known and for its medicinal properties also. The species is distributed in tropical and subtropical Asia, Far East Asia and Africa. In India, most of the popular commercial varieties are clonal selections from traditional cultivars.

The genus *Zingiber* display diversity in habitat, ethno-botanical use, and morphology (Syamkumar and Sasikumar, 2007). Very little is known about genetic diversity and phylogenetic relationship among the taxa. Relying much on the morphological characters alone in species identification has its own limitations since they are not always completely representative of the genetic structure (Noli *et al.*, 1997). Conventional taxonomic techniques in conjunction with molecular biology tools may go a long way in providing accurate and powerful ways of analyzing genetic relationship in the genus *Zingiber*. However, not much has been done on molecular characterization in ginger. Molecular markers assume great significance, as these methods detect polymorphisms by assaying subsets of the total amount of DNA sequence variation in a genome. Similar studies have been reported in Zingiberaceae by using only RAPD and ISSR markers (Paisooksantivatana *et al.*, 2001; Sasikumar, 2005; Jiang, 2006; Syamkumar and Sasikumar, 2007; Hussain, 2008).

DNA fingerprinting is an efficient tool for genotype identification, assessing genetic diversity and protecting plant varieties. Central Seed Committee established under the Seed Act, 1996 stipulates the necessity of DNA fingerprint data for the varieties released / proposed to be released.

For the newly released ginger varieties and selected superior somaclones in pipeline for release, no fingerprint data are available. The proposed study will help to make fingerprint data for the two newly released varieties and four selected superior somaclones of ginger. The specific fingerprint data will serve as an identifying mark for the varieties / clones so as to avoid biopiracy. Comparision of fingerprint data of

varieties / clones with source parent cultivars will help to assess the extent of somaclonal variability in the varieties / clones as compared to the source cultivars.

5.1 Morphological Characterization

Morphological characterization of the two released varieties and four selected somaclones of ginger was attempted in the present investigation.

a) Vegetative Characters

Morphological characters were found to vary in the different varieties / somaclones studied. The somaclone 292R could be distinguished based on its dwarf plant stature and dark green leaves. The variety Karthika had more plant height, numbers of tillers per plant and petiole length. Maran had least number of tillers and number of leaves on main tiller and the somaclone 478R had the highest number of leaves on main tiller. Nybe and Nair (1982) suggested that morphological characters are not reliable to classify the types, although some of the types can be distinguished to a certain extent based on rhizome characters. Mohandas *et al.*, (2000) also found that all the cultivars differed significantly in tiller number and leaf number. Even though morphological characters may vary as per growing conditions viz., open vs. shade, the clone 292R exhibited such a dwarf habit which could be regarded as a distinguishing character.

The selected somaclones showed improvement in characters such as height of plant, number of tillers and number of leaves on main tiller. This improvement might be for continuous longer period of evaluation and selection for diserable trait (Desclaux, 2005). Frietz *et al.*, (2003), Babu *et al.*, (2005) and Paul (2006) also reported better vegetative characters in ginger somaclone compared to source parent cultivars. Similar observations on improvement of quantitative characters in black pepper somaclones was reported by Sanchu, 2000 and Shylaja *et al.*, 2012.

b) Rhizome Characters

Significant variation had also been seen in the rhizome characters of the ginger varieties / somaclones. All the selected somaclones and improved varieties showed improvement in the yield contributing characters such as, number of layers of rhizomes, number of fingers and girth of secondary fingers, as compared to the source parent cultivars.

The size of rhizomes was bold and shape flat in Athira, 292R, 478R and Rio-de-Janeiro but in Karthika and B3 the rhizome was medium bold and round, whereas, in Maran and 88R rhizomes were medium bold and flat.

The selected somaclones and improved varieties showed increased number of primary, secondary and tertiary fingers as compared to the source parent cultivars. This may be one of the characters contributing to high yielding nature of the selected somaclones and the improved varieties.

The varieties showing bold rhizomes viz., Athira and 292R exhibited higher girth of secondary fingers and more thickness of flesh and inner core.

Babu *et al.*, (2005), Salvi *et al.*, (2002) and Paul (2006) also reported similar observations on superiority of somaclones in yield contributing characters of rhizomes over source parent cultivars in ginger and turmeric somaclones.

5.2 Molecular Characterization

5.2.1 Isolation, purification and quantification of DNA

Young, tender and pale green leaves of the varieties / somaclones of ginger were collected early in the morning on ice from individual plants grown in net house. The protocol suggested by Roger and Bendich, (1994) yielded good quality DNA. The electrophoresed DNA showed distinct bands without shearing.

Grinding in liquid nitrogen was found to improve the quality of DNA isolated. The addition of antioxidant like β-mercaptoethanol in the extraction buffer or during grinding was found effective. Similar results on use of liquid nitrogen, β-mercaptoethanol for isolation of good quality DNA were also reported in ginger and turmeric by Syamkumar *et al.*, (2003).

The detergent used in the extraction buffer in CTAB (Cetyl Trimethyl Ammonium Bromide), which helps in the disruption of the cell membrane thereby releasing nucleic acid into the extraction buffer and prevents co-precipitation of polysaccharides with nucleic acid by acting as a selective precipitant of nucleic acids. CTAB is a cationic detergent, which solubilises membrane and form a complex with DNA (Sghaier and Mohammed, 2005).

The advantageous effect of the CTAB along with PVP on the quality of DNA isolated was also reported by Gallego and Martinez (1996) and Sreenath *et al.* (1992). It effectively disrupts the cell membrane and together with NaCl separates the polysaccharides. The EDTA in the extraction buffer protects the DNA from endonuclease by chelating the Mg^{2+} ions of DNA. Double treatment with chloroform: isoamylalcohol mixture and centrifugation effectively removes the pigment and proteins. The addition of chilled isopropanol precipitates the DNA and washing the pellet with 70 per cent alcohol followed by absolute alcohol remove the traces of CTAB. TE buffer rehydrates the DNA and dissolves it (Wettasinghe and Peffley, 1998; Babu, 2000).

Problems encountered in the isolation and purification of high molecular weight DNA from certain plant species include: degradation of DNA due to endonuclease, polyphenols and other secondary metabolites that directly and indirectly interfere with subsequent enzymatic reactions as reported by Weishing *et al.* (1999) and Matasyoh *et al.* (2008). EDTA is also a major component of TE buffer in which the DNA was dissolved and stored. RNase treatment was given in order to remove RNA contamination from the isolated DNA samples.

A DNA sample was reported as high quality if it had a band of high molecular weight with little smearing and a low amount of RNA (Wettasinghe and Peffley, 1998). Use of RNase A was reported by several workers (Raval *et al.*, (1998), Wettasinghe and Peffley (1998) and Gallego and Martinez, (1996). In the present investigations, the RNase treated DNA sample on electrophoresis showed a high molecular weight DNA, which formed a single band just below the well. This indicated that the DNA under test was of good quality.

The absorbance ratio was calculated as OD 260/280, for the various samples, using spectrophotometer. Those DNA samples with ratio between 1.8 and 2.0 were considered to be of high quality. If the value goes beyond 2.0, it indicates RNA contamination and if less than 1.8, it indicates protein contamination. All molecular analysis was done using good quality DNA.

5.2.2 Molecular marker analysis

Molecular marker technology provides novel tools for DNA fingerprinting. Most of the molecular markers are developed by the PCR (Polymerase Chain Reaction) technology and amplifies unique regions on the genomic DNA based on the primers designed for DNA amplification. Two such PCR based marker systems (RAPD and ISSR) were utilized for fingerprinting ginger varieties / somaclones in the current study.

5.2.2.1 RAPD analysis

The RAPD technique was developed by Williams *et al.*, (1990) and the technique relies on the differential enzymatic amplification of small DNA fragments using PCR with arbitrary decamer primers. 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.

The RAPD technique (Parani *et al.*, 1997) is a simple technique which detects polymorphism and has been used for molecular characterisation in several crop plants such as *Oryza sativa* L. (Chakravarthi and Naravaneni, 2006), *Piper nigrum* L.(Keshavachandran *et al.*, 2005), *Manihot esculenta* crantz. (Santha, *et al.*, 2005), *Ficus carica* L. (Khadari *et al.*, 1995), Hybrid rice (Seyyed *et al.*, 2010) and *Mangifera indica* L. (Adato *et al.*, 1995).

The RAPD marker system could bring out unique bands in the variety Karthika and somaclones B3, 292R and 478R with primer OPA 12 in Karthika and B3, OPA 04 in 292R and OPA 28 in 478R.

Aptness of RAPD markers in diversity analysis of ginger has been reported in various studies (Rout *et al.*, 1998; Nayak *et al.*, 2005; Paul, 2006; Palai and Rout, 2007).Therefore, RAPD seems to be a useful tool for identification of ginger genotypes.

5.2.2.2 ISSR analysis

The choice of a molecular marker technique depends on its reproducibility and simplicity. The best marker for genome mapping, marker assisted selection, phylogenic studies, and crop conservation should have low cost and labour requirements and high reliability. Since 1994, a molecular marker technique called Inter Simple Sequence Repeat (ISSR) has been available and is being exploited (Zietkiewicz *et al.*, 1994). ISSRs are semiarbitrary markers amplified by PCR in the presence of one primer complementary to a target microsatellite.

The ISSR markers are found useful in the fingerprinting of cultivated and wild species germplasm, and in understanding the evolutionary relationships of various crops such as Oryza *spp.* (Joshi *et al.*, 2000), *Solanum lycopersicon* Mill., (Aguilera *et al.*, 2011) and *Mangifera indica* L. (Luo *et al.*, 2011).

ISSR marker system could also bring out unique band in the variety Athira with primer ISSR 06.

5.2.2.3 PIC values

The PIC values indicate the extent of polymorphism detected by the primer among the varieties studied. In the present study the Polymorphic Information Content (PIC) calculated ranged between 0.67 to 0.88 for RAPD primers and 0.67 to 0.88 for ISSR primers indicating the suitability of primers to detect polymorphism. RAPD primers OPA 12, OPD 15, OPP 16 and S 11 and ISSR primers UBC 840 and SPS 03 reported highest PIC values. The PIC values reported in various other crops for the primers used for characterisation is in conformity to the values observed in the present study (Karihaloo *et al.*, 2003; Kelley *et al.*, 2004; Dongre and Kharbikar, 2004, Hollman *et al.*, 2005), thus confirming the suitability of the primers selected for DNA fingerprinting in the present study.

5.2.2.4 Fingerprinting of ginger varieties / somaclones

Two KAU released varieties of ginger (Athira and Karthika), four superior selected somaclones (292R, 478R, 88R and B3) and source parent cultivars (Maran and Rio-de-Janeiro) maintained at CPBMB, College of Horticulture, Vellanikkara were used for the study. These somaclones were produced by single plant selection derived through *in vitro* adventitious bud regeneration after passing through several (ten to twelve) subculture cycles and field evaluation and selection for eight years. Athira, Karthika and B3 were produced from the cultivar Maran and 292R, 478R and 88R were produced from the cultivar Rio-de-Janeiro. The varieties / clones studied have different morphological parameters. Some of the morphological characters viz., height of the pseudostem, tiller number, number of leaves and rhizome characters like size and shape of rhizomes were found different in varieties / somaclones and were able to distinguish the clones / varieties.

The variation seen in the varieties / selected somaclones might be due to continuous multiplication during subculturing cycles which might have caused genetic and epigenetic changes to the cultures. The desirable changes occurred were selected during field evaluation trials and they were fixed through vegetative propagation. The genetic and epigenetic changes occuring in the somaclones were reviewed by several workers, Larkin and Scowcroft (1981); Evans (1989); Jain (2001) and Bairu *et al.*, (2011).

The amplification patterns observed in RAPD and ISSR assays were utilized for developing fingerprint for the selected varieties which were also supported by morphological data.the unique amplification patterns in RAPD and ISSR assays and distinguishing morphological characters of the varieties / selected somaclones are described below:

1. Variety Athira

Athira is a high yielding, high quality somaclone developed from the local cultivar Maran. It is a single plant selection developed through *in vitro* adventitious bud regeneration and field evaluation for eight years. The clone is characterized by bold rhizomes with less crude fibre and high quality volatile oil with high zingiberine (35.76 per cent) in volatile oil. It is tolerant to soft rot and bacterial wilt diseases. The number of layers of rhizomes, number of fingers and thickness of flesh are highest in Athira.

By utilizing the molecular markers the variety Athira can be easily identified. The ISSR primer ISSR 06 generated a unique band of size 900bp for the variety. The fingerprint generated with RAPD profiles showed that it shared 3.03 per cent of the bands with the variety Karthika and somaclone B3. With the ten RAPD primers studied in the present investigations, Athira could not be distinguished with any unique bands.

However, the sharing of bands with other varieties / somaclones does not make the variety loose in its identity. The RAPD, ISSR and the combined fingerprint developed for the variety Athira are unique and different from all others.

2. Variety Karthika

Karthika is a high yielding, high pungency somaclone developed from the local cultivar Maran. It is a single plant selection developed through *in vitro* adventitious bud regeneration and field evaluation for eight years. The clone is characterized by medium bold rhizomes with high recovery of volatile oil and oleoresin and high gingerol in oleoresin (21.30 per cent). It is tolerant to soft rot and bacterial wilt diseases. The variety Karthika can be highlighted using some of the salient morphological characters such as more plant height (84cm), more numbers of tillers per plant (18) and more petiole length (0.55cm).

The RAPD fingerprint can be effectively utilized to identify this variety as it produced a unique band of 2000bp with random primer OPA 12. It was also noticed that 2.94 per cent of the bands in fingerprint profile were shared with the variety Athira and somaclone B3.

However, sharing of bands with other varieties / somaclones does not make the variety loose in its identity. The RAPD, ISSR and the combined fingerprint developed for the variety Karthika are unique and different from all others.

3. Somaclone B3

B3 is a high yielding somaclone developed from the local cultivar Maran. It is developed through *in vitro* adventitious bud regeneration. The clone is characterized by medium bold rhizomes with high recovery of volatile oil and

oleoresin. It is tolerant to soft rot and bacterial wilt diseases.The colour of flesh in rhizome was pale yellow with bluish tinge in the somaclone B3.

The fingerprint developed with RAPD primers revealed that a unique band of 1400bp was produced with random primer OPA 12 and this could be used as a distinguishing DNA marker for the somaclone. 2.98 per cent of the bands in RAPD marker system were shared with the varieties Athira and Karthika. There is no unique band in B3 for ISSR marker system for the primers selected.

However, sharing of bands with other varieties / somaclones does not make the somaclone loose in its identity. The RAPD, ISSR and the combined fingerprint developed for the somaclone B3 are unique and different from all others.

4. Somaclone 292R

292R is a high yielding, high quality somaclone recommended for fresh ginger developed from the exotic cultivar Rio-de-Janeiro. It is a single plant selection developed through *in vitro* adventitious bud regeneration and field evaluation for eight years. It is characterized with dwarf strature, dark green leaves and high recovery of volatile oil and oleoresin. It is tolerant to soft rot and bacterial wilt and *Phyllostreta* leaf spot diseases. The somaclone has bold and flat rhizomes.

The fingerprint generated by RAPD profile revealed that the primer OPA 04 produced a unique band of 400bp in this somaclone which can efficiently distinguish it from other genotypes.

However, sharing of bands with other varieties / somaclones does not make the somaclone loose in its identity. The RAPD, ISSR and the combined fingerprint developed for the somaclone 292R are unique and different from all others.

5. Somaclone 478R

478R is a high yielding, high quality somaclone developed from the exotic cultivar Rio-de-Janeiro characterized by bold rhizomes. It is a single plant selection developed through *in vitro* adventitious bud regeneration and field evaluation for eight years. The unique morphological character noticed in the somaclone 478R was that it had the highest number of leaves on main tiller.

The fingerprint developed by the RAPD profile showed that the primer OPA 28 produced a unique band of size 300bp in the somaclone 478R which could be effectively be utilized for identifying the somaclone. In the fingerprint generated by ISSR profile there was no unique band for the somaclone and it shared a band of 300bp produced by the primer ISSR 06 with the source cultivar Rio-de-Janeiro.

However, sharing of bands with other varieties/somaclones does not make the somaclone loose in its identity. The RAPD, ISSR and the combined fingerprint developed for the somaclone 478R are unique and different from all others.

6. Somaclone 88R

88R is a high yielding, high quality somaclone developed from the exotic cultivar Rio-de-Janeiro characterized by medium bold rhizomes. It is a single plant selection developed through *in vitro* adventitious bud regeneration and field evaluation for eight years. The internodal length of secondary fingers recorded was more in the somaclone 88R.

No unique band was noticed in the fingerprints generated by both RAPD and ISSR marker systems. However, the fingerprints generated by RAPD, ISSR and combined marker systems are unique for the clone.

Sharing of bands with other varieties / somaclones does not make the somaclone loose in its identity. The RAPD, ISSR and the combined fingerprint developed for the somaclone 88R are unique and different from all others.

7. Cultivar Maran

Maran is an indigenous cultivar characterized by medium bold rhizomes. It is susceptible to soft rot and bacterial wilt diseases. Maran had least number of tillers and number of leaves on main tiller.

No unique band was noticed in the fingerprints generated by both the marker systems. However, the fingerprint generated by RAPD, ISSR and combined marker systems were unique with respect to percentage of bands produced by RAPD and ISSR primers. The fingerprint generated by ISSR profile showed that it shared a band of 1200bp produced by ISSR primer UBC 835 with the somaclone B3.

However, sharing of bands with other varieties / somaclones does not make the variety loose in its identity. The RAPD, ISSR and the combined fingerprint developed for the cultivar Maran are unique and different from all others.

8. Cultivar Rio-de-Janeiro

Rio-de-Janeiro is an exotic cultivar from Brazil characterized by medium bold rhizomes. It is susceptible to soft rot and bacterial wilt diseases. The source cultivar Rio-de-Janeiro recorded lowest number of layers of rhizomes and lowest number of fingers and internodal length.

No unique band was noticed in the fingerprints generated by both the marker systems, however, the fingerprint generated by the combined RAPD and ISSR markers presented a unique profile in respect of percentage of bands produced by RAPD and ISSR primers that could distinguish it from other genotypes.

However, sharing of bands with other varieties / somaclones does not make the variety loose its identity. The RAPD, ISSR and the combined fingerprint developed for the cultivar Rio-de-Janeiro is unique and different from all others

In the present investigations, ginger cultivars Maran and Rio-de-Janeiro and the somaclone 88R could not be distinguished with unique bands in the two marker systems. In the present study, only ten RAPD and eleven ISSR primers were used. If attempted with more number of primers, could bring out unique bands in the cultivar / somaclone. However, the cultivar / somaclone could be fingerprinted using RAPD, ISSR and combined profiles.

The fingerprints generated for the varieties / somaclones from Athira to Rio-de-Janeiro were unique and distinct. Though individual primers of each marker system showed several bands shared among varieties, the pattern obtained with all the bands irrespective of the marker system (RAPD and ISSR) considered together was unique for each variety which formed the fingerprint of the particular variety. The fingerprint thus generated could very well be utilized to prove varietal identity. Similar DNA fingerprinting reports utilizing RAPD and ISSR were obtained in ginger by Nayak *et al.* (2005), Harisaranraj *et al.* (2009), Palai and Rout (2007), Prem *et al.*, (2008) and Sajeev *et al.* (2011) and in black pepper (*Piper nigrum* L.) by Mogalayi (2011).

In the present investigations RAPD marker system was more efficient in fingerprinting of varieties / somaclones as it produced more number of unique bands in the different varieties and somaclones studied as compared to ISSR marker system. The use of RAPD markers in cultivar identification and diversity analysis has been

reported in ginger by Rout *et al.*, (1998), Nayak *et al.*, (2005), Palai and Rout, (2007) and use of both RAPD and ISSR markers has been reported by Prem *et al.*, (2008) and Kizhakkayil and Sasikumar, (2010).

5.2.2.5 Cluster analysis

The amplification pattern observed in RAPD and ISSR analysis was scored and analysed for relatedness / variability in the varieties / somaclones and to assess the extent of variability from the source parent cultivars. The computer package NTSYS-PC (Rohlf, 2005) was used for cluster analysis. Dendrograms generated using RAPD, ISSR and combined profiles (Fig. 14, 15 and 16) separated the eight ginger varieties / somaclones into two major clusters. The first cluster included the clones derived from the cultivar Maran viz., Athira, Karthika, B3 and the source parent cultivar Maran. The second cluster included the clones derived from the cultivar Rio-de-Janeiro viz., 292R, 478R, 88R and and source parent cultivar Rio-de-Janeiro. The clustering of Athira, Karthika, B3 and Maran in the same group is in agreement with their parentage, as the somaclones Athira, Karthika and B3 have been regenerated from the source parent cultivar Maran and the clustering of 292R, 478R, 88R and Rio-de-Janeiro the same group is in agreement with their parentage, as the somaclones 292R, 478R and 88R have been regenerated from the source parent cultivar Rio-de-Janeiro. A similar pattern of clustering of the source cultivar with the somaclones was also reported by Sheidai *et al.*, (2008) in banana. The grouping of the exotic introduction Rio-de-Janeiro along with the somaclones derived from it and the indigenous cultivar Maran along with the somaclones derived from it in two seperate clusters is also of high relevance. Hence, both the marker systems viz., RAPD and ISSR either individually or combined can effectively be utilized in determination of genetic relationships among the ginger varieties / somaclones.

The dendrogram revealed that the variety Athira was most diverse from the source parent cultivar Maran and the somaclone 292R was most diverse from the source parent cultivar Rio-de-Janeiro. Patterns of sub-clustering was different in the dendrograms generated by different marker systems which may be due to difference in genome coverage and sequence type recognised by each marker system (Powell *et al.*, 1996; Sehgal and Raina 2005; Ikegami *et al.*, 2009).

In quantitative clustering for vegetative characters, plant height and number of tillers per plant showed more divergence between the clusters. Ravindran *et al.*, 1994 also observed highest variability in tiller number per plant when they characterized 100 accessions of ginger based on morphological parameters. In Mahalanobis D^2 analysis, the improved varieties / somaclones were distantly clustered from source parent cultivar Maran. The same observation was recorded in qualitative clustering attempted in the present study with the two molecular marker systems viz., RAPD and ISSR. The dendrogram with molecular marker analysis grouped Maran and Rio-de-Janeiro clones in two separate clusters and the variety Athira was found more diverse from source parent cultivar Maran exhibiting 15 per cent variability in the ISSR marker system.

For rhizome characters, the improved variety Athira and somaclone 292R exhibiting better rhizome characters were grouped in a cluster. The improved variety Karthika was grouped with source parent cultivar Maran in cluster III. Similar type of observation was seen in qualitative clustering with RAPD marker system in which Karthika and Maran were found near in the same cluster and the same was true for combined dendrogram also.

The present investigation could fingerprint the released varieties / selected superior somaclones using two marker systems viz., RAPD and ISSR. The investigations could also highlight the variability among the varieties / somaclones and could assess the extent of variability from source parent cultivars. The specific

fingerprints generated in the study will serve as identifying marks for the varieties / clones so as to protect them and to avoid biopiracy.

Future lines of work include the development of more unique bands in ginger varieties / clones utilizing more number of primers and advanced marker systems. Further investigations on unique bands for isolation and characterization of trait specific genes and development of fingerprints for other superior genotypes in the germplasm are other areas suggested.

Summary

6. SUMMARY

The study entitled "DNA fingerprinting of released varieties and selected superior somaclones of ginger (*Zingiber officinale* Rosc.)" was undertaken at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture during the period from January 2012 to March 2013.

The research works included mainly the morphological and molecular characterization of two released varieties of KAU (Athira and Karthika), four selected superior somaclones (292R, 478R, 88R and B3) and source parent cultivars (Maran and Rio-de-Janeiro). Two marker systems viz., RAPD and ISSR were used to develop fingerprints of the varieties / somaclones.

The salient findings of the present study are as follows:

1. Morphological characters were found to vary in the varieties / somaclones studied. The somaclone 292R could be distinguished based on its dwarf plant stature and dark green leaves, the variety Karthika had more plant height, more numbers of tillers per plant and more petiole length, the cultivar Maran had lowest number of tillers per plant and lowest number of leaves on main tiller and the somaclone 478R had the highest number of leaves on main tiller.

2. Quantitative clustering for vegetative characters was done using Mahalanobis D^2 analysis and the ginger varieties / somaclones studied were grouped into three clusters. Of the seven vegetative characters analysed, plant height and number of tillers showed more divergence. Seventy five per cent of the improved somaclones were distantly clustered from the source parent cultivar Maran.

3. Rhizome characters also varied in the varieties / somaclones. The size of rhizome was bold and shape flat in the variety Athira and somaclones 292R and 478R. In the variety Karthika and somaclone B3, the rhizomes were medium bold and round and in the somaclone 88R, rhizome was medium bold and flat.

4. Quantitative clustering for rhizome characters attempted as per Mahalanobis D^2 analysis could group the varieties and somaclones into three separate clusters. The improved variety Athira and the somaclone 292R which exhibited better rhizome characters were grouped in cluster II. The number of fingers, girth of primary and secondary fingers, thickness of flesh and inner core were the characters which exhibited more divergence.

5. The protocol suggested by Rogers and Bendich (1994) was found good for isolation of DNA from young and immature leaves of ginger varieties / somaclones. The RNA contamination was completely removed through RNase treatment.

6. The quality and quantity of DNA were analyzed by NanoDrop® ND-1000 spectrophotometer. The absorbance ratio ranged from 1.80-1.90, which indicated good quality DNA. The recovery of DNA was high and was suitable for RAPD and ISSR analyses.

7. Thirty five RAPD and thirty ISSR primers were screened for their ability to amplify genomic DNA fragments from ginger varieties / somaclones. Out of the primers screened, ten RAPD and eleven ISSR primers were selected for DNA fingerprinting based on the number of the bands and nature of amplicons.

8. DNA fingerprints of the varieties / somaclones of ginger were developed utilizing the clear and distinct bands generated in RAPD and ISSR profiles and size of the bands and by assigning colour codes for band sharing. Fingerprints specific to each variety / somaclone studied were generated.

9. The RAPD marker system could bring out unique bands in the variety Karthika and somaclones B3, 292R and 478R with primer OPA 12 in Karthika and B3, OPA 04 in 292R and OPA 28 in 478R.

10. ISSR marker system could also bring out unique band in the variety Athira with primer ISSR 06.

11. The dendrogram generated using RAPD and ISSR data assessed the variability in the varieties and selected superior somaclones and the extent of variability from the source parent cultivars.

12. ISSR marker system could bring about more variability (20 per cent) than RAPD marker system (11 per cent).

13. The dengrogram seperated the Maran and the Rio-de-Janeiro somaclones in two separate clusters. The variety Athira was more diverse from the source parent cultivar Maran exhibiting 15 per cent variability in the ISSR marker system.

14. The somaclone 292R was more diverse from the source parent cultivar Rio-de-Janeiro exhibiting nine per cent variability in the RAPD and ISSR combined marker system.

15. The Resolving Power (Rp) of the RAPD and ISSR primers was calculated and the values ranged between 6.00 and 16.25 for RAPD primers and 6.00 to 15.25 for ISSR primers. The RAPD primer OPA 12 and ISSR primer UBC 840 showed high resolving power.

16. The Polymorphic Information Content (PIC) calculated ranged between 0.67 to 0.88 for RAPD primers and 0.67 to 0.88 for ISSR primers indicating the suitability of primers to detect polymorphism. RAPD primers OPA 12, OPD 15, OPP 16 and S 11 and ISSR primers UBC 840 and SPS 03 reported highest PIC values.

17. In Mahalanobis D^2 analysis also the improved varieties / somaclones were distantly clustered from source parent cultivar Maran.

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Annexures

ANNEXURE I

Details of laboratory equipments used for the study

1) High speed refrigerated centrifuge : Kubota 6550, Japan

2) Horizontal electrophoresis system : BIO-RAD, USA

3) Thermal Cycler : Applied Biosystem, Viriti

4) NanoDrop$^{R.}$ ND-1000 spectrophotometer : NanoDrop$^{R.}$ Technologies Inc. USA

5) Gel documentation : Gel Documentation System, BIORAD, USA

6) Water purification system : Milipore, Germany

7) Ice flaking machine : F100 Compact, Ice matics

8) Laminar Air Flow : HML- 104, Thermadyne

ANNEXURE II

Reagents for DNA isolation by CTAB method as per Rogers and Bendich (1994)

Reagents:

A. 2X CTAB Buffer:

- 2% CTAB (w/v)
- 100mM Tris (pH 8)
- 0.5 M EDTA (pH 8)
- 1.4 M Nacl

B. 10% CTAB solution:

- 10% CTAB (w/v)
- 0.7 M NaCl.

C. TE buffer:

- 10mM Tris (pH 8)
- 1mM EDTA (pH 8)

ANNEXURE III

Composition of buffers and dyes used for agarose gel electrophoresis

1. 50X TAE buffer (pH 8.0)

— 242g Tris base

— 57.1 ml glacial acetic acid

— 100ml 0.5 Mm EDTA (pH 8.0)

2. Tracking / 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/ml in water and was stored at room temperature in dark bottle.

DNA FINGERPRINTING OF RELEASED VARIETIES AND SELECTED SUPERIOR SOMACLONES OF GINGER (*Zingiber officinale* Rosc.)

By

PUJAITA GHOSH
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ABSTRACT OF THE THESIS

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CENTRE FOR PLANT BIOTECHNOLOGY AND MOLECULAR BIOLOGY
COLLEGE OF HORTICULTURE
VELLANIKKARA, THRISSUR - 680 656
KERALA, INDIA

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Abstract

ABSTRACT

Ginger (*Zingiber officinale* Rosc.), one of the widely cultivated and consumed spices worldwide, is well known for its medicinal properties also. As the natural variability stands limited in the crop, induction of variability through tissue culture techniques was attempted at College of Horticulture, Vellanikkara from 1996 onwards. After indepth investigations on the somaclones regenerated, two varieties viz., "Athira" and "Karthika" were released during 2010 and four clones viz. B3, 292R, 88R and 478R were selected as superior somaclones. For the newly released ginger varieties and selected superior somaclones in pipeline for release, no fingerprint data are available for genotype identification and protecting the plant varieties / clones.

The investigations on "DNA fingerprinting of released varieties and selected superior somaclones of ginger (*Zingiber officinale* Rosc.)" were carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Kerala Agricultural University, Thrissur during the period from January 2012 to March 2013. The objectives of the study were to characterize two released varieties and four selected superior somaclones using molecular markers and to develop a DNA fingerprint specific to each variety / somaclone.

Morphological characters like growth habit and size and shape of the rhizomes were found to vary in the varieties / somaclones studied. The somaclone 292R could be distinguished based on its dwarf plant stature and dark green leaves. The variety Athira has bold and flat rhizomes while the variety Karthika has medium bold and round rhizomes.

Quantitative clustering for vegetative and rhizome characters attempted as per Mahalanobis D^2 analysis could group the varieties and somaclones into three separate clusters. Of the seven vegetative characters analysed, plant height and number of tillers showed more divergence. The number of fingers, girth of primary and secondary fingers, thickness of flesh and inner core were the characters which exhibited more divergence for the rhizome characters.

For molecular characterization, good quality genomic DNA extracted from ginger varieties / somaclones using CTAB (Rogers and Bendich, 1994) method was used. Thirty five RAPD and thirty ISSR primers were screened for amplification of genomic DNA and ten RAPD and eleven ISSR primers were selected based on the amplification pattern.

DNA fingerprints of the varieties / somaclones were developed utilizing the clear, distinct bands generated in RAPD and ISSR profiles and size of the bands. Different colour codes were assigned for sharing of bands between varieties / clones to generate specific fingerprints. The RAPD marker system could bring out unique bands in the variety Karthika and somaclones B3, 292R and 478R. The RAPD primer, OPA 12 produced unique band in Karthika and B3, the primer OPA 04 in 292R and the primer OPA 28 in 478R. ISSR marker system could also bring out unique band in the variety Athira with primer ISSR 06. The RAPD, ISSR and combined fingerprints developed for each variety / somaclone were unique.

Variability in the somaclones and the extent of variability from source parent cultivars were analysed using cluster analysis. The dendrogram seperated Maran and Rio-de-Janeiro somaclones in two separate clusters. Somaclones derived from cultivar Maran exhibited more variability than somaclones from Rio-de- Janeiro. The variety Athira was more diverse from the source parent cultivar Maran. Similarly, the somaclone 292R was more diverse from the source parent cultivar Rio-de-Janeiro.

The Resolving Power (Rp) of RAPD and ISSR primers ranged from 6.00 to 16.25, indicating the ability of the selected primers to distinguish the varieties / clones most efficiently. The Polymorphic Information Content (PIC) ranged from 0.67 to 0.88, indicating the suitability of the selected primers for DNA fingerprinting.

RAPD, ISSR and combined fingerprints developed specific for the ginger varieties / somaclones could be utilized for registration, documentation of varieties and for settling IPR issues.