VARIABILITY ANALYSIS IN GINGER (*ZINGIBER OFFICINALE* ROSC.) SOMACLONES USING MOLECULAR MARKERS

By

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(2011-11-107)

THESIS

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DECLARATION

I, hereby declare that this thesis entitled **"Variability analysis in ginger (*Zingiber officinale* Rosc.) somaclones using molecular markers"** is a bonafide record of research work done by me during the course of research and that it has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other university or society.

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Gavande Sharda Shivaji

Dedicated

To my

Beloved

Aai-Dada

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ABBREVIATIONS

%	Percentage
β	Beta
γ	Gamma
µg	Microgram
µl	Microlitre
^{0}C	Degree Celsius
AFLP	Amplified Fragment Length Polymorphism
bp	Base pairs
CPBMB	Centre for Plant Biotechnology and Molecular biology
CTAB	Cetyl Trimethyl Ammonium Bromide
DNA	Deoxyribo Nucleic Acid
dNTPs	Deoxyribo Nucleoside Triphosphate
EDTA	Ethylene Diamine Tetra Acetic acid
g	Gram
Gy	Grey
ha	hectare
ISSR	Inter Simple Sequence Repeats
KAU	Kerala Agricultural university
l	Litre
mA	Milli Ampere
MB	Maran bud
MC (10 Gy)	Maran indirect organogenesis (irradiated with γ rays10 Gy)
MC (20 Gy)	Maran indirect organogenesis (irradiated with γ rays 20 Gy)
MC	Maran indirect organogenesis
Min	Minutes
ml	Millilitre
mM	Milli mole
MSe (10 Gy)	Maran indirect somatic embryogenesis (irradiated with γ rays 10 Gy)
MSe (20 Gy)	Maran indirect somatic embryogenesis (irradiated with γ rays 20 Gy)
MSe	Maran indirect somatic embryogenesis

ABBREVIATIONS

MSP	Maran source parent
ng	Nanogram
OD	Optical Density
PCR	Polymerase Chain Reaction
pH	Hydrogen ion concentration
PIC	Polymorphism Information Content
PVP	Poly vinyl pyrolidine
RAPD	Random Amplified Polymorphic DNA
RB	Rio-de-Janeiro bud
RC (10 Gy)	Rio-de-Janeiro indirect organogenesis (irradiated with γ rays 10 Gy)
RC (20 Gy)	Rio-de-Janeiro indirect organogenesis (irradiated with γ rays 20 Gy)
RC	Rio-de-Janeiro indirect organogenesis
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribo Nucleic acid
RNase	Ribonuclease
rpm	Rotations per minute
RSe (10 Gy)	Rio-de-Janeiro indirect somatic embryogenesis (irradiated with γ rays 10 Gy)
RSe	Rio-de-Janeiro indirect somatic embryogenesis
RSP	Rio-de-Janeiro source parent
SAHN	Sequential Agglomerative Hierarchical Non-overlapping
sec	Second (s)
SSR	Simple Sequence Repeats
TAE	Tris Acetate EDTA
TE	Tris EDTA
U	Unit
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
UV	Ultra violet
V	Volts
v/v	Volume by Volume

Introduction

1. INTRODUCTION

Ginger (*Zingiber officinale* Rosc.), an important spice crop, is much valued for its flavour and medicinal properties. This herbaceous rhizomatous perennial plant is believed to have been originated in South East Asia probably India or China.

It is commercially cultivated in India, China, Thailand, Taiwan, Philippines, Jamaica, Fiji, Brazil, Mexico, Japan and Indonesia. The area under cultivation in India is 1,55,063 ha with production of 7,55,618t (www.indianspices.com). Karnataka is the leading state in cultivation (46511 ha area) with a production of 1,68,310t. India is also the largest exporter of ginger, accounting for 19,850t of ginger valued at Rs. 16,863.10 lakhs (www.indianspices.com).

Crop improvement through selection and hybridization are not effective in ginger due to lack of variability and absence of natural seed set. The earlier crop improvement programmes were hence focussed on mutation breeding using γ rays and ethyl methyl sulfonate (EMS). The mutants thus isolated were low yielders and the effect of mutagen treatment vanished in subsequent generations (Giridharan, 1984; Dutta and Biswas, 1985; Jayachandran 1989). Hence investigations were made to induce variability in ginger through biotechnological tools like *in vitro* pollination and exploitation of somaclonal variation.

Research on somaclonal variation in ginger was initiated at College of Horticulture, Kerala Agricultural University from 1996 onwards to exploit the tissue culture induced variability. Studies were undertaken in two cultivars viz. Maran and Rio-de-Janeiro. Regenerants were produced through various modes of regeneration viz. bud culture, indirect organogenesis, and embryogenesis. *In vitro* induction of mutation through γ irradiation was also attempted and regenerants produced were maintained in the germplasm.

Currently, molecular marker techniques are widely employed to detect and assess somaclonal variation in several crops as they are stable, detectable in all tissues and are not confounded by environment, pleiotropic and epistatic effects.

RAPD markers could detect somaclonal variation in beet (Munthali *et al.*, 1996), garlic (Zahim *et al.*, 1999), date palm (Saker *et al.*, 2000), banana (Gimenez *et al.*, 2001 and Mohamed, 2007), tomato (Soniya *et al.*, 2001) and potato (Ehsanpour *et al.*, 2007) in varying degrees.

Similarly, microsatellite markers were able to detect somaclonal variation in grapevine (Welter *et al.*, 2007), sugarcane (Singh *et al.*, 2008), cauliflower (Leroy *et al.*, 2001) and potato (Karacsonyi *et al.*, 2011). AFLP analysis has been used to study somaclonal variation in coffee (Sanchez-Teyer *et al.*, 2003) and eucalyptus (Mo *et al.*, 2009).

Use of various molecular marker techniques like AFLP (Wahyuni *et al.*, 2003), ISSR (Prem *et al.*, 2008; Kizhakkayil and Sasikumar 2010) and RAPD (Sajeev *et al.*, 2011; Paul *et al.*, 2012) have been reported in ginger for variability analysis.

The present investigations on "variability analysis in ginger (*Zingiber officinale* Rosc.) somaclones using molecular markers" were conducted at Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Kerala Agricultural University, Thrissur during the period February 2012 to May 2013 to assess somaclonal variation in ginger at molecular level, to study the influence of genotype and mode of regeneration on somaclonal variation, to assess the extent of variability in somaclones from the original source parent cultivars and to select out the variants.

Review of Literature

2. REVIEW OF LITERATURE

Ginger is an important commercial spice crop esteemed for its aroma and pungency. As natural variability is limited in ginger, tissue culture induced variability is being utilized for improvement programmes. Though crop improvement research in ginger started as early as 1950, the earlier efforts were mainly focused on evaluation of germplasm and selection of high yielding clones (Nybe, 1978; Rattan, 1994) and mutation breeding.

The lack of variability for resistance to major diseases (Shankar, 2003) and extremely rare seed set make conventional breading programmes ineffective in ginger. The crop improvement through biotechnological interventions was attempted in ginger. Valsala (1994) tried *in vitro* pollination and fertilization in ginger, she could obtain seed set through *in vitro* placental pollination but the seed germination was yet to be refined. Under these circumstances, *in vitro* culture induced variation (somaclonal variation) constitutes an important source of variability for improvement of this valuable spice crop.

2.1 Somaclonal variation

Somaclonal variation is defined as variation originating in cell and tissue cultures (Larkin and Scowcroft, 1981). Presently, the term somaclonal variantion is universally used for all forms of tissue culture derived variants (Bajaj, 1990). It has been exploited in many crops for isolation of desirable plants types showing improvement in yield quality and ressistance / tolerance to biotic and abiotic stresses (Bairu *et al.,* 2011). Many factors are found to influence the rate of somaclonal variation. They include the mode of regeneration (Evans, 1988; Karp, 1989; Skirvin and Janick, 1996; Rani and Raina 2000; Saker *et al.,* 2012), type and concentration of growth regulators (Shoemaker *et al.,* 1991; Martin *et al.,* 2006; Jin *et al.,* 2008), explants source (Tsai *et al.,* 1997; Kawiak and Łojkowska, 2004; Chuang *et al.,* 2009) Number and duration of subcultures (Bairu *et al.,* 2006; Smykal *et al.,* 2007) Effect of stress and genotype (Lee and Phillips, 1988; Etienne and Bertrand, 2003)

Somaclonal variation was effectively utilized for production of useful phenotypic variants in banana (Tan *et al.,* 1993), rye (Trojanowska, 2002) and pearl millet (Srivastav *et al.,* 2003) for improving yield in sugarcane (Dhumale *et al.,* 1994), barley (Li *et al.,* 2001), banana (Nwauzoma *et al.,* 2002), rice (Rasheed *et al.,* 2003), wheat (Arun *et al.*, 2003) and sunflower (Encheva *et al.,* 2004) for upgrading quality in geranium, (Ravindra 2004), chilli (Anu *et al.,* 2004) for disease tolerance in black paper (Shylaja, 1996; Sanchu, 2000).

Several techniques of molecular biology are available now a days for detection of genetic polymorphism in crop plants. Molecular marker have been widely used for estimating genetic diversity, varietal finger printing, linkage mapping, and identification of somatic hybrids and for analyzing genetic stability of plants derived through tissue culture.

The present investigations entitled "variability analysis in ginger (*Zingiber officinale* Rosc.) somaclones using molecular markers" were carried out to assess somaclonal variation in ginger at molecular level and to study the influence of genotype and mode of regeneration on the extent of somaclonal variability. The study also aimed to assess variability in somaclones from the original source parent cultivars and to select out the variants. The relevant literature on various aspects of the investigations are reviewed in this chapter.

2.2. Isolation of genomic DNA

One pre-requisite for molecular work is the isolation of good quality genomic DNA. Doyle and Doyle (1987) reported a protocol for rapid DNA isolation procedure from small quantities of fresh leaf tissue.

Porebski *et al.* (1997) standardized DNA isolation protocol for plant material containing large quantities of polyphenols, tannins and polysaccharides. The method involved a modified CTAB extraction protocol, employing high salt concentration to remove polysaccharides and use of polyvinyl pyrrolidone (PVP) to remove polyphenols.

Turmeric and ginger are used as spices and in alternative systems of medicine. They are rich in polysaccharides, polyphenols, and alkaloids. A simple and rapid method for isolating good quality DNA with fairly good yields from mature rhizome tissues of turmeric and ginger has been perfected. Isolated DNA was amenable to restriction digestion and PCR amplification Syamkumar *et al.* (2003).

Sharma *et al.* (2008) modified DNA extraction method in tuber crops by inactivating protein contaminants by using CTAB or proteinase K and precipitating polysaccharides in presence of high concentration of salt.

Amani *et al.* (2011) optimized a simple, rapid and efficient method for leaf DNA extraction. This method involved the use of small amount of plant material to reduce inhibitory agents (alkaloids and phenol). The procedure involved homogenization of the plant leaf in extraction buffer, incubation at 60°C, extraction by chloroform: iso-amyl alcohol and finally DNA precipitation by cold isopropanol. The results showed that the extracted DNA could be used directly for PCR.

2.3. DNA bulking

Dulson *et al.* (1997) examined the usefulness of bulking equal quantities of DNA from 14 to 20 individuals of a cultivar in a study using RAPD marker to distinguish between *Brassica napus* cultivars of varying genetic complexity. For the four cultivars assessed (Quantum, OAC Springfield, Innovator and AC Excel), consistent presence / absence scores were obtained from bulked DNA samples of three different RAPD markers despite a significant degree of variation among samples from individuals. Use of bulked DNA samples thus may enable identification of a distinguishing profile of RAPD markers whose presence / absence was uniform and stable even in complex cultivars.

The use of bulked leaf samples from individual plants for amplified fragment length polymorphism (AFLP) analysis was evaluated as a tool for assessment of genetic diversity in white clover (*Trifolium repens* L.). Bulking of leaf samples produced slightly simpler AFLP profiles compared to the combined profiles of individual plants from the same cultivar. Approximately 90% of bands which were

present in individual plants were present in bulked samples of the same cultivar. Cluster analysis of AFLP data derived from individual plants resulted in a phenogram similar to that produced from data derived from bulked samples of the same plants. AFLP analysis of bulked samples detected significant amounts of genetic variability among 52 cultivars and accessions with genetic similarity values ranging from 0.42 to 0.92 (Kolliker *et al.*, 2001).

Fu *et al.* (2003) assessed effectiveness of several bulking strategies in detecting RAPD variations and determining genetic relationships of five flax (*Linum usitatissimum* L.) landrace accessions. Bulking ten individuals before and after DNA isolations generated consistent RAPD variations. About 30 per cent of the polymorphic RAPD loci observed in the plant-by-plant (PBP) sample were difficult to score and / or undetected in the bulked samples of the same accession. Heterogeneity among the six bulked samples of the same accession was observed at 5.6 per cent of the loci scored. The frequency of a specific RAPD band present in those individuals used to form a bulk was at least 1/11 for its detection in the bulked sample. In spite of these limitations, bulking still generated compatible genetic relationships of the five accessions from its PBP sampling.

Genetic variability analysis using bulked DNA samples for Random Amplified Polymorphic DNA analysis have been conducted in alfalfa (Yu and Pauls, 1993), common buckwheat (Kump and Javornik, 1996) and tea (Goo *et al.*, 2002).

2.4. Variability analysis

Genetic variability is usually measured as the amount of genetic diversity among individuals of a variety or population of a species (Brown, 1983). There are several methods for determining genetic variability in somaclones such as morphological, biochemical and molecular methods.

2.4.1. Morphological markers

Morphological markers generally correspond to the qualitative traits that can be scored visually. They have been found in nature or generated as the result of

mutagenesis. Morphological markers are usually dominant or recessive (Chawla, 2010).

Morphological characters have long been used to identify variants in clonal micropropagation. Variants can be easily detected based on characters such as difference in plant stature, leaf morphology and pigmentation abnormality (Israeli *et al.*, 1991). Off-types in banana can be visually detected during acclimatization of micropropagated plants in the green house before transplanting to the field. In the field, it is also possible to detect dwarf off-types by observing the stature and leaf index (leaf length/width) 3-4 months after establishment (Rodrigues *et al.*, 1998). Similarly, in date palm, the production of offshoots, excessive vegetative growth and leaf whitening are common morphological traits used in detecting somaclonal variants (Zaid and Al Kaabi, 2003).

However, morphological traits are often strongly influenced by environmental factors and may not reflect the true genetic composition of a plant (Mandal *et al.*, 2001). In addition, morphological markers used for phenotypic characters are limited in number, often developmentally regulated and easily affected by environmental factors (Cloutier and Landry, 1994). Likewise, Jarret and Gawel, (1995) raised concerns about the irregular responses of the genomes under *in vitro* manipulation that can result in over or under estimations of the degree of closeness among somaclones. In most of the cases major changes of the genome, as a result of *in vitro* manipulation may not be expressed as an altered phenotype and vice versa. Furthermore, the detection of variants using morphological features is often mostly feasible for fully established plants either in the field or greenhouse. This is not an ideal technique for commercial application due to cost implications (Israeli *et al.*, 1995).

2.4.1.1. Variability analysis using morphological markers

In ginger, variability for morphological, yield, quality and disease reaction was reported by several workers. Mohanty and Sarma (1979) reported that the expected genetic advance and heritability estimates were high for the secondary rhizome and total root weight. Genetic coefficient of variation was high for weight of root tubes.

Positive correlation of rhizome weight with plant height, number of tillers and leaf number was reported by Sreekumar *et al.* (1982).

Mohanty *et al.* (1981) observed significant varietal differences for all the characters except the number of tillers per plant and number of leaves per plant.

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 from rhizome characters.

Sasikumar *et al.* (1992) studied 100 accessions of ginger germplasm for variability. They found that rhizome yield was correlated with plant height, tiller and leaf number leaf number leaf length and width. Pandey and Dobhal (1993) observed a wide range of variability for most of the characters such as plant height, number of fingers, and yield / plant studied by them.

Ravindran *et al.* (1994) characterized 100 accessions of ginger germplasm based on morphological, yield and quality parameters. Moderate variability was observed for many yield and quality traits. Tiller number per plant had the highest variability, followed by rhizome yield per plant. Among the quality traits the shogaol content recorded high variability followed by crude fiber and oleoresin.

Yadav (1999) reported high genotypic variation all the characters like length and weight of secondary rhizomes, weight of primary rhizomes number of primary and secondary rhizomes and rhizome yield / plant. Mohandas *et al.* (2000) found that all the cultivars differed significantly in tiller number and leaf number.

Morphological markers have limited application in breeding as they are few in number as well as dependant on the season and developmental stage of the plant and are influenced by the environment (Krishna and Singh, 2007).

Aragaw *et al.* (2011) assessed genetic variability of thirty-six ginger (*Zingiber officinale* Rosc.) accessions for morphological and some quality attributes. The experiment was laid out in a 6x6 simple lattice design with two replications during 2009-2010 main cropping seasons at locations i.e., Tepi and Bahir Dar in Ethiopia.

Variances component method was used to estimate genetic variation, broad sense heritability and genetic advance. Number of plants per plot, fresh rhizome yield and dry rhizome yield showed high Genetic Coefficient of Variation (GCV) and Phenotypic Coefficient of Variation (PCV) at both locations. Relatively high heritability and genetic advance was obtained for oleoresin content, volatile oil content, fiber content, fresh rhizome yield and dry rhizome yield. The D^2 analysis showed the 36 genotype grouped into 7 and 11 clusters at Tepi and Bahir Dar, respectively.

Jan *et al.* (2012) estimated genetic variability in turmeric (*curcuma longa* L.) germplasm using agro-morphological traits. The study was conducted to determine the extent of genetic variation and relationship among turmeric genotypes using 21 qualitative and quantitative traits. A total of 20 genotypes collected from three eco-geographical areas (Bannu, Haripur and Kasur) of turmeric cultivation in Pakistan were studied under field conditions. In qualitative traits light green leaf color, light orange yellow rhizome color and greenish white flower color were found in abundance in most of the genotypes. The leaves, rhizomes and flowers of turmeric plants collected from Bannu and Haripur were light green, yellow and yellowish-white in color, while those of Kasur area were dark green, dark orange and whitish green in color, respectively. A considerable level of variability was displayed by various genotypes for some of the quantitative traits measured. Pattern of variation among the genotypes was different for different agro-morphological traits. The largest variation was observed for plant height, leaf length, leaf width, total and fresh number of leaves, whereas relatively low level of variability was detected in most of the remaining quantitative traits. Agro-morphological data was also analyzed by numerical taxonomic techniques using two complementary procedures: cluster and principal component analysis (PCA). Phenogram based on Euclidean distance coefficients placed 20 genotypes into two main clusters with three sub-groups in the 2nd cluster. Genotype groups were primarily associated with morphological differences among the collections and secondly with the consumer preference and horticulture use.

2.4.1.2. Variability of ginger against pest and diseases

Ginger germplasm were screened for variability to the pest and disease incidences by many scientists. Nybe and Nair (1979) reported that cultivar Maran showed least infection for rhizome rot disease while Bajpai, Maran and Nadia cultivars showed most tolerance against leaf spot disease. Nybe *et al.* (1980) reported that cultivat Rio-de-Janeiro is tolerant to the shoot borer pest.

Paul and Shylaja (2012) studied screening of ginger (*Zingiber officinale* Rosc.) somaclones derived through indirect organogenesis against soft rot and bacterial wilt diseases. Their study indicated that somaclones of cultivar Maran showed more tolerance to both diseases as compared to somaclones of cultivar Rio-de-Janeiro.

2.4.2. Biochemical markers

The physiological responses and / or biochemical markers for detecting variants are faster and can be carried out at juvenile stages to lower the possible economic loss (Israeli *et al.,* 1995). The response of plants to physiological factors such as hormones and light can be used as basis to differentiate between normal and variant somaclones (Peyvandi *et al.,* 2009).

Biochemical markers, such as allozymes are used to detect variability. Allozymes are allelic variants of enzymes encoded by structural genes. Enzymes are proteins consisting of amino acids, some of which are electrically charged. As a result, enzymes have a net electric charge, depending on the stretch of amino acids, comprising the protein. When a mutation in the DNA results in an amino acid being replaced, the net electric charge of the protein may be modified, and the overall shape (conformation) of the molecule can change. Because changes in electric charge and confirmation can affect the migration rate of proteins in an electric field, allelic variation can be detected by gel electrophoresis and subsequent enzyme-specific stains that contain substrate for the enzyme, cofactors and an oxidized salt. Usually two, or sometimes even more loci can be distinguished for an enzyme and these are termed isoloci. Therefore, allozyme variation is often also referred to as isozyme variation (Kephart, 1990; May 1992). Allozyme analysis is simple, quick and easy to use.

Allozymes are codominant markers that have high reproducibility. Zymograms can be readily interpreted in terms of loci and alleles but they are less abundant, show low level of polymorphism and they are affected by environmental conditions.

Allozymes found applications in many fields such as diversity analysis (Lamboy *et al.,* 1994; Hamrick and Godt, 1999), population genetics studies (Erskine and Muehlenbauer, 1991), population structure and divergence (Freville *et al.,* 2001), interspecific relationships (Garvin and Weeden, 1994), fingerprinting purposes (Maass and Klaas, 1995).

2.4.2.1. Biochemical variability in ginger

Oleoresin is the extract of ginger containing all the flavoring principles as well as the pungent constituents. The oleoresin contains two important compounds-gingerol and shagoal that contributes to the pungency of ginger. On long term storage, gingerol is converted to shagol. The quality of ginger thus depends on the content of gingerol and shagoal. Zachariah *et al.* (1993) classified 86 ginger accessions based on quality and grouped them into high medium and low quality types.

Shamina *et al.* (1997) reported biochemical variability in ginger. They studied twenty-five accessions of ginger 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.

2.4.3. Variability analysis using molecular markers

Molecular techniques are valuable tools for testing genetic fidelity of *in vitro* micropropagated plants. At the molecular level, variations observed in tissue culture derived plants are changes in chromosome number or structure, or more stable changes in the DNA (Gostimsky *et al.,* 2005). The work by Botstein *et al.* (1980) on the construction of genetic maps using restriction fragment length polymorphism (RFLP) was the first reported molecular marker technique in the detection of DNA

polymorphism. Presently, a number of molecular techniques are available to detect sequence variation between closely related genomes including differences between source plants and somaclones. These techniques involve the use of molecular markers which are useful in comparing the DNA from different samples for the differentiation in plants due to sequence variation by identifying random polymorphisms (Cloutier and Landry, 1994).

A molecular marker is a DNA sequence which is readily detected and whose inheritance can be easily monitored. They are based on naturally occurring polymorphism in DNA sequences. The molecular markers are directly linked to the genome. Their number is potentially unlimited in the genome and dispersion across the genome is complete. Molecular tools provide valuable data on variability through their ability to detect variation at the DNA level. Glaszmann *et al.* (2010) reported that molecular markers have clarified the structure of genetic diversity in a broad range of crops.

Molecular markers have the advantages of being abundant, phenotypically neutral, show absence of epistasis and are not influenced by the developmental stage or tissue of the plant or environmental conditions (Mohapatra, 2007). Many molecular markers are utilized for numerous purposes such as characterization of germplasm, varietal identification and clonal fidelity testing, assessment of genetic diversity, validation of genetic relationships and marker-assisted selection (Hoogendijk and Williams, 2001; Sing, 2008). Molecular markers are highly heritable, obtainable at a high number and frequency and display enough polymorphism in closely related genotypes (Stuber *et al.*, 1999; Archak *et al.*, 2003; Weising *et al.*, 2005).

Markers based on differences in DNA sequences between individuals generally detect more polymorphisms than morphological and proteins based on markers and constitute a new generation of genetic markers (Tanksley *et al.*, 1989). Techniques which are particularly promising in assisting selection for desirable characters involve the use of two types of molecular markers such as hybridization based molecular markers such as Restriction Fragment Length Polymorphisms (RFLP) (Botstein *et al.*, 1980) and Polymerase Chain Reaction (PCR) based molecular markers.

Polymerase chain reaction (PCR) based techniques make use of random or specific primers to amplify DNA fragments from the genome. They are simple to perform, easily amenable for automation and be used to assay a large number of samples. Polymerase chain reaction was invented by Mullis and coworkers in 1983, and is based on the enzymatic *in vitro* amplification of DNA (Weising *et al.*, 2005). In PCR based techniques, a DNA sequence of interest is exponentially amplified with the aid of primers and a thermostable DNA polymerase. The reaction involves repeated cycles, each consisting of denaturation, primer annealing and elongation steps. Use of random primers eradicated the limitation of prior sequence information for PCR analysis (Agarwal *et al.*, 2008). PCR based analytical techniques using various molecular markers provide an essential tool needed to reveal polymorphism at the DNA sequence level and solve the problems of introgression as well as lineage (Gostimsky *et al.*, 2005; Simmons *et al.*, 2007).

Polymerase chain reaction (PCR) based techniques include Randomly Amplified Polymorphic DNA (RAPD) (Welsh and McClelland, 1990; Williams *et al.*, 1990), Amplified Fragment Length Polymorphisms (AFLP) (Vos *et al.*, 1995), Simple Sequences Repeats (SSR) (Tautz, 1989 and Hearne *et al.*, 1992), Inter Simple Sequences Repeats (ISSR) (Zietkiewicz *et al.*, 1994), DNA Amplification Fingerprinting (DAF), Sequence Tagged Sites (STS) (Fukuoka *et al.*, 1994), Sequences Characterized Amplified Regions (SCAR) (Williams *et al.*, 1990), and Expressed Sequences Tags (EST). Of these, RFLP and micro satellites markers are co-dominant markers while RAPD and AFLP markers are largely dominant markers. The utility of molecular markers in crop breeding is reviewed by Mohan *et al.* (1997) and Gupta and Roy (2002).

2.4.3.1. Molecular markers in variability analysis

2.4.3.1.1. Restriction fragment length polymorphism (RFLP)

Restriction fragment length polymorphism (RFLP) was developed by Botstein *et al.* (1980) Restriction fragment length polymorphism is a technique used for genome analysis of organisms, thereby providing a molecular basis for any observed

differences. In RFLP, extracted DNA is digested with restriction enzymes and the resultant fragments are separated by gel electrophoresis according to their sizes (Karp *et al.,* 1996). Molecular variations in the organisms are revealed by the resultant length of the fragments after digesting with the restriction enzymes. This differential profile is generated due to nucleotide substitutions or DNA rearrangements like insertion or deletion or single nucleotide polymorphisms (Agarwal *et al.,* 2008).

Restriction fragment length polymorphism (RFLP) was one of the first techniques used to study somaclonal variation and has been widely used in several species. Jaligot *et al.* (2002) described methylation-sensitive RFLP markers that differentiated between normal and abnormal embryogenic calli of oil palm. The markers were useful for the early detection of somaclonal variation. Generally, RFLP markers are relatively highly polymorphic, co-dominantly inherited and highly reproducible (Agarwal *et al.,* 2008). Although RFLP markers are useful for sampling various regions of the genome and are potentially unlimited, the technique is time consuming, costly and a large amount of plant tissue is required for analyses (Piola *et al.,* 1999). In addition, it involves the use of radioactive reagents, it requires the development of cDNA or genomic DNA probes when heterologous probes are unavailable (Karp *et al.,* 1996). These limitations led to the development of new set of less technically complex methods which are Polymerase chain reaction (PCR)-based.

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

Halward *et al.* (1991) studied genetic variation among twenty-five unadapted germplasm resources of cultivated groundnuts from south America, Africa and China, using RFLP and reported high polymorphism among wild arachis species but very little among cultivated groundnut.

Davis *et al.* (1998) reported a study to reveal genetic relationship among cultivated Avocado through RFLP analysis. They reported high level of polymorphism in 36 cultivars and all cultivars were assigned a unique genotype based on 14 genetic loci and grouped into three major clusters corresponding to the three major racial grouping in Avacado.

2.4.3.1.2. Randomly Amplified Polymorphic DNA (RAPD) markers

Random Amplified Polymorphic DNA markers are DNA fragments amplified by the Polymerase Chain Reaction (PCR) using short synthetic primers (generally 10bp) of random sequences. These oligonucleotides serve as both forward and reverse primer, and are usually able to amplify fragments from 1-10 genomic sites simultaneously. Amplified fragments, usually within the 0.5-5 kb size range, are separated by agarose gel electrophoresis, and polymorphisms are detected, by ethidium bromide staining, as the presence or absence of bands of particular sizes. These polymorphisms are considered to be primarily due to variation in the primer annealing sites, but they can also be generated by length differences in the amplified sequence between primer annealing sites. They are quick and easy to assay. Only low quantities of template DNA are required, usually 5-50 ng per reaction. Since random primers are commercially available, no prior sequence data for primer construction are needed. Moreover, RAPDs have a very high genomic abundance and are randomly distributed throughout the genome but they are less reproducible (Schierwater and Ender, 1993).

Random Amplified Polymorphic DNA (RAPD) has been used for many purposes, ranging from studies at the individual level (genetic identity) to studies involving closely related species. In 1990, molecular markers generated using Arbitrarily Primed Polymerase Chain Reaction (AP-PCR) was used to identify Randomly Amplified Polymorphic DNA (RAPD) in several organisms including plants (Welsh and McClelland, 1990; Williams *et al.*, 1990). Random Amplified Polymorphic DNA (RAPD) are commonly inherited as dominant markers, where the presence of a particular band is dominant, and its absence is recessive (Tingley and Tufo, 1993). RAPD analysis has found applications in population studies (Welsh *et*

al., 1991), biosystematics (Stiles *et al.*, 1993), gene tagging (Ranade *et al.*, 2001) and fingerprinting (Moglayi, 2011). RAPD has also been successfully applied in molecular ecology (Hadrys *et al.*, 1992), genetic analysis (Williams *et al.*, 1990), assessing variation in plants (Newbury and Ford-Lloyd, 1993) to study the extent of diversity within plant germplasm (Virk *et al.*, 1995) and combining ability in rice (Radhidevi *et al.*, 2002).

Rout *et al.* (1998) studied genetic stability of 14 mericlones of ginger cultivar V_3 S_{18} using RAPD markers. Out of 15 decamer primers tried for DNA amplification, three produced amplification. Amplification products were monomorphic for all the clones analaysed.

Li *et al.* (1999) reported rapid identification of tomato somaclonal variation with Random Amplified Polymorphic DNA (RAPD) marker. Total DNA was extracted from the leaves of in vitro-regenerated tomato (*Lycopersicon esculentum*) plants selected after treatment with fusaric acid. Four out of 60 random primers were adapted for the identification of somaclonal variation by RAPD marker. Using those primers, physiologically tolerant clones were identified.

Polymorphism in banding pattern was detected in regenerants of upland rice cultivar IAC 47 regenerated through indirect organogenesis (Araujo *et al.*, 2001). The somaclones showed morphological variants and exhibited differences in reaction to rice blast.

Soniya *et al*, (2001) studied genetic stability of regenerants in tomato variety Shakti derived through indirect organogenesis using RAPD markers. They observed 90 to 99 per cent genetic similarity between the calliclones and mother plants.

Salvi *et al.* (2001) reported polymorphism in RAPD banding pattern in regenerants of turmeric cultivar Elite derived through indirect organogenesis. In their further studies with adventitious bud regenerants, they observed no RAPD polymorphism between somaclones and CP plants (Salvi *et al.*, 2002).

Mandal & Chand (2002) characterized 17 somaclones of tea along with control plant using RAPD markers. Four somaclones exhibited polymorphism in banding pattern while 13 somaclones and the control plant showed monomorphism in banding pattern.

Suja (2002) and Nirmal Babu *et al.* (2003) used RAPD profiles amplified by 11 operon primers as an index for estimating genetic fidelity of selected variants among micropropogated and callus regenerated plants. They found high variability among selected plants.

Riji (2003) attempted RAPD analysis of 22 regenerants of turmeric derived through indirect organogenesis along with CP plant. Nine variants were detected which exhibited polymorphism in RAPD profiles. None of the caliclones showed similarity towards the CP plants. The clones TSC-1 showed highest polymorphism with CP.

Prakash *et al.*, (2004) reported polymorphism in RAPD banding pattern in regenerants of mango ginger derived through indirect organogenesis.

The genetic variation among three *Zingiber officinale* cultivars, i.e. *Z. officinale* var. *officinale*, *Z. officinale* var. *rubrum* (halia bara) and *Z. officinale* var. *rubrum* (halia padi) was studied using RAPD analysis. The cultivars are useful in traditional medicine and as food flavorings in Peninsular Malaysia. Analysis was carried out on the RAPD profiles generated by 16 arbitrary primers to determine genetic differences between the cultivars. The clearest polymorphic bands were obtained from the OPA1, OPA8, OPA9, OPA10, OPA13, OPA16 and OPA20 primers. A total of 104 bands were scored and analysed (Muda *et al.*, 2004).

Kuriakose *et al.* (2005) conducted RAPD analysis of embryo derived clones of vanilla using 15 primers. Genetic variability to the extent of 58 per cent was observed.

Nazeem *et al.* (2005) studied the variability and relatedness among 49 accessions of black pepper (*Piper nigrum* L.) using molecular marker RAPD and AFLP. The similarity matrix was subjected to cluster analysis and dendrogram

generated using the software NTSYS pc 2.1. The dendrogram revealed an average similarity of 63 per cent among accessions. Two selections from the variety Karimunda, namely Subhakara and Shreekara, together form a single cluster with almost 90 per cent similarity. The dissimilarity observed between the varieties Panniyur-1 and Panniyur-3, the progenies of the same parentage, Uthirankotta and Cheriyakaniakkadan was only 18 per cent.

Nayak *et al.* (2005) studied the existing variation in 16 promising cultivars of ginger. The karyotypic analysis revealed a differential distribution of A, B, C, D and E type of chromosomes in different cultivars as represented by different karyotype formulas. A significant variation of 4C DNA content was recorded in ginger at an intraspecific level with values ranging from 17.1 to 24.3 pg. RAPD analysis revealed a differential polymorphism of DNA showing a number of polymorphic bands ranging from 26 to 70 in 16 cultivars. The RAPD primers OPC02, OPA02, OPD20 and OPN06 showing strong resolving power were able to distinguish all 16 cultivars. The genetic variation thus detected in promising cultivars of ginger has significance for ginger improvement programmes.

Palai and Rout (2007) carried out RAPD assay for identification and genetic variation within eight high yielding varieties of ginger. 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. The first major cluster had only one variety 'S-558' with 43 per cent similarity with other seven varieties. Second major cluster having seven varieties and divided into two minor clusters. One minor cluster had six varieties ('Jugijan', 'Turia local', 'Nadia', 'Zo-17', 'Nahfrey' and 'Gurubathan) and other having only one variety 'Surabhi'. The second minor cluster further divided into two sub-minor clusters. 'Nadia' and 'ZO-17' had 78 per cent similarity among themselves and 70 per cent similarity with 'Jugijan' and 'Turia local'. 'Jugijan' and 'Turia local' were having 81 per cent similarity among themselves. However, 'Nahfrey' had 64 per cent similarity with 'Jugijan', 'Turia local', 'Nadia' and 'ZO-17'. The study showed the distant variation within the varieties.

Sheidai *et al.* (2008) used 30 decamer RAPD primers to study somaclonal variation among the parental plants as well as regenerated banana plants of the first, third, fifth, seventh and ninth sub-cultures. Eighteen out of thirty primers produced 289 bands in all the genotypes studied. 140 bands (48.95%) were common in the parental genotype and the regenerated plants while 147 bands were polymorphic (51.40%). In total 74 specific bands were observed in the parental genotype and the regenerated plants of the sub-cultures. Grouping of the parental cultivar and their regenerated plants indicate the genetic distinctness of the genotypes studied as they are placed in different clusters/groups far from each other.

The genetic relatedness among 51 accessions, 14 species of the genus *Zingiber* and genetic variability of a clonally propagated species, *Zingiber montanum* (Koenig) Link ex Dietr., from Thailand were studied using random amplified polymorphic DNA profiles (Bua-in and Paisooksantivatana, 2010). In their study, twenty-nine random primers gave reproducible amplification banding patterns of 607 polymorphic bands out of 611 scored bands accounting for 99.40 per cent polymorphism across the genotypes. Jaccard's coefficient of similarity varied from 0.119 to 0.970, indicative of distant genetic relatedness among the genotype studied. UPGMA clustering indicated eight distinct clusters of *Zingiber*, with a high cophenetic correlation ($r = 1.00$) value. Genetic variability in *Z. montanum* was exhibited by the collections from six regions of Thailand. High molecular variance (87%) within collection regions of *Z. montanum* accessions was displayed by AMOVA and also explained the significant divergence among the sample from six collection regions. Their results indicated that RAPD technique is useful for detecting the genetic relatedness within and among species of *Zingiber* and that high diversity exists in the clonally propagated species, *Z. montanum*.

Sen *et al.* (2010) analysed genetic diversity in *Piper* species using RAPD markers. Among 22 decamer RAPD primers screened, 11 were selected for comparative analysis of different species of *Piper*. High genetic variations were found among different species of *Piper*.

Identification of genotypic variations is a pre-requisite for ginger (Zingiber officinale Rosc.) improvement programmes. Genetic diversity analysis was carried out in a set of forty-nine ginger clones cultivated in North-East India using random amplified polymorphic DNA (RAPD) markers. 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. Cluster V included four clones traditionally cultivated in the Indian state of Meghalaya known for production of high-quality ginger indicating that the clones were a good candidate for ginger improvement. 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 (Sajeev *et al.*, 2011).

Paul *et al.* (2012) reported molecular characterization of selected somaclones in ginger using RAPD markers. In their study, twelve selected somaclones of ginger cultivars Maran and Rio-de-Janeiro were characterised using RAPD markers. Five primers of the series OPAH and OPP gave good DNA amplification. The primer OPAH 3 exhibited highest polymorphism (33.75%). The molecular characterization of somaclones revealed the occurrence of genetic variability in the clones evaluated. The extent of genetic variation was more in somaclones of cultivar Maran than clones of cultivar Rio-de-Janeiro. Genetic variation from the source parent cultivar was also more in somaclones of cultivar Maran.

Singh *et al.* (2012) evaluated genetic diversity in turmeric (Curcuma longa L.). They examined the genetic diversity in turmeric accessions from 10 different agro-climatic regions comprising 5 cultivars and 55 accessions. Two DNA-based molecular marker techniques, viz., RAPD and ISSR were used to assess the genetic diversity in turmeric genotypes. A total of 17 polymorphic primers (11 RAPDs and 6 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. Number of amplified fragments with RAPD primers ranged from 3 to 13 with the size of amplicons ranging from 230 to 3000bp in size. The polymorphism ranged from 45

to 100 with an average of 91.4 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 number of amplified bands varied from 1 to 14 with size of amplicons ranging from 200 to 2000bp. 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.

Afrasiab and Iqbal (2012) analyzed somaclonal variants and gamma induced mutants of potato (*Solanum tuberosum* L.) cv. Diamant using RAPD-PCR technique. In their work, all the four selected somaclonal variants and five gamma induced mutants were differentiated by banding patterns obtained from 22 primers that generated 140 polymorphic bands. The presence of polymorphic bands in variants and mutants suggest that genetic variation occurred in all the treatments as compared to control. Similarity and clustered analysis were conducted using Jaccard's coefficients and the un-weighted pair-group method using arithmetic averages. The dendrogram showed genetic diversity among the variants and mutants. The study showed that RAPD markers were efficient in discriminating somaclonal variants and induced mutants of potato.

Chinmayee *et al.* (2012) assessed genetic fidelity of micropropogated banana using RAPD. Plants showing normal and off-type morphology were selected from green house and field. Two arbitrary decemer primers have been used to amplify genomic DNA from selected plants (primer OPH-09 and OPJ-04), OPJ-04 showed polymorphism in the pattern as compared to normal plants. A total of six amplified products were obtained, out of which five were polymorphic.

Saker *et al.* (2012) conducted RAPD assay in potato using five selected primers to detect somaclonal variation. All the morphological variants were excluded. One of the regenerated plantlet derived from leaf-explants was true-to-type to the main *in vitro* plantlet, so it would be used as a source of explants for transformation

experiments. The other regenerated plantlets derived from leaf explants and tuber discs were variant.

Sharma *et al.* (2012) conducted RAPD-PCR analysis in apple regenerants using 80 decamer primers initially. Out of these, 26 primers yielded consistent and reliable differences during repeated analysis. Total 134 scorable bands were observed with 58.2 per cent polymorphism. Similarity coefficient values were higher among regenerants than between regenerants and the mother plant. The dendrogram obtained from all 14 samples revealed four clusters and one separated regenerant which did not cluster with others.

Esmaiel *et al.* (2012) detected genetic polymorphism in carnation using RAPD technique. They used five primers to amplify DNA of the two selected carnation cultivars and their sixteen somaclones. A total of 62 amplification products were obtained, out of which 96.15 per cent showed polymorphism. Genetic similarity among the eighteen genotypes ranged from 0.32 to 0.91. In this study the regenerated somaclonal variant lines and their parents were classified into two clusters: The cultivar Lia and its 8 somaclones were grouped in one cluster while the cultivar White Liberty and its somaclones were included in another cluster. The study indicated that the use of RAPD technique was sensitive and powerful to detect genetic variation at the level of DNA among carnation variants.

Udayakumar *et al.* (2013) carried out RAPD analysis to study the genetic variations among 20 different populations of *Withania somnifera* (L.) Dunal collected from different habitats (locations) of South India. Out of 40 primers, 11 selected primers produced 96 consistent RAPD markers; out of which 75 were polymorphic. Similarity indices were estimated using the Dice coefficient of similarity and cluster analyses were carried out on the similarity estimates using the unweighted pairgroup method to produce a dendrogram using arithmetic average (UPGMA) in the NTSYS pc-version 1.80 software. The similarity coefficient ranges from 0.53 to 0.98, suggesting that the pronounced genetic variations exist among populations of *W. somnifera* in South India. The cluster analysis indicates that the 20 populations of *W. somnifera* were divided into five major groups, regardless of geographical locations.

The RAPD analysis indicates existence of genetic variations in natural populations and it may influence and produce changes in phytochemical constituents of *W. somnifera* populations.

The genetic variation of 20 banana accessions (including 3 clones obtained by mutagenesis and somaclonal variation) was determined using random amplified polymorphic DNA markers. PCR products were analysed by electrophoresis in 1.5 per cent agarose gel and TBE buffer stained with ethidium bromide. Each genotype was reported taking the most intense bands creating one matrix of binary values analysed by UPGMA method using Jaccard's similarity coefficients. The analysis of main components was conducted by SPSS software for Windows and taxonomic classification (Peteira *et al.*, 2003).

Detection of somaclonal variation using RAPD markers was attempted in several crops species such as beet (Munthali *et al.*, 1996), garlic (Zahim *et al.*, 1999), date palm (Saker *et al.*, 2000), pear and apple (Caboni *et al.*, 2000), asparagus (Raimondi *et al.*, 2001), banana (Gimenez *et al.*, 2001 and Mohamed, 2007), chilli pepper (Hossain *et al.*, (2003) and potato (Ehsanpour *et al.*, 2007) in varying degrees.

Paul (2004) characterized selected somaclones in ginger using RAPD markers and detected somaclonal variation in the clones evaluated.

2.4.3.1.3. Inter Simple Sequence Repeats (ISSR)

Inter Simple Sequence Repeats (ISSR) are DNA fragments of about 100-3000bp located between adjacent, oppositely oriented microsatellite regions. ISSRs are amplified by PCR using microsatellite core sequences as primers with a few selective nucleotides as anchors into the non-repeat adjacent regions (16-18bp). About 10-16 fragments from multiple loci are generated simultaneously, separated by gel electrophoresis and scored as the presence or absence of fragments of particular size. The main advantage of Inter Simple Sequence Repeats (ISSR) is that no prior sequence information for primer construction is needed. Because the analytical procedures include PCR, only low quantities of template DNA are required (5-50 ng per reaction). Inter Simple Sequence Repeats (ISSR) are largely distributed throughout

the genome. It is a multi locus molecular technique based on PCR that identifies insertions and deletions in DNA. It is highly sensitive, highly reproducible, provides Mendelian segregation and has been successfully applied in genetic and evolutionary studies of many species.

It can also be applied in studies involving genetic identity, percentage, clone and strain identification, and taxonomic studies of closely related species. It can be used to assess the genetic stability of micropropogated plants like ginger (Mohanty *et al.*, 2011; Mohanty *et al.*, 2012). In addition, Inter Simple Sequence Repeats are considered useful in gene mapping studies (Godwin *et al.*, 1997; Zietkiewicz *et al.*, 1994; Gupta *et al.*, 1994) and diversity analysis in many crops including ginger (Kizhakkayil and Sasikumar, 2010).

Leroy *et al.*, (2001) detected *in vitro* culture-induced instability in cauliflower through inter-simple sequence repeat (ISSR) analysis.

Ngezahayo, *et al.* (2007) studied the nature of somaclonal variation in rice cv. Nipponbare at the nucleotide sequence level. First, they investigated genomic variations by using two molecular marker systems: RAPD and ISSR. This was followed by sequencing of selected bands that represented genomic variations, and pairwise sequence analysis taking advantage of the whole genome sequence of rice. In addition, transpositional activity of the active MITE, mPing, was analysed by locus-specific PCR amplifications. The two-year-old calli and their regenerated plants, analysed with 24 RAPD and 20 ISSR primers, showed moderate levels of genomic variation (20.83% and 17.04%, respectively). Nevertheless, dendrograms constructed according to the Jaccard coefficient calculated by UPGMA of the ISSR bands revealed that the 5-azacytidine-treated and untreated somaclones were grouped into two distinct clusters, suggesting a possible indirect effect of the treatment on the genomic changes, depending on the marker used. Sequence analysis indicated a low level of variation (0.31%), with single-base-pair substitutions predominating.

Prem *et al.* (2008) conducted molecular characterization of ginger genotypes using RAPD and ISSR markers. They developed molecular fingerprints of elite, exotic

and primitive ginger genotypes to characterize and protect the accessions. 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.

Genetic diversity analysis of ginger germplasm was undertaken by Kizhakkayil and Sasikumar (2010) using ISSR and RAPD marker. In this study global collections of ginger germplasm consisting of 46 accessions were characterized using two types of molecular markers, RAPD and ISSR. UPGMA dendrograms constructed based on three similarity coefficients, i.e., Jaccard's, Sorensen-Dice and Simple Matching using the combined RAPD and ISSR markers grouped the accessions in four similar clusters in all the three dendrograms revealing the clustering patterns among the similarity coefficients and a rather less genetic distance among the accessions. Improved varieties/ cultivars are grouped together with primitive types. Moreover, in the clustering pattern of the accessions, a geographical bias was also evident implying that germplasm collected from nearby locations especially with vernacular identity may not be genetically distinct. The clustering of the accessions was largely independent of its agronomic features.

Mohanty *et al.* (2011) reported genetic stability of micropropagated *Zingiber rubens* Roxb. They evaluated clones periodically at an interval of six months up to 30 months in culture using random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) analysis and genetic uniformity in all regenerants was confirmed.

Mohanty *et al.* (2012) suggested that the micropropagation protocol developed by them is suitable for clonal micropropagation of *Curcuma amada,* when they studied genetic stability assessment of micropropagated mango ginger (*Curcuma amada* Roxb.) using RAPD and ISSR markers.

Twenty seven bread wheat genotypes comprised two parental varieties (Gemmisa-1 and Sakha-69), six somaclonal variant lines derived from the parental cultivar (Gemmisa-1) and seventeen somaclonal variant lines derived from the parental cultivar (Sakha-69) and two local check varieties were analysed using RAPD and ISSR marker systems. All generated dendrograms from standardized morpho-agronomic, ISSR, RAPD and the combined dendrogram (ISSR + RAPD + morpho-agronomic) data separated the twenty seven wheat genotypes into two main groups which diverged at similarity index of averages 0.479, 0.488, 0.501 and 0.282, respectively (Abouzied, 2012).

Rai *et al,* (2012) evaluated genetic homogeneity of one year old guava (*Psidium guajava* L.) plants developed through *in vitro* somatic embryogenesis. Seven randomly selected plants along with the mother plant were subjected to molecular analysis. A total of six SSR primer pairs produced reproducible and clear bands ranging from 100 to 300bp in size. Of the ten ISSR primers screened, six produced resolvable, reproducible and scorable bands. All the ISSR primers produced a total of 25 bands, ranging between 300 and 1,200bp length, and the number of scorable bands, for each primer varied from three to six with an average of 4.1 bands per primer. The amplification products were monomorphic across all the micropropagated plants produced by all SSR and ISSR primers applied. The monomorphic banding pattern in micropropagated plants and the mother plant confirms the genetic homogeneity of the *in vitro* raised plants and demonstrates the reliability of *in vitro* somatic embryogenesis for clonal micropropagation of guava.

Mishra-Rawat *et al.* (2013) investigated genetic differentiation and clonal identification of eight tea clones using morphological and molecular markers (RAPD and ISSR). Random Amplified Polymorphic DNA analysis produced 82.14 per cent polymorphism in the clones. Two main groups were recognized from cluster analysis, similarity within the clones ranged from 0.33 to 0.91. ISSR data also revealed a pattern similar to that of RAPD markers. ISSRs were found to be more discriminative in cultivar identification than RAPDs since clones that could not be distinguished by RAPD marker were easily differentiated by the ISSR markers.

2.4.3.1.4. Microsatellites or Simple Sequence Repeats (SSR)

Microsatellites are tandem repeats of 1-6 base pairs. Microsatellites and their flanking sequences can be identified by constructing a small-insert genomic library, screening the library with a synthetically labeled oligonucleotide repeat and sequencing the positive clones. Alternatively, microsatellites may be identified by screening sequence databases for microsatellite sequence motifs from which adjacent primers may then be designed. In addition, primers may be used that have already been designed for closely related species. Polymerase slippage during DNA replication, or slipped strand mispairing, is considered to be the main cause of variation in the number of repeats units of a microsatellite, resulting in length polymorphisms that can be detected by gel electrophoresis.

They are codominant, highly abundant in eukaryotic genomes and are randomly distributed throughout the genomes with preferential association in low copy regions (Morgante *et al.,* 2002). Only less quantities of template DNA is required (10-100 ng per reaction). It is highly reproducible and do not require high quality DNA. Although microsatellite analysis is, in principle, a single-locus technique, multiple microsatellites may be multiplexed during PCR or gel electrophoresis if the size ranges of the alleles of different loci do not overlap (Ghislain *et al.,* 2004).

Microsatellite markers have been found immensely useful in establishing genetic stability of several micropropagated plants such as bananas (Hautea *et al.,* 2004; Ray *et al.,* 2006), grapevine (Welter *et al.,* 2007), sugarcane (Singh *et al.,* 2008), rice (Gao *et al.,* 2009) and wheat (Khlestkina *et al.,* 2010)

In black pepper, Meneze *et al.* (2009) reported nine microsatellite markers from an enriched library of *Piper nigrum* L. Twenty varieties from the Brazilian germplasm collection were analyzed, and observed and expected heterozygosity values ranged over 0.11-1.00 and 0.47-0.87. The nine microsatellite loci characterized contributed for studies on genetic diversity and conservation of *Piper nigrum.*

Karacsonyi *et al.* (2011) identified the *in vitro* somaclonal variation in potato (*Solanum tuberosum*) plantlets regenerated from callus, using simple sequence repeats (SSR markers).

Joy *et al.* (2011) used SSR markers to analyse genetic diversity among forty popular genotypes and four different species of black pepper in South India. A total of 62 alleles with an average of 15.5 alleles over 4 loci were identified. All the SSR primers showed an average Polymorphism Information Content (PIC) value of 0.85. The estimated average shared allele frequency ranged between 1.57 and 20.12 per cent. The PCA plot revealed four closely related individual groups and identified Karimunda, wild pepper and a local landrace 'local b' as the most divergent genotypes. Cluster analysis exposed the genetic relatedness between hybrids and selections with other known cultivars.

Pandey *et al.* (2012) described early assessment of clonal fidelity in sugarcane plants regenerated through direct organogenesis using RAPD and SSR markers. Analysis of RAPD banding patterns generated by PCR amplification using 20 random primers gave no evidences for somaclonal variation and the percent of polymorphic bands in a total of 110 amplicons was 0.02 per cent. Random Amplified Polymorphic DNA patterns of the plantlets were identical with the original mother plant, indicating that direct adventitious organogenesis did not induce somaclonal variation that can be detected by RAPD. Also SSR banding pattern analysis generated 15 primers (112 amplicons) gave no evidences for somaclonal variation. The genetic fidelity testing of micro-shoots based on a RAPD and SSR analysis indicated a strong genetic purity with the parent genotype. Lack of variation confirms the genetic purity of tissue culture plants of sugarcane raised through direct organogenesis and confirms the suitability of regeneration protocol for clonal micropropagation.

Eighteen ginger cultivars from Northwest Himalayan region, showing significant differences in rhizome size, texture and pungency, were selected and characterized both by chemical and genetic analyses. The genetic analysis was undertaken utilizing molecular markers (ISSR and SSR) while chemical characterization was done through HPLC of four chemical markers (gingerol

homologues and shogaol). The data revealed moderate to high diversity in the collection, clustering them broadly into two groups. Both ISSR and SSR techniques were efficient in distinguishing all the 18 ginger cultivars, however, SSR markers were observed to be better in displaying average polymorphism (77.8%) than ISSR (66.7%). Based on statistical analysis, one ISSR and two SSR primers could be identified which effectively distinguished closely related ginger cultivars. Chemical profiling and subsequent multivariate analysis distinguished five lines which were distinct from rest of the collection (Pandotra, *et al.*, 2013).

2.4.3.1.5. Amplified Fragment Length Polymorphism (AFLP)

Amplified fragment length polymorphism is a PCR-based tool for molecular analysis and DNA fingerprinting. It was developed in the early 1990s to overcome the limitation of reproducibility associated with RAPD. Theoretically, AFLP represent the ingenious combination power of RFLP and flexibility of PCR-based technology (Agarwal *et al.*, 2008). It provides a novel and very powerful DNA fingerprinting technique for DNAs of any origin or complexity. In addition, cDNA AFLP and three endonuclease AFLP (TE-AFLP) which are used to quantify differences in gene expression levels and to detect transposable element mobility, respectively are variations of AFLP technique (Van Der Wurff *et al.*, 2000; Weising *et al.*, 2005).

In AFLP-PCR, genomic DNA is digested using two restriction endonucleases, typically one with a 6-bp recognition sequence (usually EcoRI) and the other with a 4-bp recognition sequence (usually MseI). Thereafter, adapters of known sequence are ligated to complementary double stranded adaptors of the ends of the restriction fragments. A subset of the restriction fragments are then amplified using two primers complementary to the adaptor and restriction site fragments for two successive rounds of selective PCR amplification. The first round of PCR uses primers that match the adapters on the EcoRI end and MseI end of the fragments plus one extra nucleotide. The second round has an additional two nucleotides added to the +1 primer sequences. These rounds of selective amplification reduce the resulting pool of DNA fragments to a size more manageable for analysis (Vos *et al.*, 1995). The fragments are visualized on denaturing polyacrylamide gels either through autoradiography or fluorescence

methodologies (Weising *et al.,* 2005). The resultant number of fragments for each AFLP assay depends on the number of selective nucleotides in the AFLP primer combination, the selective nucleotide motif, GC content, and physical genome size as well as its complexity (Agarwal *et al.,* 2008).

AFLP techniques generate fragments of any DNA from any source even without any prior knowledge of DNA sequence and a recent study showed that it can be used to distinguish closely related individuals at sub-species level (Althoff *et al.,* 2007). AFLP analysis revealed the values of nucleotide diversity in regenerated *Arabidopsis thaliana* which was 2-3 orders of magnitude smaller than natural variations described for ecotypes of *A. thaliana* (Polanco and Ruiz 2002). This shows that AFLP is a very sensitive and reliable marker technique that could be useful for detecting specific genomic alterations associated with tissue culture variation and identifying slightly different genotypes.

AFLP analysis has also been used to study tissue culture induced somaclonal variation in species such as cork oak (Hornero *et al.,* 2001), coffee (Sanchez-Teyer *et al.,* 2003), banana (James *et al.,* 2004), and coneflower (Chuang *et al.,* 2009). However, AFLP often requires more work with optimization and is relatively more expensive than RAPD (Weising *et al.,* 2005). Also, the technique requires high quality DNA samples which are often difficult to obtain in some plants such as conifers (Piola *et al.,* 1999).

AFLP markers have been used to study the genetic diversity of various fruit species including European plum (Goulao *et al.,* 2001), sweet cherry (Zhou *et al.,* 2002), apricot (Hagen *et al.,* 2002; Geuna *et al.,* 2003), citrus (Krueger and Roose, 2003), peach (Aranzana *et al.,* 2003), olive (Montemurro *et al.,* 2005) and mango (Galvez-Lopez *et al.,* 2010).

It is essential to prospect wild *Zingiber* species for soft rot resistance, since the cultivated ginger is susceptible to the disease. Kavitha *et al.* (2007) evaluated the genetic diversity of *Zingiber* species and their response to *Pythium aphanidermatum*, the causal agent of soft rot using AFLP marker system.

Amplified Fragment Length Polymorphism (AFLP) technique was used to characterize the genetic variation in grapevine (*Vitis vinifera*) regenerated from anther

culture (Popescu *et al.*, 2002). They reported that all the analysed grapevine somaclones regenerated from in vitro-cultured anthers were genetically distinct from the original cultivars.

DNA variations of forty-eight *Eucalyptus globulus* plants, regenerated by successive culture from seven different explants were assessed by AFLP analysis using 18 primers. Analyzed plants showed 66.7 per cent variation and the number of polymorphic bands per plant ranged from 1 to 22. However, the more times of successive culture were done the more of polymorphic bands were found within the groups. On average, between 97.39 and 99.88 per cent of all fragments were shared within the same group. AMOVA analysis showed 39.33 per cent of the variation among the accessions that originated from different calli while 60.67 per cent was from same calli (Mo *et al.*, 2009).

The genetic relationship among cassumunar gingers (*Zingiber cassumunar*) in Thailand was assessed by amplified fragment length polymorphism (AFLP). 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. Pairwise similarity estimated between cassumunar gingers ranged from 0.7644 to 1.00 with an average of 0.879. Cluster analysis divided the samples into five groups. Genetic variability within and among collection regions was estimated by analysis of molecular variance (AMOVA). High molecular variance (84%) was found within samples from the same region. The genetic similarity assessed by AFLP showed the duplicate accessions in the germplasm collection (Kladmook *et al.*, 2010).

Materials and Methods

3. MATERIALS AND METHODS

The investigations on "variability analysis in ginger (*Zingiber officinale* Rosc.) somaclones using molecular markers" were carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Kerala Agricultural University, Thrissur during the period from February 2012 to May 2013. The materials used and methodologies adopted are discussed in this chapter.

3.1 MATERIALS

3.1.1 Plant materials

Ginger somaclones (180 Nos.) regenerated through various modes of regeneration viz. bud culture, indirect organogenesis / embryogenesis and *in vitro* mutagenesis, along with two source parent cultivars (Maran and Rio-de-Janeiro) were used for the present study. Germplasm of ginger somaclones were maintained as potted plants in the green house at CPBMB, College of Horticulture, Kerala Agricultural University, Thrissur.

Thirteen groups of somaclones (seven in Maran and six in Rio-de-Janeiro) based on mode of regeneration along with two source parent cultivars used for the study were as follows:

1. MB - Maran bud culture.
2. MC - Maran indirect organogenesis.
3. MSe - Maran indirect somatic embryogenesis.
4. MC (10 Gy) - Maran indirect organogenesis (irradiated with γ rays10 Gy).
5. MC (20 Gy) - Maran indirect organogenesis (irradiated with γ rays 20 Gy).
6. MSe (10 Gy) - Maran indirect somatic embryogenesis (irradiated with γ rays 10 Gy).
7. MSe (20 Gy) - Maran indirect somatic embryogenesis (irradiated with γ rays 20 Gy).
8. MSP - Maran source parent cultivar (control M).

9. RB - Rio-de-Janeiro bud culture.
10. RC - Rio-de-Janeiro indirect organogenesis.
11. RSe - Rio-de-Janeiro indirect somatic embryogenesis.
12. RC (10 Gy) - Rio-de-Janeiro indirect organogenesis (irradiated with γ rays 10 Gy).
13. RC (20 Gy) - Rio-de-Janeiro indirect organogenesis (irradiated with γ rays 20 Gy).
14. RSe (10 Gy) - Rio-de-Janeiro indirect somatic embryogenesis (irradiated with γ rays 10 Gy).
15. RSP - Rio-de-Janeiro source parent cultivar (control R).

The somaclones from bud culture of the two cultivars Maran and Rio-de-Janeiro were planted out for rhizome formation in 1999-2000 after passing through ten to twelve subculture cycles and the clones were evaluated for yield, quality and tolerance to soft rot and bacterial wilt diseases. Preliminary field evaluation, advanced variety trials, onfarm evaluation multilocational tests, large scale demonstration of selected clones were undertaken during the period from 2002 to 2010 and two selected superior somaclones from bud culture regenerants were released under the name Athira and Karthika during 2010.

The somaclones regenerated through indirect methods and regenerants from irradiated organogenic and embryogenic calli of the two cultivars were planted out for rhizome formation in 2004. Preliminary yield evaluation and evaluation for soft rot and bacterial wilt diseases in the clones were completed during 2006 to 2010. The evaluation of the clones at molecular level and variability analysis using two marker systems were attempted in the present study.

The ginger somaclones used for the study are presented in Plate 1. The list of 180 ginger somaclones belonging to the two cultivars, Maran and Rio-de-Janeiro is presented in Table 1 and Table 2 respectively.

Plate 1. Somaclones of cultivar Maran and Rio-de-Janeiro maintained in net house

Table 1. List of somaclones derived from cultivar Maran

Sl. No.	Group of somaclones	Clone numbers	No. of clones
1	MB	488M, 91M, 197M, 132M, 436M, 668M, 79M, 393M, B3, S4, FP1056, B21, B24, B13, B16, 99M, 918M, Athira, Karthika	19
2	MC	MC545, MC815, MC338, MC277, MC262, MC740, MC231, MC263, MC270	9
3	Mse	MSe78, MSe21, MSe124, MSe357, MSe957 MSe27, MSe24, MSe1074, MSe39, MSe8, MSe1050, MSe72, MSe19,	13
4	MC (10Gy.)	MC1Kr322, MC1Kr112, MC1Kr266, MC1Kr1273, MC1Kr168, MC1Kr99, MC1Kr624, MC1Kr47, MC1Kr161, MC1Kr774, MC1Kr1064, MC1Kr1273, MC1Kr329, MC1Kr138, MC1Kr190, MC1Kr315, MC1Kr110, MC1Kr174, MC1Kr108, MC1Kr330, MC1Kr102	21
5	MC (20Gy.)	MC2Kr337	1
6	MSe (10Gy.)	MSe1Kr584, MSe1Kr200, MSe1Kr42, MSe1Kr113	4
7	MSe (20Gy.)	MSe2Kr536, MSe2Kr1351, MSe2Kr54, MSe2Kr239, MSe2Kr418, MSe2Kr248, MSe2Kr246, MSe2Kr59, MSe2Kr33, MSe2Kr178, MSe2Kr260, MSe2Kr164, MSe2Kr383	13
8	Source parent cultivar	Maran	
Total No. of somaclones			80

Table 2. List of somaclones derived from cultivar Rio-de Janeiro

Sl. No.	Group of somaclones	Clone numbers	No. of clones
9	RB	RVIII, 386R, 734R, 281R, 364R, 88R, 478R, 292R	8
10	RC	RC 665, RC1007, RC429, RC432, RC 763, RC606, RC609, RC152, RC439, RC1599, RC425, RC1251, RC445, RC144, RC153, RC668, RC30, RC280, RC132, RC504, RC460, RC31, RC150, RC692, RC423	25
11	Rse	RSe32, RSe13, RSe30, RSe42, RSe24, RSe23, RSe469, RSe16, RSe448, RSe124, RSe49, RSe27, RSe41, RSe33, RSe25, RSe44, RSe46, RSe10, RSe52, RSe439, RSe38, RSe11, RSe5, RSe45, RSe14, RSe2, RSe29, RSe986, RSe982, RSe736, RSe648, RSe498, RSe311, RSe873, RSe872, RSe593, RSe1346	37
12	RC (10Gy.)	RC1Kr375, RC1Kr53, RC1Kr343, RC1Kr462, RC1Kr194, RC1Kr245, RC1Kr445, RC1Kr131, RC1Kr615, RC1Kr743, RC1Kr975, RC1Kr1265, RC1Kr667, RC1Kr740, RC1Kr73, RC1Kr1257, RC1Kr1046, RC1Kr313	18
13	RC (20Gy.)	RC2Kr1031	1
14	RSe10 (10Gy.)	RSe1Kr1053, RSe1Kr44, RSe1Kr989, RSe1Kr648, RSe1Kr650, RSe1Kr188, RSe1Kr1257, RSe1Kr48, RSe1Kr1052, RSe1Kr78, RSe1Kr239	11
15	Source parent cultivar	Rio-de Janeiro	
Total No. of somaclones			100

3.1.2 Laboratory chemicals, glass wares and plastic wares

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

3.1.3 Equipment and machinery

The present investigations were carried out using the facilities available at CPBMB, College of Horticulture. Centrifugation was done in High speed refrigerated centrifuge (KUBOTA 6500, Japan). NanoDrop^{R} ND-1000 spectrophotometer was used for the estimation of quality and quantity of DNA. The DNA amplification was carried out in Veriti (Applied Biosystems, USA). Horizontal gel electrophoresis system (BIO-RAD, USA) was used for agarose gel electrophoresis. Gel Doc - BIO-RAD was used for imaging and documenting the agarose gel.

3.2 METHODS

Variability analyses of thirteen groups of somaclones of ginger along with source parent cultivars were carried out with two different marker systems- Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeats (ISSR).

3.2.1 Genomic DNA extraction

For genomic DNA isolation, CTAB method reported by Rogers and Bendich (1994) and Sigma's GenElute^{TM} Plant Genomic DNA Miniprep kit were used. Young tender, pale green leaves (first or second fully opened leaf from the tip) were collected early in the morning from individual plants of each group of somaclones maintained in net house of CPBMB. Leaf surface was cleaned by wiping with 70 per cent alcohol and then used for DNA isolation. The leaves were then ground into a fine

powder with liquid nitrogen using pre-chilled mortar and pestle, β -mercaptoethanol (50µl) and a pinch of PVP were added before grinding.

3.2.1.1 DNA isolation by CTAB method

Reagents:

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

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

Young and tender leaf tissue (1g) was weighed and ground in liquid nitrogen using pre chilled mortar and pestle along with 50µl of β-mercaptoethanol and a pinch of Poly Vinyl Pyrrolidone (PVP). The sample was ground to a fine powder using excess of liquid nitrogen and 4 ml of extraction buffer (2x). The powder was transferred to a sterile 50 ml centrifuge tube containing 3 ml of pre-warmed extraction buffer (total 7 ml). The homogenate was incubated for 30 minutes at 65°C with occasional mixing by gentle inversion. Equal volume of chloroform: isoamyl alcohol (24:1) mixture was added to the tube, mixed gently by inversion and centrifuged at 10000 rpm for 15 minutes at 4^0C. The contents got separated into three distinct phases. The upper aqueous phase containing DNA was pipetted out into 50 ml of fresh autoclaved Oakridge tube. Then added $1/10^{th}$ volume of ten per cent CTAB and equal volume of chloroform: isoamyl alcohol (24:1) mixture, mixed gently by inversion and centrifuged at 10000 rpm for 15 minutes at 4^0C. The supernatant was collected into 50ml of Oakridge tube, then 0.6 volume of ice cold chilled isopropanol was added and the contents were mixed gently. The sample was then incubated at -20^0C for 30 minutes to precipitate the DNA completely. The DNA was then pelleted

by centrifuging at 10000 rpm for 15 minutes at 4^0C. Isopropanol was gently poured off. Washed the pellet with 70 percent ethanol by centrifuging at 10000 rpm for 10 minutes, discarded ethanol and drained well. The pellet was air dried and finally dissolved in 50 µl sterile distilled water. The isolated DNA was electrophoresed on 0.8 per cent agarose gel along with λDNA / *Eco*RI+H*ind*III double digest as molecular marker.

3.2.1. DNA isolation by Sigma's GenElute^{TM} Plant Genomic DNA Miniprep kit

Reagent preparation

Reagents were thoroughly mixed and examined for precipitation. If any reagent formed a precipitate, it was warmed at 55-65°C until the precipitate dissolved and allowed to cool at room temperature before use. The water bath was preheated to 65°C. Diluted the wash solution concentrate with 72 ml (70 prep packages) of 95-100 per cent ethanol. After each use, tightly caped the diluted wash solution to prevent the evaporation of ethanol. The elution solution was preheated to 65° C.

Disruption of cells

Fresh leaves were ground to a fine powder in liquid nitrogen using a chilled mortar and pestle to disrupt cells. Approximately 100 mg of the powder was transferred to a microcentrifuge tube. The samples were kept on ice for immediate use.

Cell lysis

Lysis solution (Part A) 350 ml and lysis solution (PartB) 50 ml were added to the tube; thoroughly mixed by vortexing and inverting. A white precipitate was formed upon the addition of lysis solution (Part B). The mixture was incubated at 65°C for 10 minutes with occasional inversion to dissolve the precipitate. RNase A (50 units) was added to the lyses mixture just prior to incubation at 65°C.

Precipitation of debris

Precipitation solution (130 ml) was added to the mixture, mixed completely by inversion and placed the sample on ice for five minutes. The sample was centrifuged at maximum speed (12,000-16,000 5g) for five minutes to pellet the cellular debris, proteins, and polysaccharides.

Filtration of debris

The supernatant from above step was carefully pipetted out onto a GenElute filtration column (blue insert with a 2 ml collection tube) to filter debris and centrifuged at maximum speed (12,000-16,000 5g) for one minute for removing any cellular debris not removed in previous step. The filtration column was discarded but the collection tube was retained.

Preparation of binding column

Binding solution (700 ml) was directly added to the flow-through liquid from above step and mixed thoroughly by inversion. Binding column was prepared by inserting a GenElute Miniprep binding column (with a red o-ring) into a microcentrifuge tube, if not already assembled. Added 500 ml of the column preparation solution to each miniprep column and centrifuged at 12,000 5g for 30 seconds to 1 minute. The flow-through liquid was discarded. (The column preparation solution maximizes binding of DNA to the membrane resulting in more consistent yields).

Loading lysate

Carefully pipetted out 700 ml of the mixture to the column prepared and centrifuged at maximum speed (12,000–16,000 5g) for one minute. The flow-through liquid was discarded but the collection tube was retained. The column was returned to the collection tube. The remaining lysate was applied to the column. Repeated the centrifugation as above and discarded the flow-through liquid as well as collection tube.

Column washing

For first column wash, the binding column was placed into a fresh 2 ml collection tube and applied 500 ml of the diluted wash solution to the column and centrifuged at maximum speed for one minute. The flow-through liquid was discarded and collection tube was retained. For second column wash, applied another 500 ml of diluted wash solution to the column and centrifuged at maximum speed for three minutes to dry the column. Prevented the flow-through liquid to contact the column; wiped off any fluid that adhered to the outside of the column.

Elution of DNA

The binding column was transferred to a fresh 2 ml collection tube, to elute DNA. Pre-warmed (65 °C) 100 ml elution solution was applied to the column and centrifuged at maximum speed for one minute. The elution was repeated. The flow-through liquid did not allow contacting the column. Eluates may be collected in the same collection tube. Alternatively, a second collection tube may be used for the second elution to prevent dilution of the first eluate. The eluate contained pure genomic DNA. For short-term storage of DNA, 2-8°C is recommended. For long-term storage of DNA, -20 °C is recommended. Avoid freezing and thawing, which causes breaks in the DNA strand. Elution solution will help stabilize the DNA at these temperatures.

3.2.2 Assessing the quality of DNA by electrophoresis

The quality of isolated DNA was evaluated through agarose gel electrophoresis.

Reagents and equipments

1. Agarose
2. 50X TAE buffer (P^H 8.0)
3. 6X Loading /Tracking dye
4. Ethidium bromide (0.5μg/ml)
5. Electrophoresis unit, power pack (BIO-RAD), gel casting tray, comb
6. UV transilluminator (Herolab ^{R})

7. Gel documentation and analysis system (BIO-RAD)

Chemical composition of buffers and dyes are given in Annexure II.

Cleaned the work area, swabbed gel casting tray and comb with 70 per cent ethanol. The open end of gel casting tray was sealed with a cello tape and kept on a horizontal surface. The comb was placed desirably. Agarose (0.8 per cent) was weighed and dissolved in TAE buffer (1X) by boiling in micro wave oven until the agarose melted completely and solution became clear. Agarose solution was allowed to cool to about 42 to 45^0C and added ethidium bromide (0.5µg/ml) and mixed well. Dissolved agarose was poured on to the tray. The gel was allowed to set for 30 minutes. The comb and tape (used for sealing the tray) were removed carefully. The tray was kept in the electrophoresis tank with well side directed towards the cathode. 1X TAE buffer was added to the tank. Then DNA sample (5 µl) along with tracking dye (1 µl) was loaded into the wells using a micropipette carefully. Suitable molecular weight marker (λDNA/*Eco*RI+*Hind*III double digest) was loaded in one lane. After closing the tank, the anode and cathode ends were connected to the power pack and the gel was run at a constant voltage (100V) and current (50 A). The power was turned off when the tracking dye reached $2/3^{rd}$ length of the gel. Then the gel was taken from the electrophoresis unit and viewed under UV transilluminator for presence of DNA. The intact DNA appeared as orange fluorescent band under UV light due to ethidium bromide dye.

3.2.3 Gel documentation

The image was documented in gel documentation system (BIO-RAD). The gel profile was examined for intactness, clarity of DNA band, presence of contamination such as RNA and proteins.

3.2.4 Purification of DNA

The DNA contained RNA as contaminant and was purified by RNase treatment.

Reagents

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

The RNase A from Sigma, USA was used for the present study. One per cent solution was prepared by dissolving RNase in TE buffer at 100^0C for 15 minutes. The solution was cooled to room temperature, dispensed into aliquots and stored at -20^0C.

RNase solution (5 µl) was added to 100 µl DNA sample and incubated at 37^0 C in dry bath (Genei, Thermocon) for one hour. Then added equal volume of chloroform: isoamyl alcohol (24: 1) and centrifuged at 10000Xg for 20 minutes at 4^0 C. The upper aqueous phase transferred to another tube. Repeated above step and finally precipitated the DNA from the aqueous phase with 0.6 volume of chilled isopropanol. The mixture was then incubated at -20^0 C for 30 minutes and centrifuged at 10000 rpm for 15 minutes at 4^0 C. The pellet of DNA was washed with 70 per cent ethanol. The pellet was air dried and dissolved in 50 to 100µl autoclaved distilled water. Electrphoresis was carried out 0.8 percent agarose gel at constant voltage of 100V to test the quality and to find whether there was any shearing during RNase treatment.

3.2.5 Assessing the quality and quantity of DNA by NanoDrop method

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

1OD at 260 nm = 50 µg DNA/ml

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

3.2.6 Bulking of DNA

Bulking of DNA was done as per the procedure reported by (Dulson *et al.*, 1998). The DNA bulk was made with equal amount (10 µl) of diluted working stock from each of one eighty somaclones of ginger. Bulked samples of the thirteen groups of the one eighty somaclones along with two source parent cultivars were subjected to RAPD and ISSR analysis with selected primers. The genotype and mode of regeneration exhibiting more variability was focused for further indepth investigations, using individual DNA of each somaclone.

3.3 Molecular markers used for the study

Two different types of marker systems viz., Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeats (ISSR) were used for variability analysis.

3.3.1 DNA amplification conditions

The PCR condition required for effective amplifications in RAPD and ISSR analyses include appropriate proportions of the various components of the reaction mixture. The reaction mixture include template DNA, assay buffer A or B, $MgCl_2$, *Taq* DNA polymerase, dNTPs and primers. The aliquot of this master mix were dispensed into 0.2 ml PCR tubes. The PCR was carried out in Veriti (Applied Biosystem, USA).

Another important factor, which affect amplification rate is the temperature profile of thermal cycle. The thermocycler is programmed for desired duration and temperature for denaturation, annealing and polymerization.

3.3.2 RAPD (Random Amplified Polymorphic DNA) analysis

The good quality genomic DNA after bulking (35 to 40ng/µl) were subjected to RAPD assay. Random decamer primers supplied by 'Operon Technologies' USA

with good resolving power was used for amplification of DNA. The decamer primers for RAPD assay were selected after an initial screening of primers.

The amplification was carried out in Veriti (Applied Biosystem, USA). PCR amplification was performed in a 20 µl reaction mixture as constituted below:

Composition of the reaction mixture for PCR (20.0 µl)

a) Genomic DNA (35ng/µl)	- 2.0 µl
b) 10X *Taq* assay buffer B	- 2.0 µl
c) $MgCl_2$	- 2.0 µl
d) dNTP mix (10mM each)	- 1.5 µl
e) *Taq* DNA polymerase (3U)	- 0.4 µl
f) Decamer primer (10 pM)	- 2.0 µl
g) Autoclaved distilled water	- 10.1 µl
Total volume	- 20.0 µl

The thermocycler was programmed as follows:

93°C for 1 minutes	-	Initial denaturation	
93°C for 1 minute	-	Denaturation	40 cycles
37°C for 1 minute	-	Primer annealing	
72°C for 2 minutes	-	Primer extension	
72°C for 8 minutes	-	Final extension	
4°C for infinity to hold the sample			

3.3.2.1 Screening of random primers for RAPD analysis

Thirty-five decamer primers in the series of OPA, OPC, OPD, OPG, OPK, OPE, OPP, OPU, OPAH, (Operon Technologies, USA), S, RN (Reverse Neo), RY were screened for RAPD analysis using the bulked DNA from three somaclones viz. RVIII, MSe1Kr200 and MC1Kr168. Details of the primers used for screening is given in Table 3. Out of the 35 decamer primers screened for RAPD analysis, primers which gave good amplification products were selected for further studies.

3.3.2.2 Random primers selected for RAPD Assay

The amplified products were run on two per cent agarose gel using 1X TAE buffer stained with ethidium bromide along with marker (100 bp). The profile was visualized under UV (312 nm) transilluminator and documented using gel documentation system Gel DOC-It ^{TM} Imaging system UVP (USA). The documented RAPD profiles were carefully examined for amplification of DNA as bands. The size of polymorphic band in kb / bp of bases was recorded in comparison with 100 bp ladder.

3.3.3 ISSR (Inter Simple Sequence Repeat) analysis

The good quality genomic DNA after bulking (35 to 40ng/µl) were subjected to ISSR assay. ISSR primers (ISSR Technologies, USA) with good resolving power were used for amplification of DNA. The ISSR primers for analysis were selected after an initial screening of primers. The amplification was carried out in Veriti (Applied Biosystem, USA). PCR amplification was performed in a 20 µl reaction mixture as constituted below:

Table 3. Decamer primers used for screening DNA samples from ginger somaclones

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

Composition of the reaction mixture for PCR (20.0 µl)

a) Genomic DNA (35ng/µl)	- 2.0 µl
b) 10X *Taq* assay buffer B	- 2.0 µl
c) $MgCl_2$	- 2.0 µl
d) dNTP mix (10mM each)	- 1.5 µl
e) *Taq* DNA polymerase (3U)	- 0.4 µl
f) Primer (10 pM)	- 2.0 µl
g) Autoclaved distilled water	- 10.1 µl
Total volume	- 20.0 µl

The thermocycler was programmed as follows:

94° C for 4 minutes	-	Initial denaturation	
94° C for 45 seconds	-	Denaturation	35 cycles
42 - 47° C for 1 minute	-	Primer annealing	
72° C for 2 minutes	-	Primer extension	
72° C for 8 minutes	-	Final extension	

4° C for infinity to hold the sample

3.3.3.1 Screening of primers for ISSR analysis

Thirty primers (ISSR Technologies, USA), were screened for ISSR analysis using the bulked DNA from three somaclones viz. RVIII, MSe1Kr200 and MC1Kr168. Details of the primers used for screening is given in Table 4. Primers which gave good amplification of the DNA sample were selected for further studies.

Table 4. ISSR primers used for screening DNA samples from ginger somaclones

Sl. No.	Name of Primer	Nucleotide Sequence
1.	UBC 840	5'GAGAGAGAGAGAGAGAYT3'
2	UBC 844	5'CTCTCTCTCTCTCTCTRC3'
3	UBC 890	5'VHVGTGTGTGTGTGTGT3'
4	UBC 811	5'GAGAGAGAGAGAGAGAC3'
5	UBC813	5'CTCTCTCTCTCTCTCTT3'
6	UBC 815	5'CTCTCTCTCTCTCTCTG3'
7	UBC354	5'CTAGAGGCCG3'
8	UBC 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'

3.3.3.2 ISSR assay of selected primers

The amplified products were run on two per cent agarose gel using 1X TAE buffer stained with ethidium bromide along with marker (100 bp). The profile was visualized under UV (312 nm) transilluminator and documented using gel documentation system Gel DOC-It ^{TM} Imaging system UVP (USA). The documented ISSR profiles were carefully examined for amplification of DNA as bands. The size of polymorphic band in kb / bp of bases was recorded in comparison with 100 bp ladder.

3.3.4 Scoring of bands and data analysis

Scoring of bands on agarose was done with the Quantity one software (BIORAD) loaded in Gel Doc. Molecular weight size marker 100 bp ladder, was used for each gel along with DNA samples. Clear and distinct bands were considered scorable and each band was scored as 1 for the presence and 0 for the absence and their size recorded in relation to the molecular weight marker used.

The results obtained from RAPD and ISSR assays were transformed into data matrix as discrete variables. Jaccard's coefficient of similarity was used to derive the dissimilarity matrix and dendrogram was generated by using Unweighted Pair Group Method with Arithmetic Mean (UPGMA) as per Sneath and Sokal (1973). Only distinct and well resolved fragments were scored. The resulting data were analysed using the software package NTSYS pc version 2.02i (Rohlf, 2005).

Resolving power (Prevost and Wilkinson, 1999) was used to identify the primers that would distinguish the somaclones most efficiently. Resolving power (Rp) of a primer was calculated as the sum of 'band informativeness' of all the bands produced by the primer. Band information (Ib) is = $1-[2(0.5-p)]$,

where p is the proportion of the somaclones containing the bands. Resolving power of the primer is represented as: $Rp= \Sigma Ib$.

Polymorphic Information Content (PIC) was used for assessing the suitability of the primers selected for variability analysis. The PIC value (Hollman *et al.*, 2005) of a

marker detects polymorphism within a population depending on the number of detectable alleles and their frequency.

PIC value was calculated according to Anderson (1993) as PIC = 1- Σpi^2, where *pi* was the frequency of the i^{th} allele.

Results

4. RESULTS

The investigations on variability analysis in ginger (*Zingiber officinale* Rosc.) somaclones using molecular markers were conducted at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Kerala Agricultural University. The objectives of the study were to assess somaclonal variation in ginger at molecular level and to study the influence of genotype and mode of regeneration on the extent of somaclonal variability. The study also aimed to assess variability in somaclones from the original source parent cultivars and to select out the variants. The results of various aspects of the investigations are presented in this chapter.

4.1 Source of DNA

Ginger somaclones (180 Nos.) regenerated through various modes of regeneration viz. bud culture indirect organogenesis / embryogenesis and *in vitro* mutagenesis along with source parent cultivars (Maran and Rio-de-Janeiro) were the experimental materials in the present study. Leaves were collected from individual plant of each group of somaclones for extraction of total genomic DNA. Leaves at different stages of maturity were tried for isolation of DNA. Young, tender, fresh, pale green leaves (1g) yielded good quality DNA in sufficient quantity in ginger somaclones (Plate 2).

4.1.1 Isolation, purification and quantification of DNA

For isolation of genomic DNA, two methods were used. Genomic DNA isolated using CTAB method reported by Roger and Bendich 1994 had RNA contamination (Plate 3a). However, RNase treatment gave good quality DNA (Plate 3b). The agarose gel electrophoresis indicated clear distinct bands without RNA contamination and spectrophotometric analysis gave ratio of UV absorbance ($A_{260/280}$) between 1.8 and 2.0. Quality and quantity of DNA isolated using CTAB method from selected ginger somaclones are indicated in Plate 3 and Table 5. Genomic DNA isolated by Sigma's GenElute^{TM} Plant Genomic DNA Miniprep kit was pure without RNA contamination and are indicated in Plate 4 and Table 6.

A - Folded, immature

B - Young, tender and pale green - (ideal stage)

C - Medium mature

D - Mature

Plate 2. Different maturity stages of leaves tried for DNA isolation in ginger somaclones

Table 5. Quality and quantity of DNA isolated by CTAB method in ginger somaclones

Sr. No.	Group of somaclones	$A_{260/280}$ (range in group)	Quantity (ng/µl) (range in group)
1	MB	1.81-1.92	986.93-1360.11
2	MC	1.81-1.96	1014.52-1210.21
3	Mse	1.81-1.92	996.77-1230.97
4	MC (10 Gy)	1.80-2.00	1011.30-1237.81
5	MC (20 Gy)	1.84	1186.01
6	MSe (10 Gy)	1.83-1.90	1086.54-1203.52
7	MSe (20 Gy)	1.82-2.00	1014.501275.88
8	Maran source parent cultivar	1.85	1213.77
9	RB	1.84-2.00	1094.59-1257.12
10	RC	1.81-1.91	998.98-1540.65
11	RSe	1.80-2.00	993.99-1339.37
12	RC (10 Gy)	1.80-2.00	997.30-1246.32
13	RC (20 Gy)	1.84	1240.48
14	RSe10 (10 Gy)	1.80-2.00	1093.21-1433.53
15	Rio-de-Janeiro source parent cultivar	1.90	1365.53

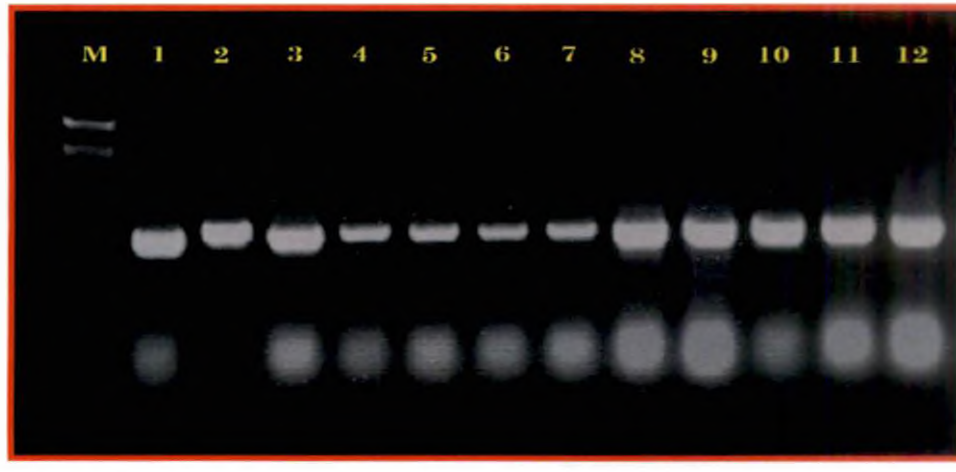


Plate 3a. DNA isolated by CTAB method before RNase treatment

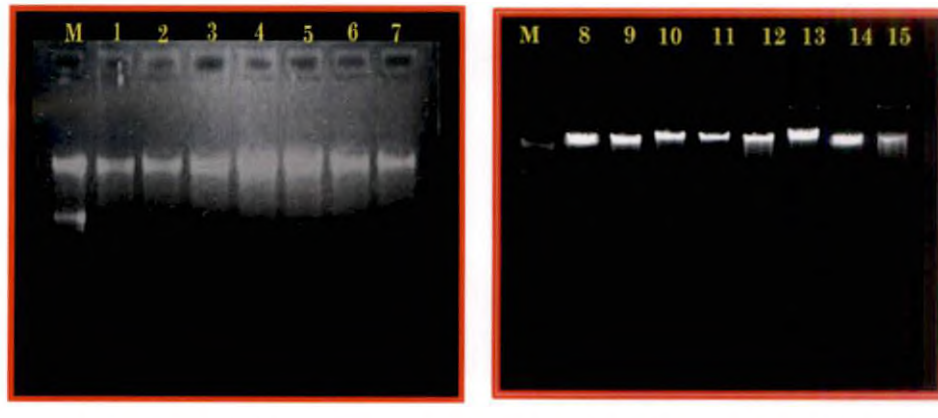
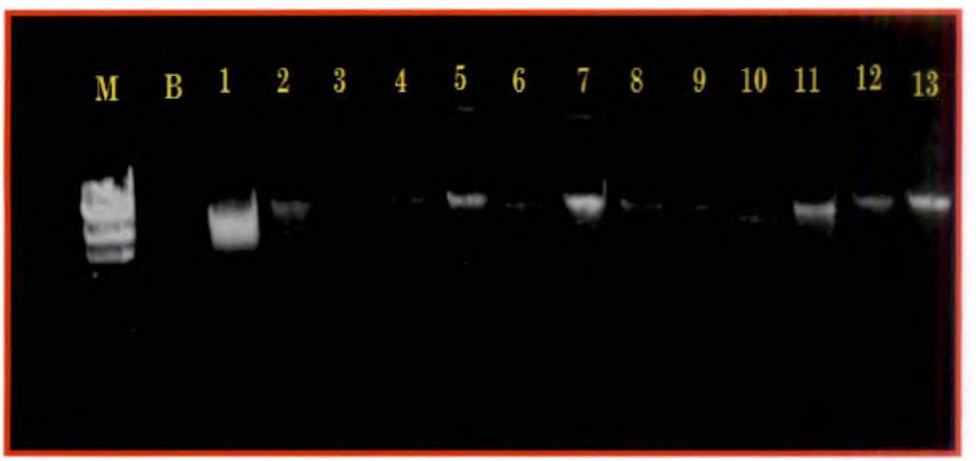


Plate 3b. DNA isolated by CTAB methods after RNase treatment

M: Molecular weight marker λ DNA/*Eco* RI+ *Hind* III double digest (100bp)

B: Blank	8: RVIII
1: 488M	9: 386R
2: 91M	10: 734R
3: 197M	11: 281R
4: 132M	12: 364R
5: 436M	13: MSe1Kr200
6: MC545	14: Mse2Kr1351
7: MC815	15: MC1Kr168

Plate 3. DNA isolation by CTAB method and removal of RNA contamination



M: Molecular weight marker λ DNA/*Eco* RI+ *Hind* III double digest (100bp)

B: Blank
1: 79M
2: 688M
3: 393M
4: B3
5: KARTHIKA
6: ATHIRA
7: MC740
8: MC263
9: MC231
10: MC270
11: MC1Kr138
12: MC1Kr190
13: MC1Kr315
14: MC1Kr1273
15: MC1Kr110
16: MC1Kr161
17: MC1Kr99

Plate 4. DNA isolated using Sigma's GenElute™ kit in ginger somaclones

Table 6. Quality and quantity of DNA isolated using Sigma's GenElute™ Kit in ginger somaclones.

Sr. No.	Group of somaclones	$A_{260/280}$ (range in group)	Quantity (ng/µl) (range in group)
1	MB	1.83-1.96	81.20-91.11
2	MC	1.80-1.91	57.99-81.09
3	MC (10 Gy)	1.81-1.91	68.66-91.42
4	RC	1.82-1.97	55.54-125.99
5	RSe	1.81-1.90	66.80-134.06
6	RC (10 Gy)	1.85-1.91	59.99-107.56

Even though the quantity of DNA isolated using Sigma's GenElute^{TM} Plant Genomic DNA Miniprep kit was less, the quality of isolated DNA was pure without RNA contamination.

4.1.2 Bulking of DNA

Good quality genomic DNA from one eighty somaclones of ginger was used for group wise bulking. Bulked samples of the thirteen groups of somaclones along with two source parent cultivars were subjected to RAPD and ISSR analyses with selected primers.

4.2 Molecular marker analysis:

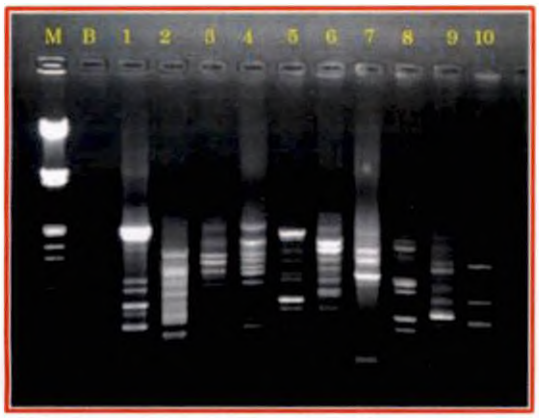
Two different marker systems viz. RAPD and ISSR were studied for variability analyses of somaclones. Different primers (35 RAPD and 30 ISSR) were screened with good quality genomic DNA bulked from RVIII, MSe1Kr200 and MC1Kr168. The best twelve primers selected based on amplification for each marker system were used for amplifying the DNA from different groups of ginger somaclones.

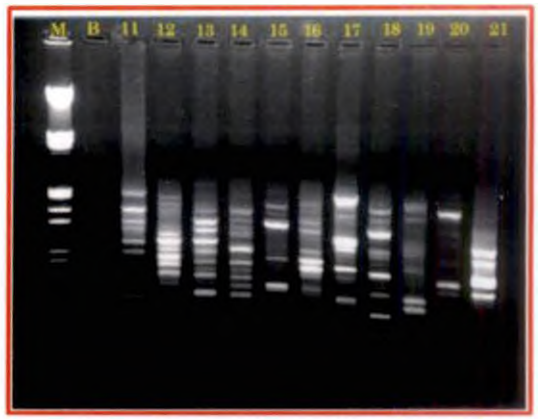
4.2.1 Random Amplified Polymorphic DNA (RAPD) analysis:

Original source parent cultivars and thirteen groups of somaclones (seven in cultivar Maran and six in cultivar Rio-de Janeiro) were subjected to RAPD analysis using bulked DNA samples.

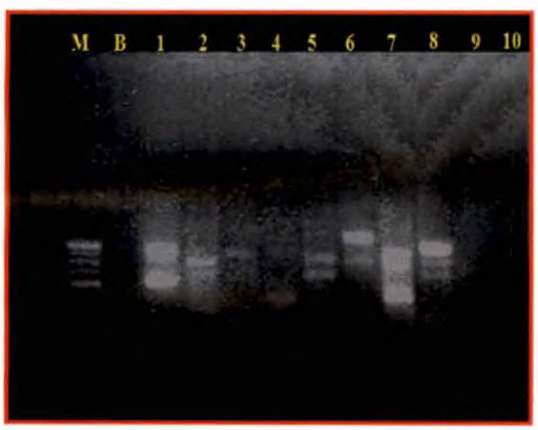
4.2.1.1 Screening of random primers for RAPD analysis

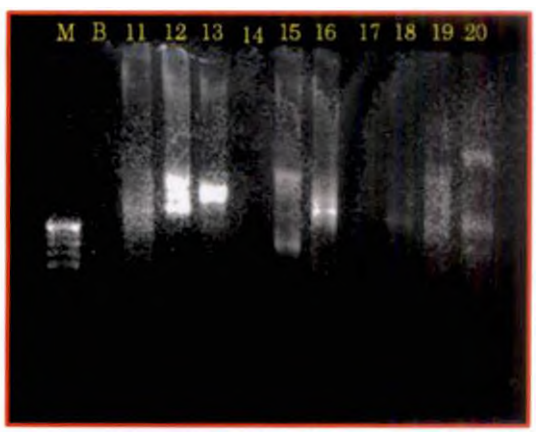
Thirty-five decamer primers were screened for RAPD analysis using good quality genomic DNA bulked from three somaclones viz. RVIII, MSe1Kr200 and MC1Kr168. The analysis by RAPD marker system described in 3.3.2 gave good amplification. The amplification patterns of screened primers are presented in Plate 5 and Table 7.





M: 100bp ladder / Marker
B: Blank
1: OPA 04
2: OPC 02
3: OPA 08
4: OPAH 3
5: OPA 12
6: OPP 17
7: OPA 02
8: OPA 27
9: OPAH 05
10: OPP16
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





M: 100bp ladder / Marker
B: Blank
1: OPK-01
2: OPU-03
3: OPG-08
4: OPE-05
5: OPU-07
6: OPU-13
7: OPAH-09
8: OPAH-06
9: OPD-15
10: OPD-10
11: OPA 10
12: OPA 28
13: OPA 27
14: OPA 06
15: OPA 12
16: OPC 01
17: OPAH 01
18: OPC 04
19: S 12
20: OPC 02.

Plate 5. Screening of RAPD primers for amplification of DNA from ginger somaclones

Table 7. Amplification pattern of primers screened for RAPD assay

Sl. No.	Primers	Amplification Pattern			
		No. of bands	Types of bands		Remarks
			Clear and 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	2	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	2	6	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	3	8	Selected
33	RY 08	12	6	6	------
34	S11	8	4	4	Selected
35	S 12	2	0	2	------

Of the 35 decamer primers screened, twelve decamer primers which gave good amplification pattern were selected for RAPD assay of thirteen groups of ginger somaclones and their original source parent cultivars. The details of the selected primers are presented in Table 8.

Overall analysis of primer screening showed that primers of the OPA series gave good amplification as compared to OPD and OPAH series. Primerwise analysis of the screened primers showed that the primer OPD 15 gave the highest number of clear and distinct bands followed by OPA 27 and OPA 28.

4.2.1.2 Variability analysis in ginger somaclones with selected RAPD primers

The gel profile of RAPD analysis in thirteen groups of somaclones with selected primers is presented in Plate 6a and 6b. Polymorphism percentage was worked out for each primer. Size of the amplicons was measured using Quantity one software. The details of amplification pattern are presented in Table 9.

The polymorphism percentage ranged from 10 to 54.54 in the selected primers. The highest polymorphism percentage was recorded by the primer OPA 28 (54.54) followed by OPD 15 (45.45) and S11 (45.45). Primerwise analysis is as shown below:

OPA 02

A total of eleven amplicons ranged in size 300 bp to 1100 bp were produced by the primer OPA 02. They were clear, distinct and reproducible. It could generate four polymorphic bands out of eleven amplicons (Plate 6a. 1) and the percentage polymorphism was 36.36.

Three loci of size 700 bp, 1000 bp and 1100 bp were found in all the groups except RC20 Gy. One loci of size 900 bp was found in all groups except RC20 Gy and RSe10 Gy.

Table 8. Details of selected RAPD primers

Sl. No.	Name of Primer	Sequence
1	OPA-02	5'TGCCGAGCTG3'
2	OPA-04	5'AATCGGGCTG3'
3	OPA -12	5' TCGGCGATAG3'
4	OPA-27	5'GAAACGGGTG3'
5	OPA-28	5'GTGACGTAGG3'
6	OPD-15	5'CATCCGTGCT3'
7	OPD-20	5'ACCCGGTAAC3'
8	OPP-16	5'CCAAGCTGCC3'
9	OPU-03	5'CTATGCCGAC3'
10	RN-08	5'ACCTCAGCTC3'
11	S-11	5'GTAGACCCGT3'
12	OPAH-03	5'GGTTACTGCC3'

OPA 04

The primer OPA 04 could generate a total of eight clear, distinct and reproducible bands (150 bp to 1500 bp) out of which only two were polymorphic. The percentage polymorphism was 25. The amplification profile is given in Plate 6a.2.

Two loci of size 800 bp and 1500 bp were found in all groups except RC20 Gy and RSe10 Gy.

OPA 12

Twelve clear, distinct and reproducible amplicons were produced by the primer OPA 12 (Plate 6a. 3) ranged in size 300 bp to 1600 bp. It could detect five polymorphic amplicons out of twelve amplicons. The polymorphism percentage calculated was 41.66 per cent.

Three loci of size 1200 bp, 1300 bp and 1500 bp were found in all groups except RC20 Gy and RSe10 Gy. One loci of size 1550 bp was found in regenerants of irradiated group viz. MC10 Gy, MC20 Gy, MSe10 Gy, RC20 Gy and RSe10 Gy. One loci of size 300 bp was found only in somaclones of Maran group.

OPA 27

OPA 27 generated ten clear, distinct and reproducible amplicons ranged in size from 400 bp to 1600 bp and only one of them was polymorphic (Plate 6a. 4). The percentage polymorphism was very less (10%) as compared to other primers.

One loci of size 1500 bp was found only in somaclones of Maran group.

OPA 28

Amplification of thirteen groups of ginger somaclones with the selected primer OPA 28 produced eleven clear, distinct and reproducible amplicons ranged in size 300 bp to 1500 bp (Plate 6a. 5). Six amplicons were polymorphic and the percentage polymorphism was very high as compared to other primers (54.54%).

Two amplicons of size 1150 bp and 1200 bp were found in all groups of somaclones except RC20 Gy. One loci of size 1500 bp was found in all groups except MC20 Gy, MSe20 Gy, RC20 Gy and RSe10 Gy. One loci of size 1300 bp was found in all groups except RC20 Gy and RSe10 Gy. One amplicon of size 1250 bp was found in all groups except MC20 Gy, RC20 Gy and RSe10 Gy. One amplicon of size 1100 bp was found in all the groups except MC20 Gy and RC20 Gy.

OPD 15

The primer OPD 15 was able to generate eleven clear, distinct and reproducible amplicons (Plate 6a. 6). The amplicons were ranged in size 300 bp to 1500 bp. Five amplicons were polymorphic giving 45.45 per cent polymorphism.

Three amplicons of size 800 bp, 1350 bp and 1500 bp were found in all the groups except RC20 Gy and RSe10 Gy. Two amplicons of size 600 bp and 1000 bp were found in all groups except RC20 Gy.

OPD 20

The decamer primer OPD 20 could generate ten clear, distinct and reproducible amplicons ranged in size 200 bp to 1800 bp, three of them were polymorphic (Plate 6b. 7). The percentage polymorphism was 30.

Two amplicons of size 1500 bp and 1800 bp were found in all the groups except RC20 Gy and RSe10 Gy. One loci of size 400 bp was found in all the groups except RC20 Gy.

OPP 16

Ten clear, distinct and reproducible amplicons were produced by the primer OPP 16 (Plate 6b. 8) ranged in size 400 bp to 1400 bp. The polymorphism per centage was less (20) as it could detect only two polymorphic amplicons out of ten amplicons.

Two amplicons of size 900 bp and 1400 bp were found in all the groups except RC20 Gy and RSe10 Gy.

OPU 03

OPU 03 could generate a total of ten clear, distinct and reproducible amplicons (400 bp to 1500 bp) and out of which two were polymorphic (Plate 6b. 9). The polymorphism percentage recorded was 20.

One loci of size 1400 bp was found in irradiated group viz. MC10 Gy, MC20 Gy, MSe10 Gy and MSe20 Gy. One loci of size 1500 bp was present only in somaclones of Maran group.

RN 08

Twelve amplicons were produced by RN 08 (300 bp to 1700 bp) and four of them were polymorphic. The bands were clear, distinct and reproducible (Plate 6b. 10). The polymorphism percentage calculated was 33.33.

Three amplicons of size 1100 bp, 1300 bp and 1700 bp were found in all the groups except RC20 Gy and RSe10 Gy. One loci of size 800 bp was found in MSe, MSe10 Gy, MSe20 Gy and Maran source parent cultivar (control M).

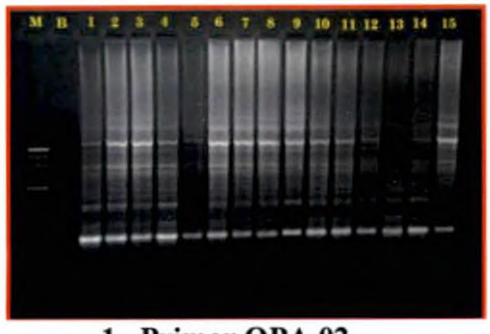
S 11

The primer S 11 was able to generate eleven amplicons ranged in size 300 bp to 1800 bp, five of them were polymorphic. The bands were clear, distinct and reproducible (Plate 6b. 11). The polymorphism percentage was 45.45.

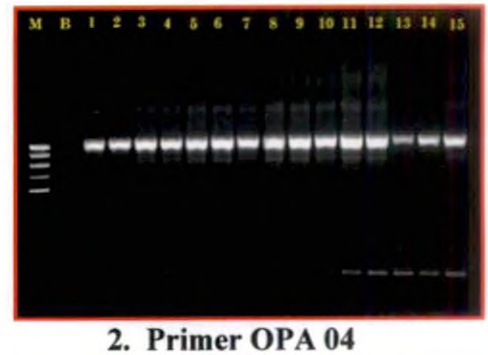
Four amplicons of size 1100 bp, 1300 bp, 1500 bp and 1800 bp were found in all the groups except RC20 Gy and RSe10 Gy. One loci of size 300 bp was present only in somaclones of Rio-de-Janeiro group.

OPAH 03

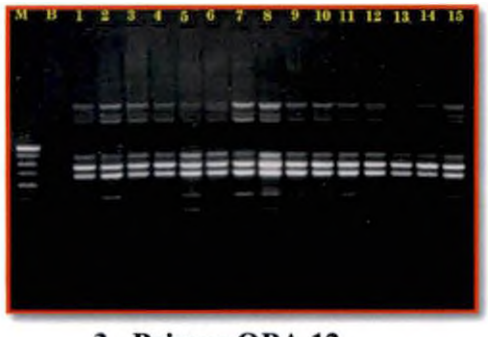
OPAH 03 produced thirteen clear, distinct and reproducible amplicons (200 bp to 1200 bp) and out of which five amplicons were polymorphic giving 38.46 per cent polymorphism. The profile showing the amplification pattern of OPAH 03 was presented in Plate 6b. 12.



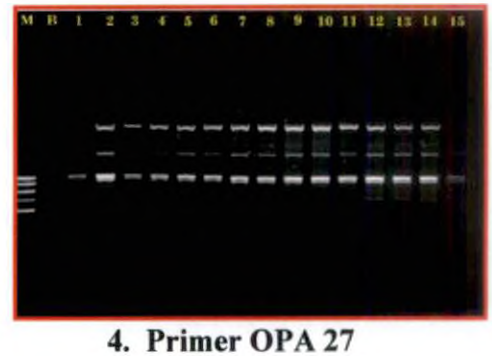
1. Primer OPA 02



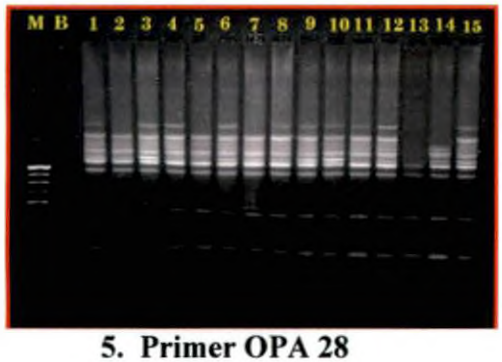
2. Primer OPA 04



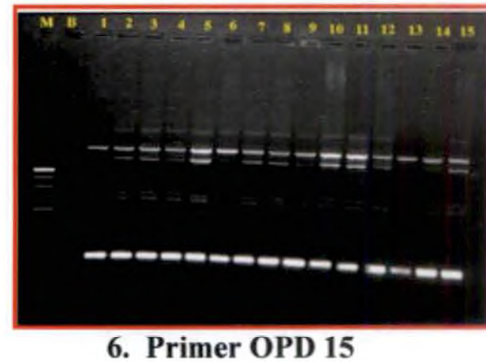
3. Primer OPA 12



4. Primer OPA 27



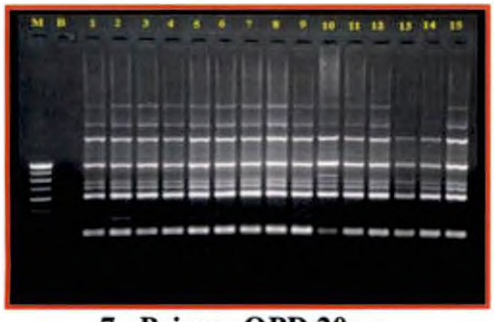
5. Primer OPA 28



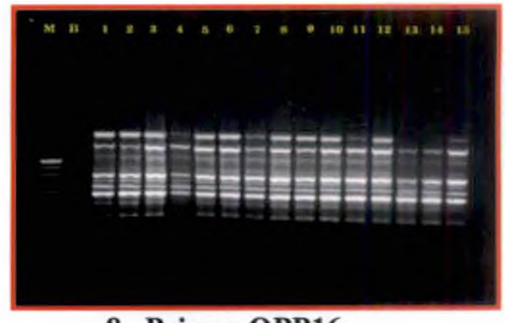
6. Primer OPD 15

M: 100bp ladder / Marker
B: Control
1: Maran Bud
2: MC
3: Mse
4: MC (10 Gy)
5: MC (20 Gy)
6: Mse (10 Gy)
7: Mse (20 Gy)
8: Control M
9: Rio Bud
10: RC
11: Rse
12: RC (10 Gy)
13: RC (20 Gy)
14: Rse (10 Gy)
15: Control R

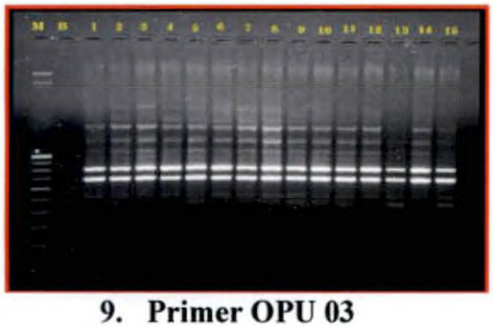
Plate 6 a. Amplification patterns in different groups of ginger somaclones with selected RAPD primers



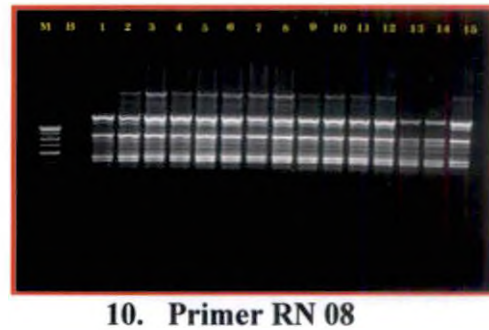
7. Primer OPD 20



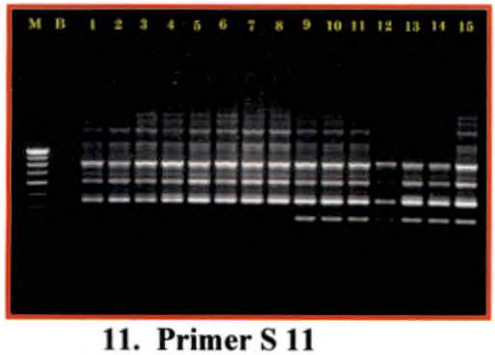
8. Primer OPP16



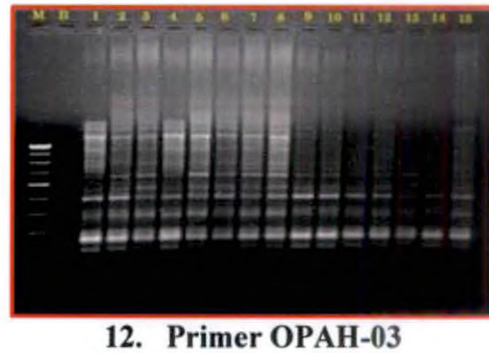
9. Primer OPU 03



10. Primer RN 08



11. Primer S 11



12. Primer OPAH-03

M: 100bp ladder / Marker
B: Control
1: Maran Bud
2: MC
3: Mse
4: MC (10 Gy)
5: MC (20 Gy)
6: Mse (10 Gy)
7: Mse (20 Gy)
8: Control M
9: Rio Bud
10: RC
11: Rse
12: RC (10 Gy)
13: RC (20 Gy)
14: Rse (10 Gy)
15: Control R

Plate 6 b. Amplification patterns in different groups of ginger somaclones with selected RAPD primers

Three amplicons of size 700 bp, 900 bp and 1100 bp were found in all the groups except RC20 Gy and RSe10 Gy. Two amplicons of size 1000 bp and 1200 bp were present only in somaclones of Maran group.

4.2.1.3 RAPD data analysis

Reproducible, well resolved fragments were scored using Quantity one software (Biorad) and each scorable band was scored for presence (1) or absence (0). RAPD analysis using twelve selected primers produced a total of one twenty nine markers in the thirteen groups of ginger somaclones (Table 9). The number of scorable markers produced by each primer ranged from eight (OPA 04) to thirteen (OPAH 03) with an average of 10.75 markers per primer. The molecular weight of these markers ranged 150 bp to 1800 bp. The polymorphic bands were 34.10 per cent of the total, each primer detecting on an average 3.66 polymorphic bands.

The numerical series for the presence or absence of bands was entered into a binary data matrix and used for calculating the similarity coefficient using Jaccard's coefficient (Jaccard, 1908). The mean similarity coefficient for each accession pair was calculated and used for cluster analysis using the UPGMA method and a dendrogram generated using the software package NTSYS pc version 2.02i (Rohlf, 1993). The dendrogram generated using NTSYS is given in Figure 1 and details of clusters are given in Table 10.

The dendrogram generated using NTSYS grouped the somaclones into two main clusters. Cluster I includes eleven groups of somaclones (MB, MC, MSe, MC10 Gy, MC20 Gy, MSe10 Gy, MSe20 Gy, RB, RC, RSe, RC10 Gy) and two source parent cultivars. Rio-de-Janeiro regenerants from calli irradiated with 20 Gy and somatic embryo regenerants from embryogenic calli irradiated with 10 Gy formed the second main cluster.

Cluster I was divided into two major sub clusters, with somaclones of Maran in first subcluster and somaclones of Rio-de-Janeiro in second subcluster.

Table 9. Amplification pattern of selected primers for RAPD assay in ginger

Sr. No.	Primer	Total no. of amplicons	No. of polymorphic amplicons	No. of monomorphic amplicons	Size of amplicons (range-bp)	Polymorphism (%)
1	OPA-02	11	4	7	300-1100	36.36
2	OPA-04	8	2	6	150-1500	25
3	OPA -12	12	5	7	300-1600	41.66
4	OPA-27	10	1	9	400-1600	10
5	OPA-28	11	6	5	300-1500	54.54
6	OPD-15	11	5	6	300-1500	45.45
7	OPD-20	10	3	7	200-1800	30
8	OPP-16	10	2	8	400-1400	20
9	OPU-03	10	2	7	400-1500	20
10	RN-08	12	4	8	300-1700	33.33
11	S-11	11	5	6	300-1800	45.45
12	OPAH-03	13	5	8	200-1600	38.46
Total		129	44	84		34.10
Average		10.75	3.66	7		

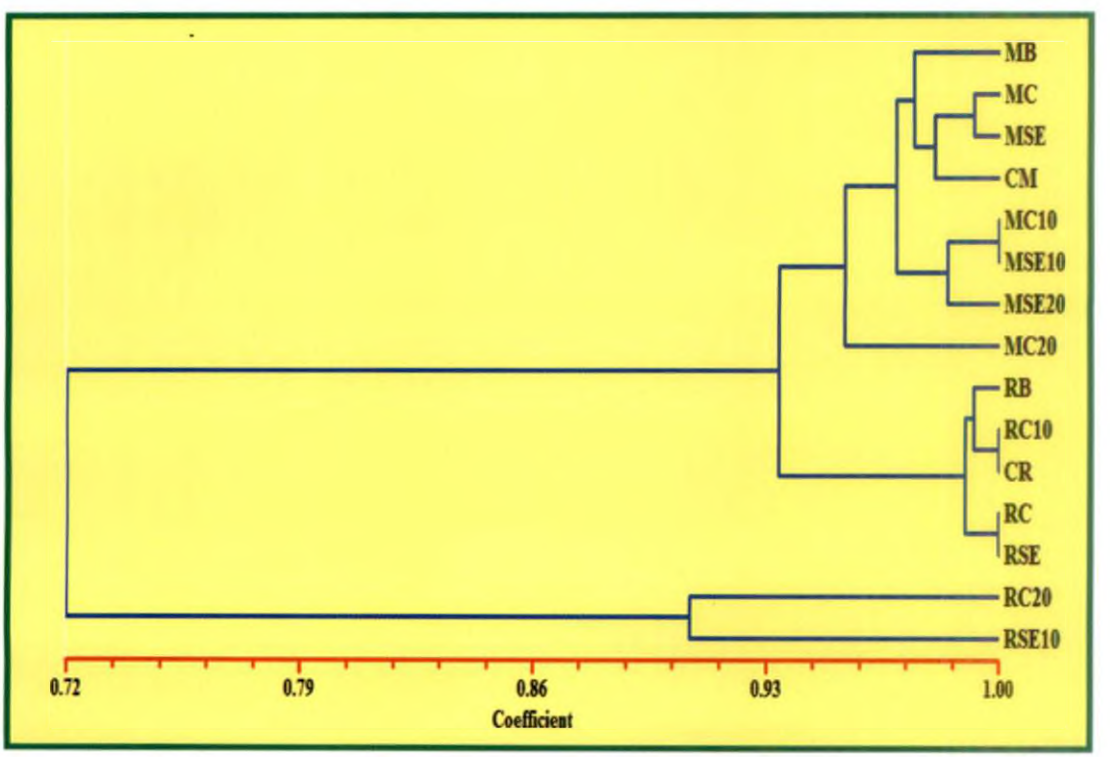


Figure 1. Dendrogram generated with RAPD profile in different groups of ginger somaclones

Table 10. Grouping of ginger somaclones based on RAPD data

Cluster number	Details of groups in each cluster	Name of the groups of somaclones
Cluster I	Eleven groups of somaclones and two parent cultivar	MB, MC, MSe, MC10 Gy, MC20 Gy, MSe10 Gy, MSe20 Gy, Maran source parent cultivar, RB, RC, RSe, RC10 Gy, Rio-de-Janeiro source parent cultivar
Cluster II	Two groups of somaclones	RC20 Gy and RSe10 Gy

Table 11. Jaccard's similarity coefficient matrix with RAPD data in ginger somaclones

	MB	MC	MSE	MC10	MC20	MSE10	MSE20	CM	RB	RC	RSE	RC10	RC20	RSE10	CR
MB	1.0000														
MC	0.9690	1.0000													
MSE	0.9767	0.9922	1.0000												
MC10	0.9535	0.9690	0.9767	1.0000											
MC20	0.9225	0.9535	0.9457	0.9690	1.0000										
MSE10	0.9535	0.9690	0.9767	1.0000	0.9690	1.0000									
MSE20	0.9535	0.9845	0.9767	0.9845	0.9690	0.9845	1.0000								
CM	0.9767	0.9767	0.9845	0.9767	0.9457	0.9767	0.9612	1.0000							
RB	0.9302	0.9457	0.9535	0.9457	0.9147	0.9457	0.9302	0.9535	1.0000						
RC	0.9302	0.9457	0.9535	0.9302	0.8992	0.9302	0.9302	0.9380	0.9845	1.0000					
RSE	0.9302	0.9457	0.9535	0.9302	0.8992	0.9302	0.9302	0.9380	0.9845	1.0000	1.0000				
RC10	0.9225	0.9380	0.9457	0.9380	0.9070	0.9380	0.9225	0.9457	0.9922	0.9922	0.9922	1.0000			
RC20	**0.6589**	0.6744	0.6667	**0.6589**	0.6899	**0.6589**	**0.6589**	0.6667	0.7132	0.7132	0.7132	0.7209	1.0000		
RSE10	0.7364	0.7519	0.7442	0.7209	0.7209	0.7209	0.7364	0.7287	0.7752	0.7907	0.7907	0.7829	0.9070	1.0000	
CR	0.9225	0.9380	0.9457	0.9380	0.9070	0.9380	0.9225	0.9457	0.9922	0.9922	0.9922	**1.0000**	0.7209	0.7829	1.0000

MB: Maran bud culture, MC: Maran indirect organogenesis, MSe: Maran indirect somatic embryogenesis, MC10: indirect organogenesis (irradiated with γ rays10 Gy), MC20: Maran indirect organogenesis (irradiated with γ rays 20 Gy), MSE10: Maran indirect somatic embryogenesis (irradiated with γ rays 10 Gy), MSE20: Maran indirect somatic embryogenesis (irradiated with γ rays 20 Gy), CM: Maran source parent cultivar (control M), RB: Rio-de-Janeiro bud culture, RC: Rio-de-Janeiro indirect organogenesis, RSE: Rio-de-Janeiro indirect somatic embryogenesis, RC10: Rio-de-Janeiro indirect organogenesis (irradiated with γ rays 10 Gy), RC20: Rio-de-Janeiro indirect organogenesis (irradiated with γ rays 20 Gy), RSE10: Rio-de-Janeiro indirect somatic embryogenesis (irradiated with γ rays 10 Gy), CR: Rio-de-Janeiro source parent cultivar.

A genetic similarity matrix of different groups of ginger somaclones based on the proportion of shared RAPD fragments was also generated (Table 11). The pairwise similarity coefficient values varied between 0.6589 and 1.0000 indicating 34 per cent variability in somaclones and induced mutants of different groups.

The extent of variability in somaclones from the source parent cultivar Maran was found more in MC20 Gy (5.4%) followed by MSe20 Gy (4%), MSe10 Gy (2.5%), MC10 Gy (2.5%) MB (2.5%) and callus regenerants (2.5%) while somatic embryo regenerants without irradiation showed less variability (1.5%).

In Rio-de-Janeiro, the highest variability was recorded by RC20 Gy (28%) followed by RSe10 Gy (22%). However, the indirect organogenic and embryogenic regenerants without irradiation showed less variability (1.1%) as compared to irradiated groups.

4.2.2 Inter Simple Sequence Repeat (ISSR) analysis

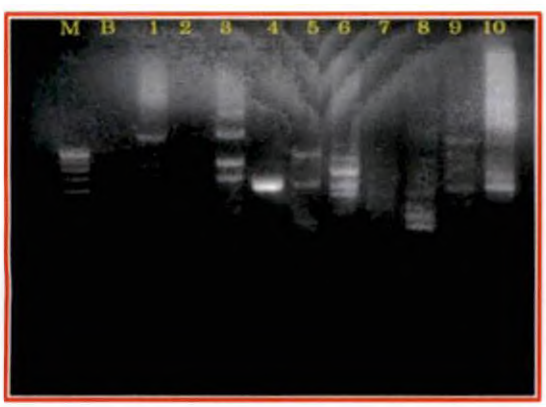
Original source parent cultivars and thirteen groups of somaclones (seven in cultivar Maran and six in cultivar Rio-de-Janeiro) were subjected to ISSR analysis using bulked DNA samples of each group.

4.2.2.1 Screening of primers for ISSR analysis

Thirty primers of which 20 belonging to UBC (University of British, Columbia) series, eight to ISSR (ISSR Technologies, USA) series and two to SPS series were screened for ISSR analysis using good quality genomic DNA bulked from three somaclones viz. RVIII, MSe1Kr200 and MC1Kr168.

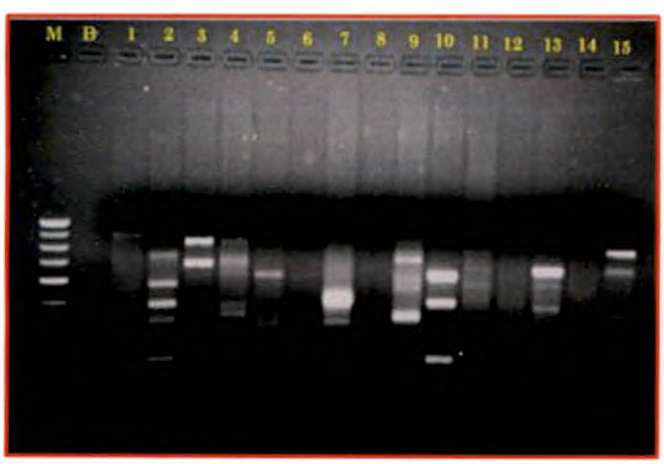
ISSR analysis with the thermal settings described in 3.3.3 gave good amplification. The amplification pattern of screened primers is presented in Plate 7 and Table 12.

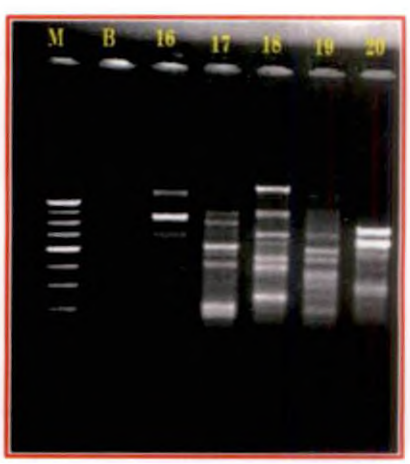
Out of 30 ISSR primers, twelve primers showed good amplification and hence they were selected for variability analysis in ginger somaclones. The details of the selected primers are presented in Table 13.



M- 100bp ladder / Marker

B- Blank	7: UBC 807	14: UBC 820
1: UBC 811	8: UBC 834	15: UBC 840
2: UBC 813	9: UBC 835	16: UBC 826
3: UBC 815	10: UBC 836	17: UBC 845
4: UBC S2	11: UBC 890	18: UBC 848
5: UBC 844	12: UBC 817	19: UBC 868
6: UBC 866	13: UBC 818	20: UBC 354





M- 100bp ladder / Marker

B- Blank	7: UBC 868	14: SPS 08
1: UBC 811	8: UBC 354	15: ISSR 05
2: UBC 840	9: UBC 866	16: ISSR 09
3: UBC 815	10: UBC 844	17: ISSR 10
4: UBC 835	11: ISSR 07	18: ISSR 06
5: UBC 834	12: ISSR 15	19: ISSR 04
6: UBC 890	13: SPS 03	20: ISSR 08

Plate 7. Screening of ISSR primers for amplification of DNA from ginger somaclones

Table 12. Amplification pattern of primers screened for ISSR assay

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 13. Details of 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	SPS-03	5'GACAGACAGACAGACA3'	43
6	ISSR-04	5'ACACACACACACACACC3'	42
7	ISSR-05	5'CTCTCTCTCTCTCTTG3'	42
8	ISSR-06	5'GAGAGAGAGAGAGAGAC3'	47
9	ISSR-08	5'GAGAGAGAGAGAGAGAT3'	45
10	ISSR-09	5'CTCTCTCTCTCTCTCTG3'	42
11	ISSR-10	5'ACACACACACACACACG3'	47
12	ISSR-15	5'TCCTCCTCCTCCTCC3'	42

Primers of the ISSR series used for screening gave good amplification as compared to UBC and SPS series. The primers UBC 840, UBC 844 and ISSR05gave highest number of clear and distinct bands followed by ISSR 04 and ISSR 08.

4.2.2.2 Variability analysis in ginger somaclones with selected ISSR primers

The amplification pattern in thirteen groups of ginger somaclones and source parent cultivars using selected ISSR primers is depicted in Plate 8a and 8b. The percentage of polymorphism was worked out for each primer. The details of amplification pattern are presented in Table 14.

The polymorphism percentage ranged from 12.5 to 40 in the selected primers. The highest polymorphism percentage was recorded by the primer ISSR 05 (40) followed by UBC 835 (30.76), UBC 834 (36.36) and ISSR 06 (33.33). Primerwise analysis is as shown below:

UBC 834

A total of eleven amplicons ranged in size 200 bp to 1000 bp were produced by the primer UBC 834. They were clear, distinct and reproducible. It could generate four polymorphic bands out of eleven amplicons (Plate 8a. 1) and the percentage polymorphism was 36.36.

Two amplicons of size 700 bp and 1000 bp were found in all the groups except RC20 Gy and RSe10 Gy. Two amplicons of size 300 bp and 500 bp were found in all groups except MC20 Gy and MSe20 Gy.

UBC 835

The primer UBC 835 could generate a total of thirteen clear, distinct and reproducible bands (200 bp to 900 bp) out of which four were polymorphic. The percentage polymorphism was 30.76. The amplification profile is given in Plate 8a. 2.

Two amplicons of size 650 bp and 900 bp were found in all the groups except RC20 Gy and RSe10 Gy. One loci of size 750 bp was present only in all the somaclones of Maran group.

One amplicon of size 600 bp was found in all the groups except, MSe10 Gy, MSe20 Gy, RC20 Gy and RSe10 Gy.

UBC 840

UBC 840 generated eleven clear, distinct and reproducible amplicons ranged in size 200 bp to 1100 bp and four of them were polymorphic (Plate 8a. 3). The per cent polymorphism was 36.36.

Two amplicons of size 1000 bp and 1100 bp were found in all the groups except the irradiated group RC20 Gy and RSe10 Gy. One amplicon of size 800 bp was present in the MC 20 Gy, RC 10 Gy and Maran source parent cultivar (control M). One loci of size 400 bp was present only in somaclones of Rio-de-Janeiro group.

UBC 844

Six clear, distinct and reproducible amplicons were produced by this primer (Plate 8a. 4) ranged in size 300 bp to 1000 bp. The polymorphism percentage calculated was less (16.16%) as it could detect only one polymorphic amplicons out of six amplicons.

One amplicon of size 1000 bp was found in all the groups except RC20 Gy and RSe10 Gy.

SPS 03

Amplification of thirteen groups of ginger somaclones with the selected primer SPS 03 produced eleven clear, distinct and reproducible amplicons ranged in size 300 bp to 1100 bp (Plate 8a. 5) three amplicons were polymorphic and the percentage polymorphism calculated was 27.27.

Two amplicons of size 350 bp and 1000 bp were found in all the groups except RC20 Gy and RSe10 Gy. One amplicon of size 800 bp was present only in somaclones of Maran group.

ISSR 04

The primer OPD 15 was able to generate thirteen clear, distinct and reproducible amplicons (Plate 8a. 6). The amplicons were ranged in size 200 bp to 1100 bp. Only two of them were polymorphic giving 15.38 per cent polymorphism.

Two amplicons of size 500 bp and 1000 bp were found in all the groups except RC20 Gy and RSe10 Gy.

ISSR 05

The primer ISSR 05 could generate ten clear, distinct and reproducible amplicons ranged in size 350 bp to 1100 bp. Four amplicons were polymorphic (Plate 8b. 7) and per cent polymorphism was high among all the other primers screened (40%).

Two amplicons of size 670 bp and 1100 bp were found in all the groups except RC20 Gy. One amplicon of size 800 bp was found in all the groups except RC20 Gy and RSe10 Gy. One amplicon of size 1000 bp was present only in somaclones of Maran group.

ISSR 06

Nine clear, distinct and reproducible amplicons were produced by the primer ISSR 06 (Plate 8b. 8) ranged in size 100 bp to 800 bp. It could detect three polymorphic amplicons out of nine. The polymorphism percentage calculated was 33.33.

Two amplicons of size 550 bp and 800 bp were found in all the groups except RC20 Gy and RSe10 Gy. One amplicon of size 100 bp was present only in somaclones of Maran group.

ISSR 08

ISSR 08 could generate a total of eleven clear, distinct and reproducible amplicons (300 bp to 1000 bp), of which only two were polymorphic (Plate 8b. 9). The polymorphism percentage recorded was 18.18.

One loci of size 1000 bp was found in all the groups except RC10 Gy, RC20 Gy and RSe10 Gy. One amplicon of size 700 bp was found in all the groups except MC20 Gy, RC20 Gy and RSe10 Gy.

ISSR 09

Eight amplicons were produced by ISSR 09 (200 bp to 1400 bp) and only one of them was polymorphic (Plate 8b. 10). The polymorphism percentage was less (12.5) among all the other primers as it could detect only one polymorphic amplicons out of eight amplicons.

One amplicon of size 900 bp was found in all the groups except MC10 Gy, RC20 Gy and RSe10 Gy.

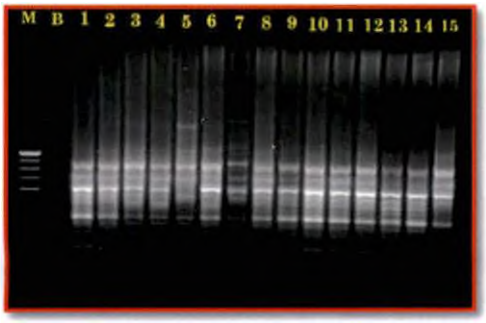
ISSR 10

The primer ISSR 10 was able to generate eleven amplicons which ranged in size from 200 bp to 900 bp and two of them were polymorphic. The bands were clear, distinct and reproducible (Plate 8b. 11). The polymorphism percentage was 18.18.

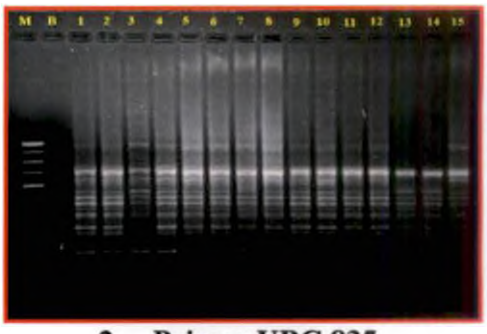
Two amplicons of size 400 bp and 600 bp were found in all the groups except RC20 Gy and RSe10 Gy.

ISSR 15

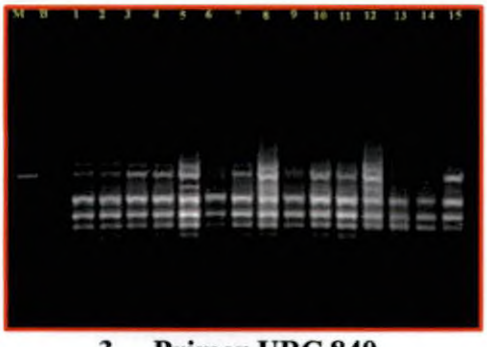
ISSR 15 produced eight clear, distinct and reproducible amplicons (400 bp to 1100 bp) and two amplicons were found polymorphic giving 25 per cent polymorphism. The profile showing the amplification pattern of ISSR 15 is presented in Plate 8b. 12.



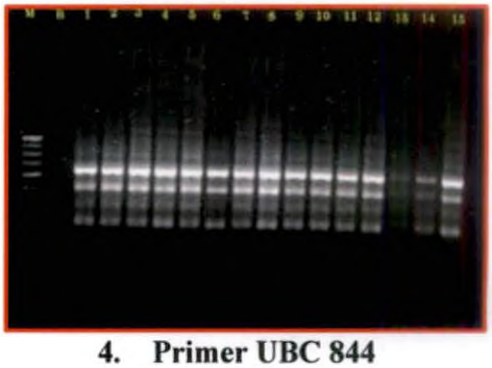
1. **Primer UBC 834**



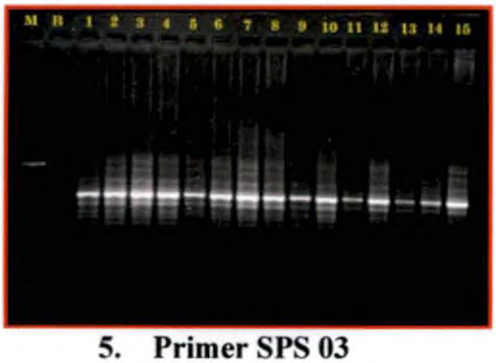
2. **Primer UBC 835**



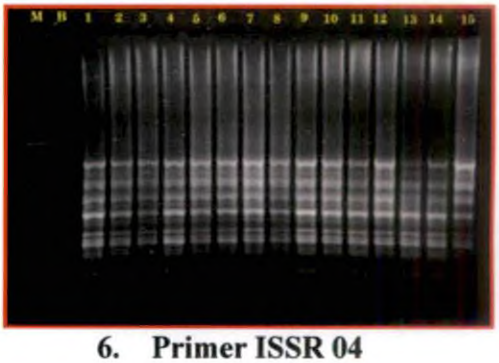
3. **Primer UBC 840**



4. **Primer UBC 844**



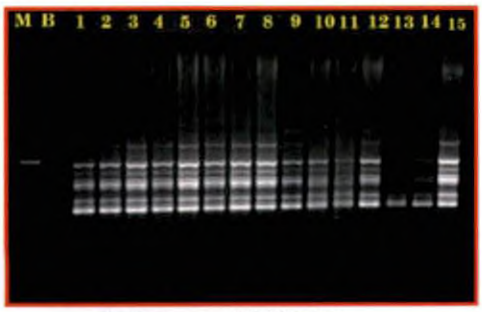
5. **Primer SPS 03**



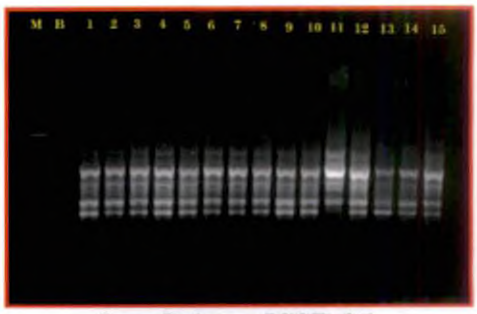
6. **Primer ISSR 04**

M: 100bp ladder / Marker
B: Control
1: Maran Bud
2: MC
3: Mse
4: MC (10 Gy)
5: MC (20 Gy)
6: Mse (10 Gy)
7: Mse (20 Gy)
8: Control M
9: Rio Bud
10: RC
11: Rse
12: RC (10 Gy)
13: RC (20 Gy)
14: Rse (10 Gy)
15: Control R

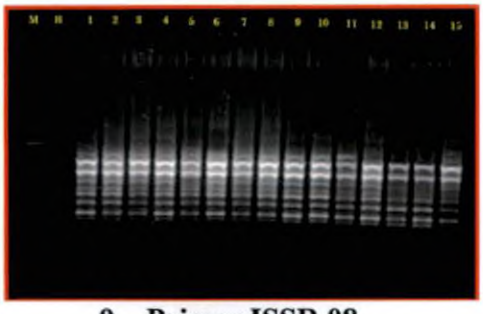
Plate 8 a. Amplification patterns in different groups of ginger somaclones with selected ISSR primers



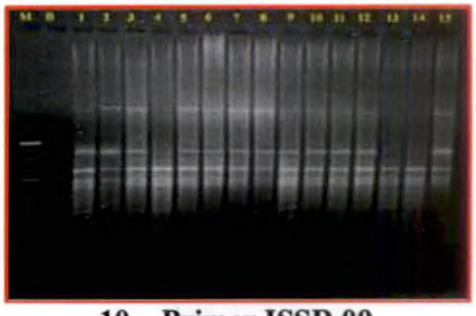
7. Primer ISSR 05



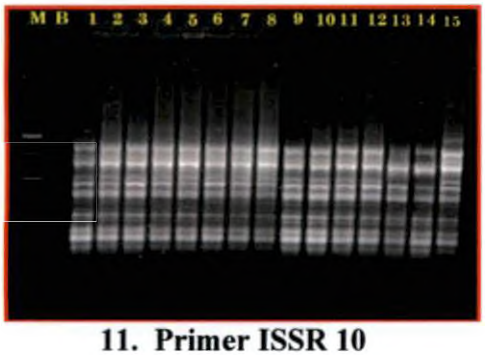
8. Primer ISSR 06



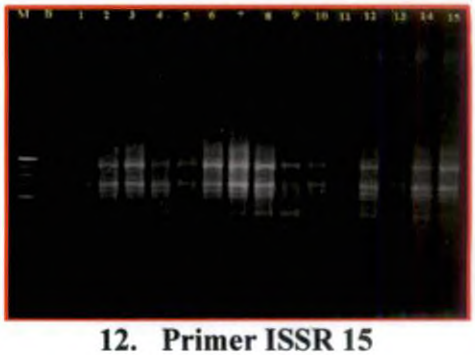
9. Primer ISSR 08



10. Primer ISSR 09



11. Primer ISSR 10



12. Primer ISSR 15

M: 100bp ladder / Marker
B: Control
1: Maran Bud
2: MC
3: Mse
4: MC (10 Gy)
5: MC (20 Gy)
6: Mse (10 Gy)
7: Mse (20 Gy)
8: Control M
9: Rio Bud
10: RC
11: Rse
12: RC (10 Gy)
13: RC (20 Gy)
14: Rse (10 Gy)
15: Control R

Plate 8 b. Amplification patterns in different groups of ginger somaclones with selected ISSR primers

One amplicon of size 900 bp was found in all groups except RC20 Gy and RSe 10 Gy. One loci of size 550 bp was present in all groups except MC20 Gy.

4.2.2.3 ISSR data analysis

Reproducible, well resolved fragments were scored using Quantity one software (BIORAD) and each scorable band was scored and assigned the series of one for presence and zero for absence.

ISSR analysis using twelve selected primers produced a total of 122 markers in thirteen groups of ginger somaclones (Table 14). The number of scorable markers produced by each primer ranged from six (UBC 840) to thirteen (UBC 835 and ISSR 05) with an average of 10.16 markers per primer. The molecular weight of these markers ranged 100 bp to 1400 bp. The overall mean of polymorphic bands was 26.23 per cent, each primer detecting on an average 2.66 polymorphic bands.

The presence or absence of bands was entered into a binary data matrix and used for calculating the similarity coefficient using Jaccard's coefficient (Jaccard, 1908). The mean similarity coefficient for each accession pair was calculated and used for cluster analysis using the UPGMA method and a dendrogram generated using the software package NTSYS pc version 2.02i (Rohlf, 1993). The dendrogram generated using NTSYS is given in Figure 2 and details of clusters are given in Table 15.

The dendrogram generated using NTSYS grouped the somaclones into two main clusters. Cluster I includes eleven groups of somaclones and two source parent cultivars. Rio-de-Janeiro regenerants from calli irradiated with 20 Gy and somatic embryo regenerants from embryogenic calli irradiated with 10 Gy formed the second main cluster.

Cluster I was divided into two major sub clusters. Somaclones of cultivar Maran were grouped in first sub cluster and somaclones of Rio-de-Janeiro were grouped in second sub cluster.

Table 14. Amplification pattern in ginger somaclones with selected ISSR primers

Sr. No.	Primer	Total no. of amplicons	No. of polymorphic amplicons	No. of monomorphic amplicons	Size of amplicon (range-bp)	Polymorphism (%)
1	UBC 834	11	4	7	200-1000	36.36
2	UBC 835	13	4	9	200-900	30.76
3	UBC 840	11	4	7	200-1100	36.36
4	UBC 844	6	1	5	300-1000	16.66
5	SPS-03	11	3	8	350-1000	27.27
6	ISSR-04	13	2	11	200-1100	15.38
7	ISSR-05	10	4	6	350-1100	40
8	ISSR-06	9	3	6	100-800	33.33
9	ISSR-08	11	2	9	300-1000	18.18
10	ISSR-09	8	1	7	200-1400	12.5
11	ISSR-10	11	2	9	200-900	18.18
12	ISSR-15	8	2	6	400-1100	25
Total		122	32	90		26.23
Average		10.16	2.66	7.5		

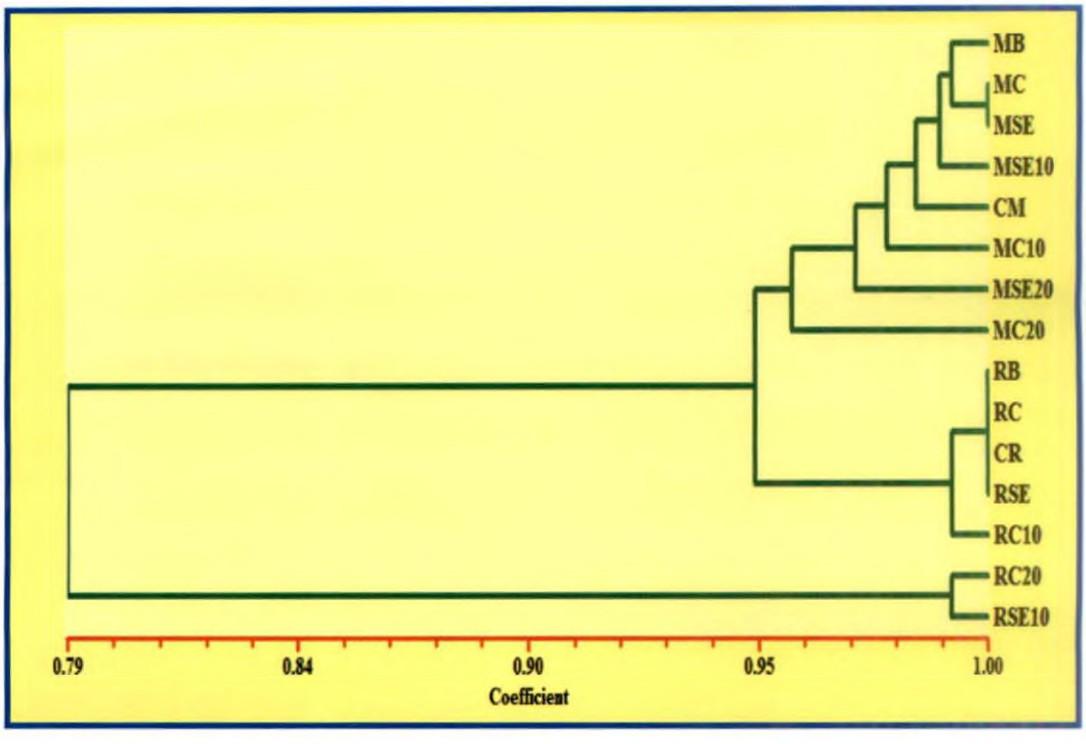


Figure 2. Dendrogram generated with ISSR profile in different groups of ginger somaclones

Table 15. Grouping of ginger somaclones based on ISSR data

Cluster number	Details of groups in each cluster	Name of the groups of somaclones
Cluster I	Eleven groups of somaclones and two parent cultivars	MB, MC, MSe, MC10 Gy, MC20 Gy, MSe10 Gy, MSe20 Gy, Maran source parent cultivar, RB, RC, RSe, RC10 Gy, Rio-de-Janeiro source parent cultivar
Cluster II	Two groups of somaclones	RC20 Gy and RSe10 Gy

Table 16. Jaccard's similarity coefficient matrix with ISSR data in ginger somaclones

	MB	MC	MSE	MC10	MC20	MSE10	MSE20	CM	RB	RC	RSE	RC10	RC20	RSE10	CR
MB															
MC	0.9917	1.0000													
MSE	0.9917	**1.0000**	1.0000												
MC10	0.9752	0.9835	0.9835	1.0000											
MC20	0.9504	0.9587	0.9587	0.9587	1.0000										
MSE10	0.9835	0.9917	0.9917	0.9752	0.9504	1.0000									
MSE20	0.9669	0.9752	0.9752	0.9587	0.9669	0.9835	1.0000								
CM	0.9917	0.9835	0.9835	0.9669	0.9421	0.9752	0.9587	1.0000							
RB	0.9669	0.9587	0.9587	0.9421	0.9174	0.9504	0.9339	0.9587	1.0000						
RC	0.9669	0.9587	0.9587	0.9421	0.9174	0.9504	0.9339	0.9587	1.0000	1.0000					
RSE	0.9669	0.9587	0.9587	0.9421	0.9174	0.9504	0.9339	0.9587	1.0000	1.0000	1.0000				
RC10	0.9587	0.9504	0.9504	0.9339	0.9091	0.9421	0.9256	0.9504	0.9917	0.9917	0.9917	1.0000			
RC20	0.7769	0.7686	0.7686	0.7851	**0.7603**	0.7769	**0.7603**	0.7686	0.8099	0.8099	0.8099	0.8182	1.0000		
RSE10	0.7851	0.7769	0.7769	0.7934	0.7686	0.7851	0.7686	0.7769	0.8182	0.8182	0.8182	0.8264	0.9917	1.0000	
CR	0.9669	0.9587	0.9587	0.9421	0.9174	0.9504	0.9339	0.9587	**1.0000**	**1.0000**	**1.0000**	0.9917	0.8099	0.8182	1.0000

MB: Maran bud culture, MC: Maran indirect organogenesis, MSe: Maran indirect somatic embryogenesis, MC10: indirect organogenesis (irradiated with γ rays10 Gy), MC20: Maran indirect organogenesis (irradiated with γ rays 20 Gy), MSE10: Maran indirect somatic embryogenesis (irradiated with γ rays 10 Gy), MSE20: Maran indirect somatic embryogenesis (irradiated with γ rays 20 Gy), CM: Maran source parent cultivar (control M), RB: Rio-de-Janeiro bud culture, RC: Rio-de-Janeiro indirect organogenesis, RSE: Rio-de-Janeiro indirect somatic embryogenesis, RC10: Rio-de-Janeiro indirect organogenesis (irradiated with γ rays 10 Gy), RC20: Rio-de-Janeiro indirect organogenesis (irradiated with γ rays 20 Gy), RSE10: Rio-de-Janeiro indirect somatic embryogenesis (irradiated with γ rays 10 Gy), CR: Rio-de-Janeiro source parent cultivar.

A genetic similarity matrix of different groups of ginger somaclones based on the proportion of shared ISSR fragments was also generated (Table 16). The pairwise similarity coefficient values varied between 0.7603 and 1.0000.

The extent of variability in somaclones from the source parent cultivar Maran was found more in MC20 Gy (5.8%) followed by MSe20 Gy (4.1%), MC10 Gy (3.3%) and MSe10 Gy (2.5%). However, callus and somatic embryo regenerants showed less variability.

In Rio-de-Janeiro somaclones the highest variability was recorded in RC20 Gy (19%) followed by RSe10 Gy (18%). However, bud, callus and somatic embryo regenerants were found similar to source parent cultivar.

4.2.3. Variability analysis in different groups of ginger somaclones using combined RAPD and ISSR data

Amplification of thirteen groups of ginger somaclones along with two source parent cultivars produced a total of 251 markers by the RAPD and ISSR assay with an average of 10.45 markers per each primer. The total polymorphic bands were 30.28 per cent, each primer detecting on an average 3.16 polymorphic bands per primer.

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

Based on the proximity matrix obtained from Jaccard's coefficients, Sequential Agglomerative Hierarchical Non-overlapping (SAHN) clustering was done using Unweighted Pair Group Method with Arithmetic averages (UPGMA) method. The dendrogram generated using NTSYS is given in Figure 3 and details of clusters are given in Table 17.

The dendrograms generated based on RAPD and ISSR profiles grouped the somaclones into two separate clusters, with somaclones of Maran in first subcluster of cluster I and somaclones of Rio-de-Janeiro in second subcluster of cluster I.

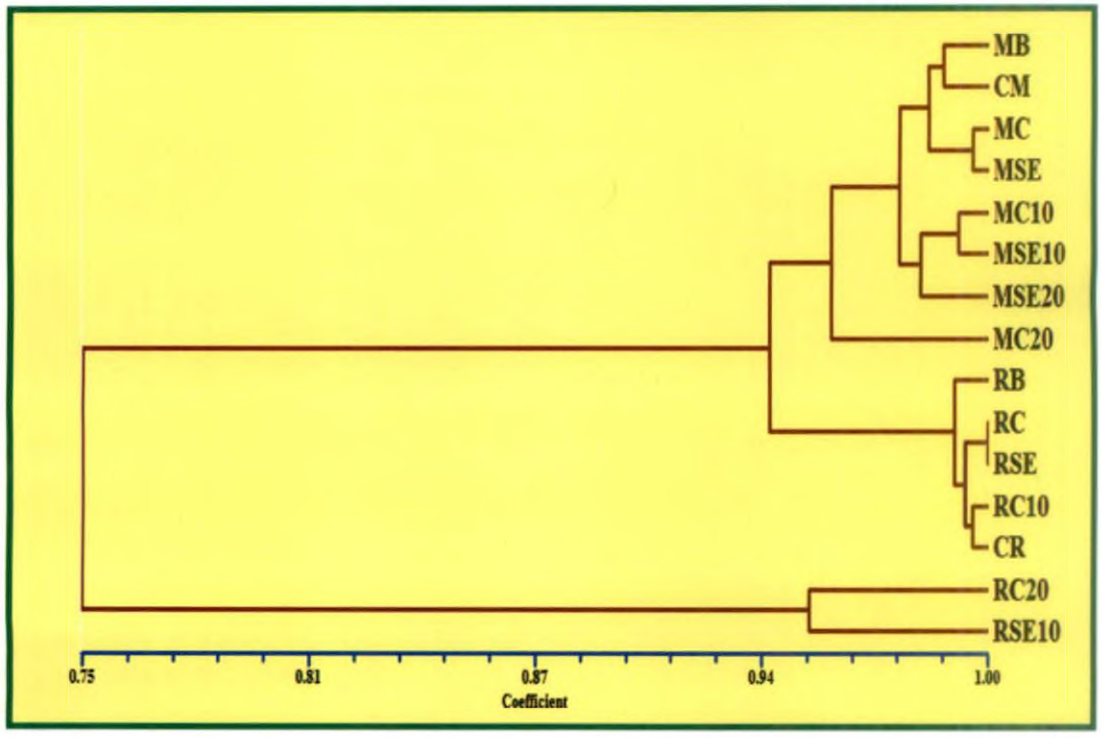


Figure 3. Dendrogram generated with RAPD and ISSR profiles in different groups of ginger somaclones

Table 17. Grouping of ginger somaclones based on combined (RAPD and ISSR) data

Cluster number	Details of groups in each cluster	Name of the groups of somaclones
Cluster I	Eleven groups of somaclones and two parent cultivars	MB, MC, MSe, MC10 Gy, MC20 Gy, MSe10 Gy, MSe20 Gy, Maran source parent cultivar, RB, RC, RSe, RC10 Gy, Rio-de-Janeiro source parent cultivar
Cluster II	Two groups of somaclones	RC20 Gy and RSe10 Gy

Table 18. Jaccard's similarity coefficient matrix with RAPD and ISSR data in ginger somaclones

	MB	MC	MSE	MC10	MC20	MSE10	MSE20	CM	RB	RC	RSE	RC10	RC20	RSE10	CR
MB	1.0000														
MC	0.9801	1.0000													
MSE	0.9801	0.9920	1.0000												
MC10	0.9641	0.9761	0.9761	1.0000											
MC20	0.9363	0.9562	0.9482	0.9641	1.0000										
MSE10	0.9681	0.9801	0.9801	0.9880	0.9602	1.0000									
MSE20	0.9602	0.9801	0.9721	0.9721	0.9681	0.9841	1.0000								
CM	0.9841	0.9801	0.9801	0.9721	0.9442	0.9761	0.9602	1.0000							
RB	0.9442	0.9482	0.9482	0.9402	0.9124	0.9442	0.9283	0.9522	1.0000						
RC	0.9482	0.9522	0.9522	0.9363	0.9084	0.9402	0.9323	0.9482	0.9880	1.0000					
RSE	0.9442	0.9482	0.9482	0.9323	0.9044	0.9363	0.9283	0.9442	0.9841	**0.9960**	1.0000				
RC10	0.9402	0.9442	0.9442	0.9363	0.9084	0.9402	0.9243	0.9482	0.9880	0.9920	0.9880	1.0000			
RC20	0.7171	0.7211	0.7131	0.7211	0.7251	0.7171	**0.7092**	0.7171	0.7570	0.7610	0.7570	0.7689	1.0000		
RSE10	0.7610	0.7649	0.7570	0.7570	0.7450	0.7530	0.7530	0.7530	0.7928	0.8048	0.8008	0.8048	0.9482	1.0000	
CR	0.9402	0.9442	0.9442	0.9363	0.9084	0.9402	0.9243	0.9482	0.9880	0.9920	0.9880	0.9920	0.7610	0.7968	1.0000

MB: Maran bud culture, MC: Maran indirect organogenesis, MSe: Maran indirect somatic embryogenesis, MC10: indirect organogenesis (irradiated with γ rays10 Gy), MC20: Maran indirect organogenesis (irradiated with γ rays 20 Gy), MSE10: Maran indirect somatic embryogenesis (irradiated with γ rays 10 Gy), MSE20: Maran indirect somatic embryogenesis (irradiated with γ rays 20 Gy), CM: Maran source parent cultivar (control M), RB: Rio-de-Janeiro bud culture, RC: Rio-de-Janeiro indirect organogenesis, RSE: Rio-de-Janeiro indirect somatic embryogenesis, RC10: Rio-de-Janeiro indirect organogenesis (irradiated with γ rays 10 Gy), RC20: Rio-de-Janeiro indirect organogenesis (irradiated with γ rays 20 Gy), RSE10: Rio-de-Janeiro indirect somatic embryogenesis (irradiated with γ rays 10 Gy), CR: Rio-de-Janeiro source parent cultivar.

The regenerants from Rio-de-Janeiro calli irradiated with 20 Gy and somatic embryogenic calli irradiated with 10 Gy formed the second main cluster.

A genetic similarity matrix of different groups of ginger somaclones based on the proportion of shared RAPD and ISSR bands was also generated (Table 18). The pairwise coefficient values varied between 0.7092 and 0.9960.

The extent of variability in somaclones from the source parent cultivar was found more in MC20 Gy (5.6%) followed by MSe20 Gy (4%), MC10 Gy (3%) and MSe10 Gy (2.5%). However, bud, callus and somatic embryo regenerants of the cultivar Maran showed less variability (2%) as compared to irradiated groups.

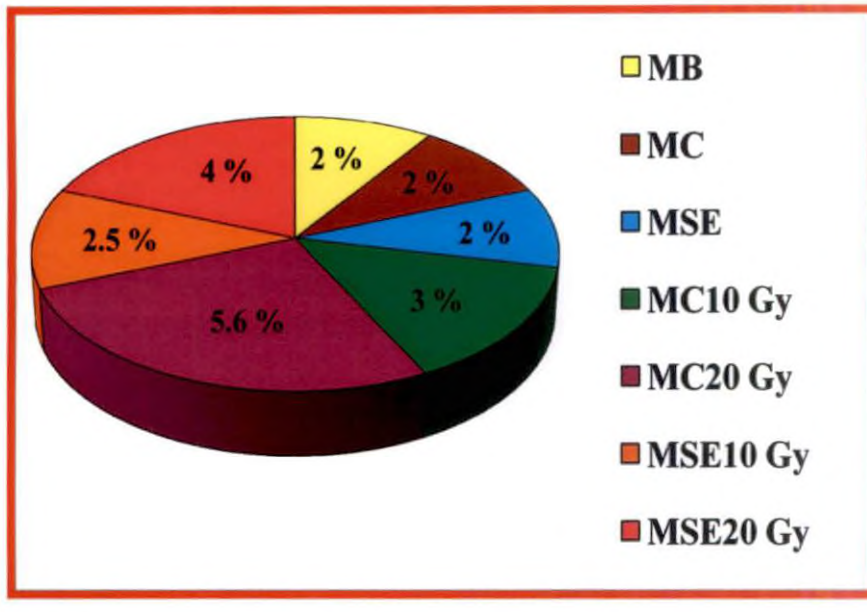
In the case of Rio-de-Janeiro somaclones, the highest variability was recorded by RC20 Gy (24%) followed by RSe10 Gy (21%). In Rio-de-Janeiro somaclones also, the irradiated group exhibited more variability as compared to non-irradiated groups.

The variability exhibited by the somaclones from source parent cultivars Maran and Rio-de-Janeiro is presented in Figure 4.

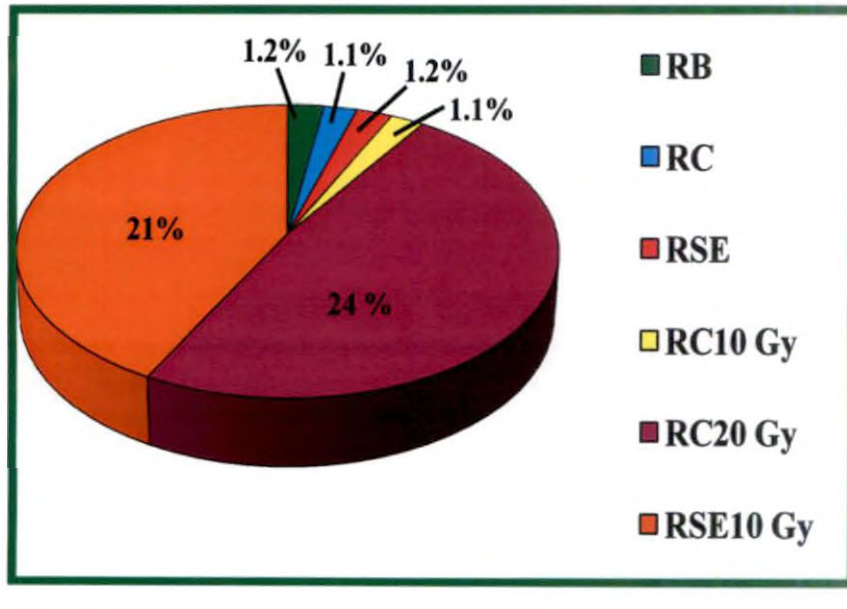
4.3. Resolving power of selected RAPD and ISSR primers

The Resolving power (Rp) was calculated for the twelve random primers are presented in Figure 5a. It ranged from 15.5 (OPA 04) to 23.3 (OPAH 03) with an average of 19.98. Primer OPAH 03 recorded highest (23.3) resolving power followed by RN 08, OPA 28 and OPD 15. Primer OPA 04 recorded lowest (15.5) resolving power compared to all RAPD primers.

Primers for ISSR assay recorded resolving power ranged from 11.7 (UBC 844) and 25.5 (ISSR 04) with an average of 19.13 (Figure 5b). The highest resolving power recorded by primer ISSR 04 (25.5) followed by UBC 835, ISSR 08 and ISSR 10. Primer UBC 844 recorded lowest (11.7) resolving power compared to all ISSR primers.

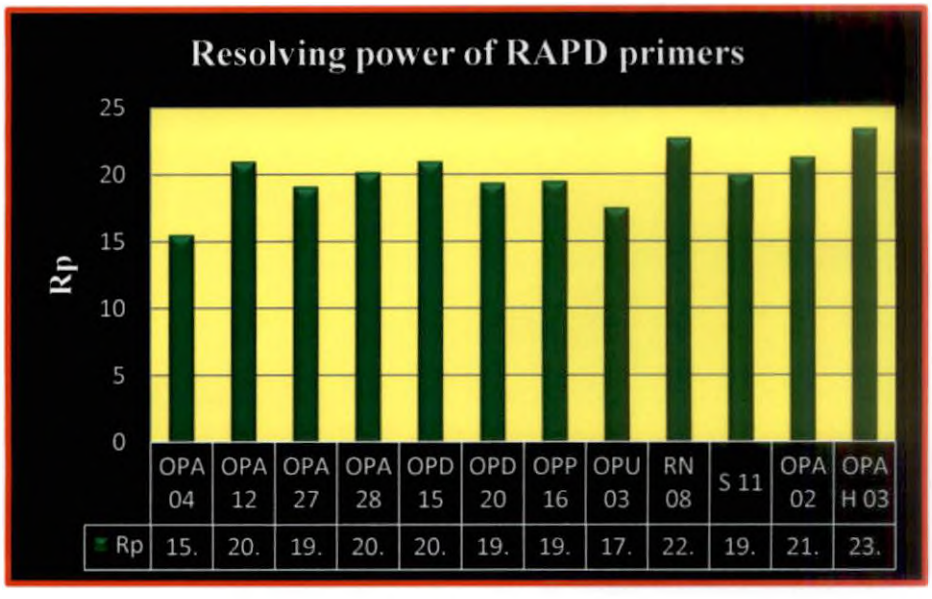


a. Variability from source parent cultivar Maran

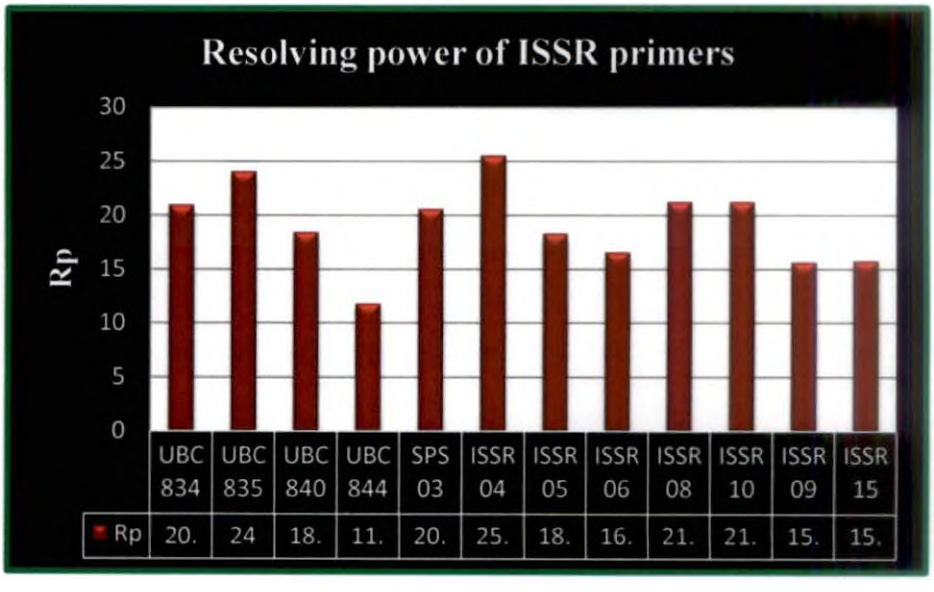


b. Variability from source parent cultivar Rio-de-Janeiro

Figure 4. Variability in groups of ginger somaclones from source parent cultivars

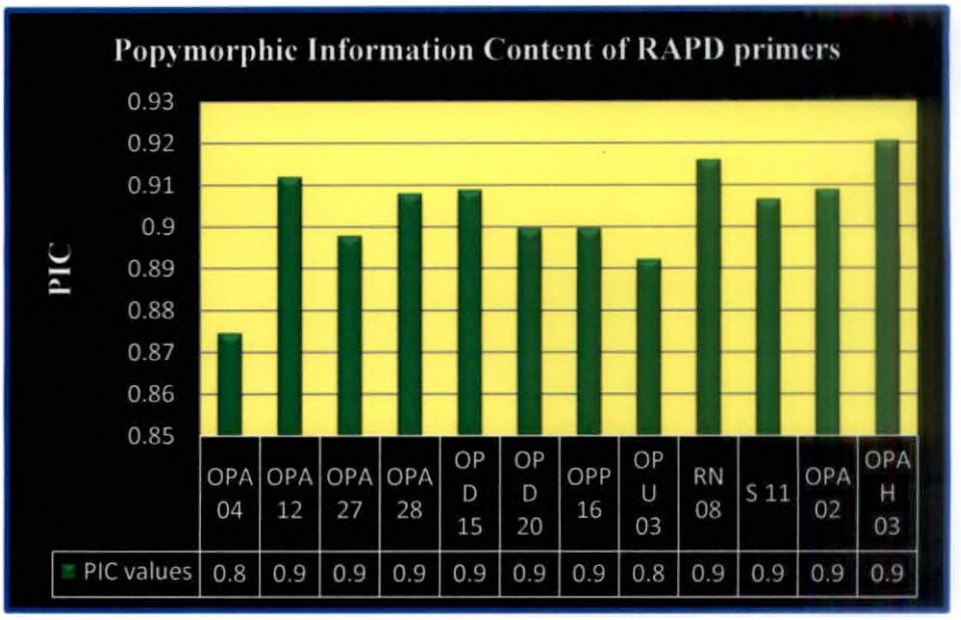


a. RAPD primers

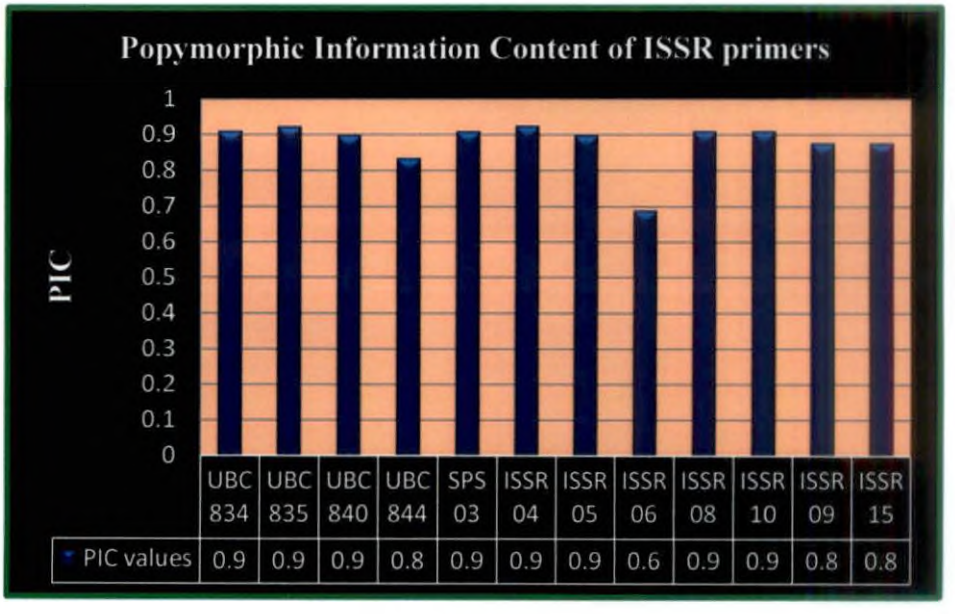


b. ISSR primers

Figure 5. Resolving power (Rp) of selected RAPD and ISSR primers



a. RAPD primers



b. ISSR primers

Figure 6. Polymorphic Information content (PIC) for selected RAPD and ISSR primers

4.4. Polymorphic Information Content (PIC) of the selected RAPD and ISSR primers

The Polymorphic Information Content (PIC) calculated for the twelve selected RAPD primers (Figure 6a) varied from 0.87 (OPU 03) to 0.92 (OPA 28 and OPAH 03) with a mean of 0.90. Primer OPA 28 and OPAH 03 recorded highest Polymorphic Information Content value (0.92) followed by RN 08 and S 11. Primer OPU 03 recorded lowest Polymorphic Information Content value (0.87).

Selected ISSR primers (Figure 6b) recorded Polymorphic Information Content values ranging from 0.69 (ISSR 06) to 0.92 (ISSR 04 and UBC 835) with an average of 0.87. The highest Polymorphic Information Content recorded by primer ISSR 04 followed by UBC 835, ISSR 05 and ISSR 08. Primer ISSR 06 recorded lowest Polymorphic Information Content value (0.69).

In the present investigations, original source parent cultivars and thirteen groups of somaclones (seven in cultivar Maran and six in cultivar Rio-de-Janeiro) were subjected to group wise RAPD and ISSR analyses using bulked DNA sample of each group. Molecular marker data obtained from both the marker systems (RAPD and ISSR) compared between modes of regeneration, genotypes and source parent cultivars. RAPD and ISSR markers helped to assess the extent of somaclonal variation in ginger at molecular level as influenced by genotype and mode of regeneration. The Rio-de-Janeiro indirect organogenesis irradiated with 20 Gy and Rio-de-Janeiro indirect embryogenesis irradiated with 10 Gy were found to be more variable among all the groups of ginger somaclones. Hence these two groups were analysed for variability.

4.5. Molecular analysis of individual ginger somaclones of groups RC20 Gy and RSe10 Gy

Regenerants of Rio-de-Janeiro indirect organogenesis irradiated with 20 Gy and Rio-de-Janeiro indirect embryogenesis irradiated with 10 Gy were found to be more variable among all the groups of ginger somaclones. Hence these two groups were selected for further indepth investigations. The group RSe10 Gy consists of eleven

somaclones and RC20 Gy consists of only one somaclone. DNAs from twelve somaclones were used for further investigation on variability analysis.

Two RAPD and one ISSR primers which recorded higher polymorphism (Table 9 and Table 14 respectively) were selected for the variability analysis of ginger somaclones of the group RC20 Gy and RSe10 Gy. The RAPD primers S 11 and OPA 28 recorded polymorphism percentage 69.23 and 64.28 respectively. The ISSR primer ISSR 05 recorded polymorphism percentage of 60. Hence OPA 28, S 11 and ISSR 05 were used for variability analysis of individual somaclones of the two groups.

4.5.1. Variability analysis of ginger somaclones of groups RC20 Gy and RSe10 Gy using selected primers

The gel profile for DNA amplification of twelve ginger somaclones (one from RC 20 Gy and eleven from RSe10 Gy groups) along with source parent cultivar (control R) with selected primers is presented in Plate 9. The polymorphism percentage was worked out for each primer. The details of amplification are presented in Table 19. The primerwise analysis is as shown below:

OPA 28

Amplification of twelve ginger somaclones along with source parent cultivar with the selected RAPD primer, OPA 28 produced fourteen clear, distinct and reproducible amplicons ranged in size from 300 bp to 1300 bp (Plate 9. a), nine amplicons were polymorphic and the per cent polymorphism calculated was 64.28.

Amplification pattern showed that one amplicon of size 1300 bp was present only in two clones (RSe1Kr 78 and RSe1Kr1257) and Rio-de-Janeiro source parent cultivar. One amplicon of size 1170 bp was present in five somaclones (RSe1Kr188, RSe1Kr78, RSe1Kr1053, RSe1Kr1257, RSe1Kr239) and source parent cultivar. One loci of size 1150 bp was present in the RSe1Kr48, RSe1Kr188, RSe1Kr78, RSe1Kr1257, RSe1Kr239 somaclones and source parent cultivar. Two loci of size 1000 bp and 1100 bp were present in all the somaclones except RC2Kr1031.

Table 19. Details of amplification with selected primers in individual ginger somaclones of group RC20 Gy and RSe10 Gy

Sr. No.	Primer	Total no. of amplicons	No. of polymorphic amplicons	No. of monomorphic amplicons	Size of amplicons (range-bp)	Polymorphism (%)
1	OPA 28	14	9	5	300-1300	64.28
2	S 11	13	9	4	300-1500	69.23
3	ISSR 05	10	6	4	400-1500	60
Total		37	24	13		64.86
Average		12.33	8	4.33		

One amplicon of size 200 bp was present only in three somaclones (RC2Kr1031, RSe1Kr650 and RSe1Kr648).

S 11

The RAPD primer S 11 was able to generate thirteen clear, distinct and reproducible amplicons (Plate 9. b). The amplicons were ranged in size 300 bp to 1500 bp. nine of them were polymorphic giving 69.23 per cent polymorphism.

The pattern of amplification showed that one amplicon of size 1500 bp was present only in the two clones (RSe1Kr48 and RSe1Kr1257) and Rio-de-Janeiro source parent cultivar. One amplicon of size 1050 bp was present in all the somaclones except RC2Kr 1031 and RSe1Kr 1052.

One amplicon of size 900 bp was present only in three somaclones (RSe1Kr48, RSe1Kr1257, RSe1Kr239) and source parent cultivar. One loci of size 650 bp was present in all the somaclones except RC2Kr 1031. One amplicon of size 550 bp was present in all the somaclones except RSe1Kr 1052.

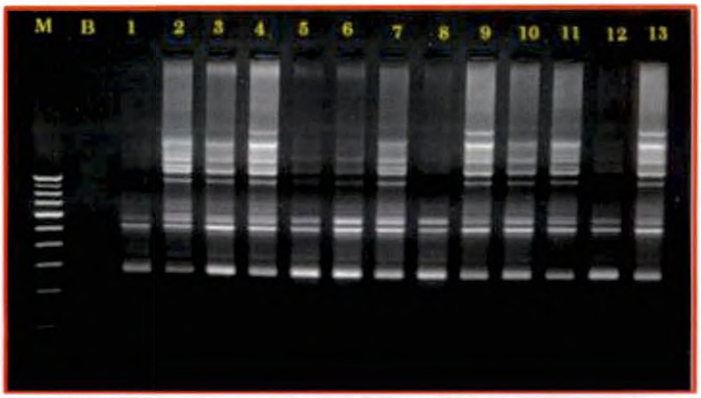
ISSR 05

The ISSR primer ISSR 05 could generate ten clear, distinct and reproducible amplicons ranged in size 400 bp to 1500 bp and six of them were polymorphic (Plate 9. c). The polymorphism per centage calculated was 60.

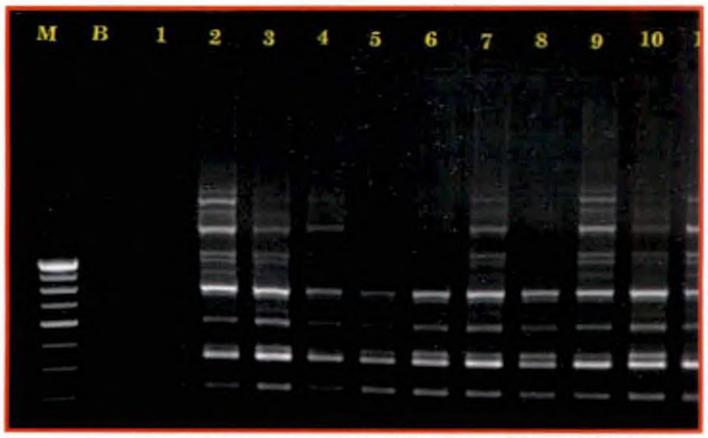
The amplification pattern showed that one amplicon of size 1500 bp was present only in two clones viz. RSe1Kr48 and RSe1Kr1257 and source parent cultivar. One loci of size 1300 bp was present in five somaclones (RSe1Kr48, RSe1Kr188, RSe1Kr78, RSe1Kr1257, RSe1Kr239) and source parent cultivar. One amplicon of size 750 bp was present in all the somaclones except three somaclones viz. RC2Kr 1031, RSe1Kr 648 and RSe1Kr 1052.

4.5.2. Molecular data analysis

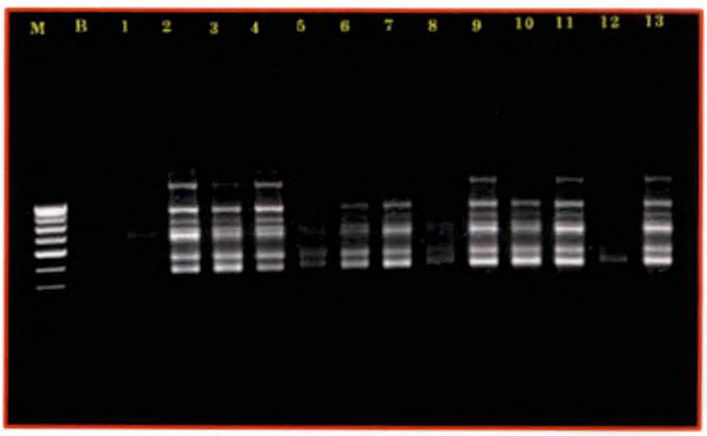
Reproducible, well resolved fragments were scored using Quantity one software (BIORAD) and each scorable band was scored for presence (1) and absence (0).



a. Amplification with RAPD primer OPA 28



b. Amplification with RAPD primer S11



c. Amplification with ISSR primer ISSR 05

M: 100bp ladder / Marker
B: Control
1: RC2Kr 1031
2: RSe1Kr 48
3: RSe1Kr 188
4: RSe1Kr 78
5: RSe1Kr 650
6: RSe1Kr 44
7: RSe1Kr 1053
8: RSe1Kr 648
9: RSe1Kr 1257
10: RSe1Kr 989
11: RSe1Kr 239
12: RSe1Kr 1052
13: Control R

Plate 9. Amplification patterns of ginger somaclones of the groups RC20 Gy and RSe10 Gy with selected primers

The numerical series for the presence or absence of bands was entered into a binary data matrix and used for calculating the similarity coefficient using Jaccard's coefficient (Jaccard, 1908). The mean similarity coefficient for each accession pair was calculated and used for cluster analysis using the UPGMA method and a dendrogram generated using the software package NTSYS pc version 2.02i (Rohlf, 1993). The dendrogram generated using NTSYS is given in Figure 7 and details of clusters are given in Table 20.

The dendrogram generated using NTSYS software grouped the somaclones into two main clusters. Cluster I includes five somaclones (RC2Kr1031, RSe1Kr1052, RSe1Kr650, RSe1Kr648 and RSe1Kr44). Cluster II includes seven somaclones (RSe1Kr49, RSe1Kr1257, RSe1Kr 239, RSe1Kr 188, RSe1Kr 78, RSe1Kr 1053, RSe1Kr 989) and Rio-de-Janeiro source parent cultivar.

Cluster was I divided into two subclusters. RC2Kr1031 and RSe1Kr1052 somaclones formed first subcluster. In second subcluster RSe1Kr650, RSe1Kr648 and RSe1Kr44 were grouped.

Cluster was II divided into two major subclusters. Somaclones RSe1Kr48, RSe1Kr1257, RSe1Kr239 and source parent cultivar (control R) were grouped in first subcluster. In second subcluster, RSe1Kr188, RSe1Kr78, RSe1Kr1053 and RSe1Kr989 were clustered together.

A genetic similarity matrix of individual ginger somaclones of RC20 Gy and RSe10 Gy groups along with the source parent cultivar (control R) using selected primers was also generated (Table 21). The pairwise coefficient values varied between 0.4167 and 1.0000.

The extent of variability of individual somaclones of the groups RC20 Gy and RSe10 Gy from Rio-de-Janeiro source parent cultivar is presented in Figure 8. RC2Kr1031 exhibited higher variability (59 %) followed by RSe1Kr1052 (53%). Two other somaclones viz. RSe1Kr650 and RSe1Kr648 also exhibited higher variability of 48 per cent each and another somaclone RSe1Kr44 exhibited a variability percentage of 37.

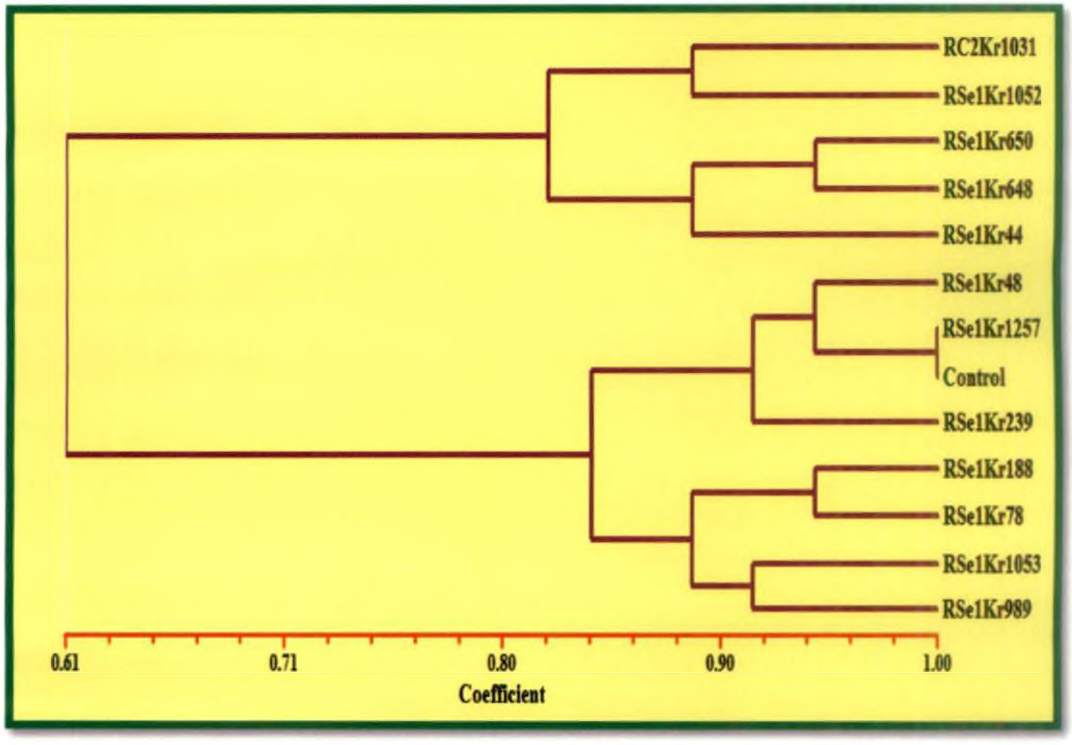


Figure 7. Combined dendrogram generated with RAPD and ISSR profiles for ginger somaclones of the groups RC20 Gy and RSe10 Gy

Table 20. Grouping of ginger somaclones of group RC20 Gy and RSe10 Gy based on RAPD and ISSR data

Cluster number	Number of somaclones in each cluster	Name of somaclones
Cluster I	Five somaclones	RC2Kr 1031, RSe1Kr 1052, RSe1Kr 650, RSe1Kr 648 and RSe1Kr 44
Cluster II	Eight somaclones and source parent cultivar	RSe1Kr 49, RSe1Kr 1257, Rio-de-Janeiro source parent cultivar, RSe1Kr 239, RSe1Kr 188, RSe1Kr 78, RSe1Kr 1053 and RSe1Kr 989

Table 21. Jaccard's similarity coefficient matrix with RAPD and ISSR data in individual ginger somaclones of group RC20 Gy and RSe10 Gy

	RC2Kr 1031	RSe1Kr 48	RSe1Kr 188	RSe1Kr 78	RSe1Kr 650	RSe1Kr 44	RSe1Kr 1053	RSe1Kr 648	RSe1Kr 1257	RSe1Kr 989	RSe1Kr 239	RSe1Kr 1052	Control R
RC2Kr1031	1.0000												
RSe1Kr48	0.4722	1.0000											
RSe1Kr188	0.5278	0.8333	1.0000										
RSe1Kr78	0.5278	0.8333	0.9444	1.0000									
RSe1Kr650	0.8333	0.5833	0.6944	0.6389	1.0000								
RSe1Kr44	0.7222	0.6944	0.7500	0.6944	0.8889	1.0000							
RSe1Kr1053	0.5833	0.8333	0.8889	0.8889	0.6944	0.8056	1.0000						
RSe1Kr648	0.8333	0.5833	0.6389	0.5833	0.9444	0.8889	0.6944	1.0000					
RSe1Kr1257	**0.4167**	0.9444	0.8333	0.8889	0.5278	0.6389	0.8333	0.5278	1.0000				
RSe1Kr989	0.6111	0.8056	0.9167	0.8611	0.7778	0.8333	0.9167	0.7222	0.7500	1.0000			
RSe1Kr239	0.5000	0.9167	0.9167	0.9167	0.6111	0.7222	0.9167	0.6111	0.9167	0.8333	1.0000		
RSe1Kr1052	0.8889	0.5278	0.5833	0.5833	0.8889	0.7778	0.6389	0.8889	0.4722	0.6667	0.5556	1.0000	
Control R	**0.4167**	0.9444	0.8333	0.8889	0.5278	0.6389	0.8333	0.5278	**1.0000**	0.7500	0.9167	0.4722	1.0000

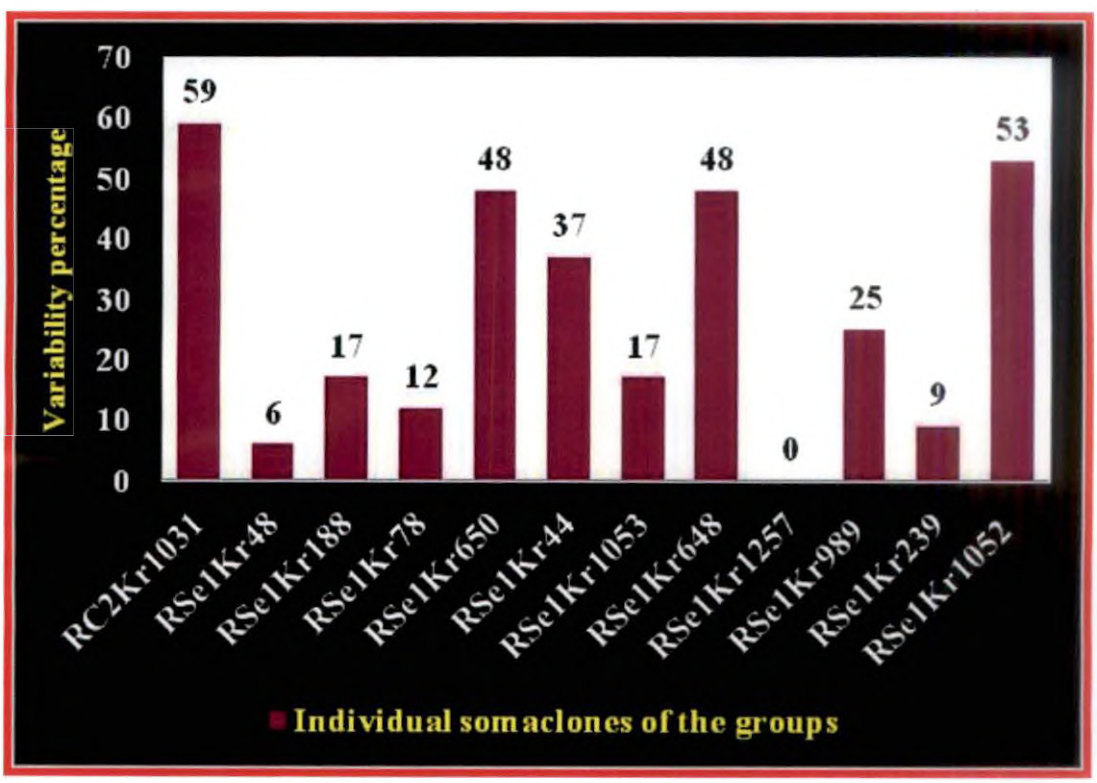


Figure 8. Variability in individual somaclones of the group RC20 Gy and RSe10 Gy

So the variability exhibited by individual somaclones from source parent cultivar was found very high (39%) as compared to groupwise analysis (25%) (Fig. 7 and Fig. 3).

The present investigations on variability analysis in ginger somaclones using molecular markers could assess the variability in somaclones of two cultivars viz. Maran and Rio-de-Janeiro regenerated through various modes of regeneration and subjected to various doses of irradiation.

Molecular analyses with two marker systems viz. RAPD and ISSR showed that somaclones derived from cultivar Maran exhibited more variability than Rio-de-Janeiro. RAPD marker system was more effective for bringing out variability. The variability observed in RAPD assay was 28 per cent while in ISSR assay it was 21 per cent and in the combined it was 25 per cent.

Of the various modes of regeneration studied, irradiated callus and somatic embryogenic regenerants showed more variability. In both the marker systems, the groups RC20 Gy and RSe10 Gy recorded higher variability from the source parent cultivar.

Callus regenerants of the irradiated group exhibited more variability as compared to somatic embryo regenerants. The clones regenerated subjected to irradiation dose of 20 Gy exhibited more variability than 10 Gy.

The groupwise variability analysis using bulked DNA gave an indication of the overall variability of the group from the source parent cultivar. However, the individual plant analysis of the variable groups gave a clear picture of the extent of variability from source parent cultivar. The variability exhibited in plantwise analysis using selected three primers (OPA 28, S11 and ISSR 05) was found very high (39%) as compared to groupwise analysis (25%). The somaclone RC2Kr1031 of the callus regenerants and RSe1Kr1052 of the somatic embryo regenerants showed more variability exhibiting 59 and 53 per cent variability respectively from source parent cultivar Rio-de-Janeiro.

Discussion

5. DISCUSSION

Ginger (*Zingiber officinale* Rosc.) is one of the major spice crops and India accounts for 50 per cent of the worlds ginger production. The ginger family is a tropical group especially abundant in Indo-Malaysia, consisting of more than 1200 plant species in 53 genera. The genus *Zingiber* includes about 85 species of aromatic herbs from East Asia and Tropical Australia. It is used as a spice on a large scale and in the preparation of medicines and confectionaries.

Zingiber officinale Rosc. belongs to the family Zingiberaceae and in the natural order Scitaminae. Several cultivars are grown and are known by the particular geographical area where they are cultivated commercially. The important cultivars could be classified as high yielding, less fiber, high oleoresin, high dry ginger and high volatile oil types.

In India, most of the popular commercial varieties are clonal selections from traditional cultivars. Breeding in ginger is seriously handicapped by poor flowering and seed set. Most of the crop improvement programmes of this species are confined to evaluation and selection of naturally occurring clonal variations (Rout *et al.*, 1998; Palai and Rout, 2007). In such species, the extent of genetic diversity is low, unless samples are drawn from diverse agro-ecological conditions (Ravindran *et al.*, 2005). Therefore, diversity analysis and identification of genetically distant clones or genotypes are very important for any ginger improvement programmes. Because of the medicinal value of ginger (*Z. officinale*), much of the available documented information are on its biochemical aspects (Singh *et al.*, 2000; Wohlmuth *et al.*, 2006). Genetic diversity analysis based on morphological characters is not always reliable. Disagreement between germplasm classification based on morphological characters and molecular data is reported in literature (Wahyuni *et al.*, 2003). Use of molecular markers for the estimation of genetic variability gives better insight into the similarity / differences at the genetic level. Several molecular markers viz., RFLP, AFLP, RAPD, ISSR and SSR have been extensively used for the variability analysis in ginger.

For crops like ginger where molecular information is limited, RAPD markers are the markers of choice because of their simplicity in use and low cost. RAPD markers have been widely used to assess the genetic diversity and phylogenetic relationship in different crop species (Isabel *et al.,* 1993; Rani *et al.,* 1995; Mimura *et al.,* 2000; Da Costa *et al.,* 2006). Using phylogenetic analysis and metabolic profiling, Jiang *et al.* (2006) studied diversity within and among *Zingiber* species and found that *Z. officinale* from different geographical origins were indistinguishable. Wahyuni *et al.* (2003) used AFLP markers to assess genetic diversity in morphologically distinct Indonesian ginger types and reported that there were no clear genetic differentiations between small and big type morphological variants of gingers.

5.1 Isolation, purification and quantification of the genomic DNA

Grinding in liquid nitrogen was found to improve the quality of DNA isolated. The addition of antioxidant like β-mercaptoethanol and sodium metabisulfite in the extraction buffer or during grinding was found effective for removal of phenolic compounds. Similar result was reported by Rogers and Bendich (1994) and (Ram and Sreenath, 1999) in coffee, which is a crop with high phenols.

The detergent used in the extraction buffer of the protocol is CTAB (Cytyl 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 action as a selective precipitant of nucleic acids. CTAB is cationic detergent, which solubilises membrane and form a complex with DNA (Sghaier and Mhammed, 2005).

The CTAB was found superior in the present study. The advantageous effect of the CTAB along with PVP on the quality of DNA was also reported by Roger and Bendich (1994), Gallego and Martinez (1996) and (Ram and Sreenath, 1999). It effectively disrupts the cell membrane and together with NaCl separates the polysaccharides. 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 percent alcohol followed by absolute alcohol removes the traces of CTAB, salt TE buffer rehydrates the DNA and dissolves it (Rogers and Bendich, 1994; Wettasingf and Peffley, 1998).

Problem 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). EDTA present in the extraction buffer could protect the DNA from the action of DNase enzyme by chelating and blocking the action of mg^{2+}ions, which are the major co-factor of DNase enzyme. EDTA was also major component of TE buffer in which the DNA dissolved and stored.

RNase treatment was given in order to remove RNA contamination from the isolated DNA samples isolated using CTAB method similar to the result of Sambrook *et al.* (1989).

Genomic DNA isolated by Sigma's GenElute™ Plant Genomic DNA Miniprep kit was pure without RNA contamination (Plate 4) but the quantity of DNA recovered was less compared to CTAB method as only 100 mg leaf sample was used for DNA isolation.

The quality of DNA was tested by subjecting it to agarose gel electrophoresis as well as by spectrophotometric method. In the former, the DNA was visualized on 0.8 percent agarose gel under UV light by ethidium bromide staining. The stain was added directly to the melted agarose before casting the gel in order to have a better resolution. Nanda and Jain (1994) also reported similar results.

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 (Wettasingf and Peffley, 1998). The DNA extracted showed high amount of RNA as a smear below it. To remove RNA, RNase A was used. Use of RNase A was reported by Raval *et al.* (1998); Wettasingf and Peffley (1998) and Gallego and Martinez (1996). In present

investigations, the RNase treated DNA sample when electrophoresed showed a high molecular weight DNA, which formed a single band just below the well. This indicted that the DNA under test was of good quality (Plate 3b).

In spectrophotometer method, the ratio of optical density at 260 and 280 nm was worked out to test the quality. The absorbance ratio was calculated as OD at 260/280, for the various samples. Those samples with ratio between 1.8 and 2.0 were considered to be of high quality. All the samples recorded a ratio between 1.8 and 2.0 (Table 5 and Table 6).

Quantification of the DNA on the basis of UV absorbance at 260nm indicated substantial yield of DNA from the tissue samples. Spectrophotometric determination of concentration of DNA was also reported by Gallego and Martinez (1996) and Ram and Sreenath (1999).

5.2. Bulking of genomic DNA

Good quality genomic DNA from one eighty somaclones of ginger was used for group wise bulking. Bulk samples of the thirteen groups of the one eighty somaclones along with two source parent cultivars were subjected to RAPD and ISSR analyses with selected primers. Dulson *et al.* (1997) examined the usefulness of bulking equal quantities of DNA from 14 to 20 individuals in cultivar identification with RAPD DNA markers. Michelmore *et al.* (1991) reported the RAPD analysis using bulked DNA samples. Kolliker *et al.* (2001) reported use of bulked leaf samples from individual plants for amplified fragment length polymorphism (AFLP) analysis and evaluated it as a tool for assessment of genetic diversity in white clover (*Trifolium repens* L.).

5.3. Molecular marker analysis

Molecular markers have been proved to be a fundamental and reliable tool for fingerprinting varieties, establishing the fidelity of progenies, germplasm characterization, and detecting somaclonal variation etc. Molecular markers provide an important technology for evaluating levels and patterns of genetic variability and

have been utilized in a variety of plant species. Molecular markers which detect variation at the DNA level overcome most of the limitations of morphological and biochemical markers. Molecular markers are independent of developmental stages of the crop and are not influenced by the varying environmental conditions. Hence it is preferred for variability analysis than traditional morphological markers (Spooner *et al.*, 2005).

Most of the molecular marker are developed by PCR (Polymerase Chain Reaction) technology and amplifies unique regions in the genomic DNA based on the primers designed for DNA amplification. In present study, two such PCR based marker systems, RAPD and ISSR were utilized for variability analyses in ginger somaclones regenerated through various mode of regeneration.

5.3.1 RAPD assay of ginger somaclones

The advent of automated PCR technology made a new set of markers available to scientist interested in comparing organisms at molecular level. Williams *et al.* (1990) were the first to use RAPD markers 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. Technical problems associated with application of RAPD technique in the field of genetic variation research have been reported by many workers (Lynch and Milligan, 1994; Rajput *et al.*, 2006). Use of high quality DNA is shown to be a key factor in obtaining reproducible RAPDs bands (Penner *et al.*, 1993). In the present study the use of high quality DNA helped in getting reproducible bands using the standardised conditions for the thermal cycler.

However, the advantages of RAPD include simplicity, rapidity, requirement for only a small quantity of DNA, and ability to generate numerous polymorphisms (Cheng *et al.*, 2007). The RAPD amplification can be classified into two types: constant (monomorphic) and vriable (polymorphic) between the genotypes. These

differences can be used to examine and establish systematic relationship (Hadrys *et al.*, 1992).

RAPD marker system for assessment of somaclonal variation was attempted in several crops. RAPD markers could detect somaclonal variation in beet (Munthali *et al.*, 1996), garlic (Zahim *et al.*, 1999), date palm (Saker *et al.*, 2000), banana (Gimenez *et al.*, 2001 and Mohamed, 2007), tomato (Soniya *et al.*, 2001) and potato (Ehsanpour *et al.*, 2007) in varying degrees. Paul (2004) characterized selected somaclones in ginger using RAPD markers and detected somaclonal variation in the clones evaluated.

5.3.1.1 DNA amplification conditions for RAPD

The amplification conditions standardized in the present study were suited to Mastercycler personal thermalcycler from Eppendorf (USA). Also, the anneling temperature was identified as the most critical with respect to number of amplified fragments and reproducibility of result. Cipriani *et al.* (1996) and Erlich *et al.* (1991) suggested 37°C as the best, and hence only time was varied for this step.

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

5.3.1.2. Screening of RAPD primers

Thirty-five decamer primers in the series of OPA, OPC, OPD, OPG, OPK, OPE, OPP, OPU, OPAH, (Operon Technologies, USA), S, RN (Reverse Neo), RY were screened for RAPD analysis using good quality genomic DNA bulked from RVIII, MSe1Kr200 and MC1Kr168 (Plate 5 and Table 7).

Out of 35 decamer primers twelve decamer primers (Table 8) which showed good amplification pattern were selected for RAPD assay of thirteen groups of ginger somaclones and their original source parent cultivars.

5.3.1.3. Variability analysis in ginger somaclones with selected RAPD primers

The RAPD markers generated using the selected primers were visualised by electrophoresis, in a two per cent agarose gel stained with ethidium bromide (Plate 6a and 6b).

The selected primers produced clear and distinct amplification pattern (Table 9) with the thirteen groups of ginger somaclones and their source parent cultivars selected for the investigation. There were 129 amplicons of which 44 were polymorphic giving a polymorphism of 34.10 per cent. The number of amplicons produced ranged from eight to thirteen with an average of 10.75 markers per primer and a mean of 3.66 polymorphic bands per primer. Paul *et al.* (2012) in the study of molecular characterization of selected somaclones in ginger using RAPD markers reported a total of 494 RAPD band positions, out of which 154 were polymorphic. Percent polymorphism exhibited by primers ranged from zero to 33.75 per cent. Kizhakkayil and Sasikumar (2010) reported a total of 269 scorable bands, out of which 126 were polymorphic in the study of genetic diversity analysis of ginger (*Zingiber officinale* Rosc.) germplasm. Sajeev *et al.* (2011) reported in the genetic diversity analysis of ginger clone a total of 109 fragments amplified, 101 (91.87%) were polymorphic and an average of 6.05 amplified fragments were produced per primer. Palai and Rout (2007) reported a total of 55 fragments were scored across eight varieties of ginger using selected primers, out of which only 25 fragments (45.5%) were polymorphic. Similar polymorphism was reported by Prakash *et al.* (2004) in mango ginger when they assessed variability in regenerants produced through indirect organogenesis.

Of the twelve primers studied, four gave polymorphism of above 40 per cent. The primer OPA 28 gave the highest polymorphism of 54.54 per cent. The polymorphism information content (PIC) of selected primers ranged from 0.87 (OPA

04) to 0.92 (RN 08 and OPAH 03) with a mean of 0.90 (Figure 6a). The Resolving power (Rp) calculated for the 12 random primer ranged between 15.5 (OPA 04) and 23.3 (OPAH 03) with an average of 19.98 for RAPD primers (Figure 5a). This shows the effectiveness of selected RAPD primers in variability analysis.

Paul *et al* (2012) reported five primers of the series OPAH and OPP gave good DNA amplification in selected somaclones of ginger. The primer OPAH 3 exhibited the highest polymorphism percentage of 33.75.

In the present investigations, the amplicons produced by selected random primers had a molecular weight ranging from 150 bp to 1800 bp. Sajeev *et al.* (2011) reported band size in the range of 300 to 2500 bp when they analysed genetic diversity in forty nine ginger clones cultivated in North East India. Similarly, Palai and Rout (2007) reported RAPD fragments ranging in size from 500 to 2400 bp when they assessed genetic variation in eight high yielding varieties using twelve selected primers.

In the present study, RAPD assay showed clear and distinct variation between two ginger cultivars (Maran and Rio-de-Janeiro). Among the selected RAPD primers, OPA 12, OPA 27 OPU 03 and OPAH 03 produced unique amplicons which was present only in the Maran groups. The primer S11 produced an amplicon present only in Rio-de-Janeiro groups. So the RAPD primers OPA 12, OPA 27, OPU 03, S11 and OPAH 03 could be utilized for identification of Maran and Rio-de-Janeiro cultivars. Similarly, to separate the irradiated group from non-irradiated group, primer OPA 12 could be employed as it produced unique amplicons only in irradiated groups of Maran and Rio-de-Janeiro. Further, the primer OPU 03 produced an amplicon specific to irradiated mutants of Maran clones which was absent in Rio-de-Janeiro clones.

5.3.1.4. Genetic distance between somaclones based on RAPD data

The binary data matrix was used for calculating the similarity coefficient using Jaccard's coefficient (Jaccard, 1908). The mean similarity coefficient was

calculated and used for cluster analysis using the UPGMA method and a dendrogram generated using the software package NTSYS pc version 2.02i (Rohlf, 1993).

The dendrogram generated using NTSYS grouped the somaclones into two main clusters. Cluster I includes eleven groups of somaclones (MB, MC, MSe, MC10 Gy, MC20 Gy, MSe10 Gy, MSe20 Gy, RB, RC, RSe, RC10 Gy) and two source parent cultivars. Rio-de-Janeiro regenerants from calli irradiated with 20 Gy and somatic embryo regenerants from embryogenic calli irradiated with 10 Gy formed the second main cluster.

Cluster I was divided into two major sub clusters, with somaclones of Maran in first subcluster and somaclones of Rio-de-Janeiro in second subcluster (Figure 1).

A genetic similarity matrix of different groups of ginger somaclones based on the proportion of shared RAPD fragments was also generated (Table 11). The pairwise coefficient values varied between 0.6589 and 1.0000. Kizhakkayil and Sasikumar (2010) reported the genetic similarity coefficients (Jaccard's) in the range of 0.76-0.97 when they studied genetic diversity in ginger (*Zingiber officinale* Rosc.) germplasm. Sajeev *et al.* (2011) reported a Jaccard's similarity coefficient of 0.57-0.96, Palai and Rout (2007) reported the similarity matrix value was ranged from 0.34 to 0.74, with a mean value of 0.54 when they assessed variability in ginger germplasm.

Dendrogam separated the somaclones derived from cultivar Maran and Rio-de-Janeiro into two separate groups. In the cluster for somaclones derived from cultivar Maran all the groups of somaclones irradiated with γ rays showed more difference from rest of the groups. The extent of variability in somaclones from the source parent cultivar Maran was found more in MC20 Gy (5.4%) followed by MSe20 Gy (4%), MSe10 Gy (2.5%), MC10 Gy (2.5%) Maran bud (2.5%) and callus regenerants (2.5%) while somatic embryo regenerants without irradiation showed less variability (1.5%).

In Rio-de-Janeiro, the highest variability was recorded by RC20 Gy (28%) followed by RSe10 Gy (22%). However, the indirect organogenic and embryogenic regenerants without irradiation showed less variability (1.1%) as compared to irradiated groups.

Among all the groups of somaclones, RC20 Gy and RSe10 Gy groups were found to be most distant ones from the rest of the groups of somaclones. This has been attributed by the varying mode of regeneration and irradiation with γ rays of both the groups.

5.3.2. Inter Simple Sequence Repeat (ISSR) analysis

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

ISSR is a multilocus molecular technique based on PCR that identifies insertions and deletions in DNA. It is highly sensitive, highly reproducible, provides Mendelian segregation and has been successfully applied in genetic and evolutionary studies of many species, including ginger. The ISSR marker requires small amounts of DNA and does not require information on DNA sequences. ISSR targets the highly variable microsatellite regions of the nuclear genome providing a large number of polymorphic fragments (Gupta *et al.,* 1994). Therefore this technique has been used extensively to evaluate genetic diversity both within and between plant populations in angiosperms and gymnosperms (Oshborn *et al.,* 2005).

5.3.2.1. DNA amplification conditions for ISSR

Both the proportion of components in the reaction mixture and the thermal profile used for ISSR assay gave good amplification of ginger samples. Good quality genomic DNA was used for ISSR analysis.

5.3.2.2. Screening of ISSR primers

Thirty primers, 20 belonging to UBC (University of British, Columbia) series, eight to ISSR (ISSR Technologies, USA) series and two to SPS series were screened for ISSR analysis using good quality genomic DNA bulked from three somaclones viz. RVIII, MSe1Kr200 and MC1Kr168 (Plate 7 and Table 12).

Of 30 ISSR primers screened in the present study, twelve ISSR primers which showed good amplification pattern were selected for ISSR assay of thirteen groups of ginger somaclones with their original source parent cultivars (Table 13). Nayak *et al.* (2005) also reported the use of RAPD primers OPA 02 and OPD 20 for distinguishing 16 promising cultivars of ginger.

5.3.2.3. Variability analysis in ginger somaclones with selected ISSR primers

The selected ISSR primers produced a total of 122 amplicons of which 32 were polymorphic giving a polymorphism of 26.23 per cent with an average of 10.16 markers per primer and a mean of 2.66 polymorphic bands per primer (Table 14). Mohanty *et al.* (2012) reported a total 66 bands were produced with an average of 8.3 bands per primer when they assessed stability of mango ginger through RAPD and ISSR markers. Kizhakkayil and Sasikumar (2010) reported a total of 160 scorable bands out of which 76 were polymorphic in the study of genetic diversity analysis of ginger (Zingiber officinale Rosc.) germplasm. Prem *et al.* (2008) in the study of molecular characterization of ginger genotypes reported out of 14 primers studied maximum (62.5%) polymorphism was observed in case of (GA)8T and seven primers produced unique bands. Ahmed *et al.* (2012) reported a total number of 780 clear DNA bands with an average of 48 bands per primer in the ISSR analysis of

somaclonal variation in date palm plantlets regenerated from callus using 16 single primers

Out of the twelve primers studied, the primer ISSR 05 gave the highest polymorphism of 40 per cent. The polymorphism information content (PIC) of selected primers ranged from 0.69 (ISSR 06) to 0.92 (ISSR 04 and UBC 835) with an average of 0.87 (Figure 6b). ISSR primers recorded Rp values ranged from 11.7 (UBC 844) and 25.5 (ISSR 04) with an average 19.13 (Figure 5b).

The amplicon size in the present investigation ranged from the molecular weight of 100 bp to 1400 bp. A similar range of amplicon size (250bp to 2900 bp) was reported by Mohanty *et al.*, 2012.

ISSR assay showed variation between somaclones derived from two ginger cultivars (Maran and Rio-de-Janeiro). Among selected ISSR primers, four primers (UBC 835, SPS 03, ISSR 05 and ISSR 06) produced unique amplicon which was present only in the Maran somaclones. The primer UBC 840 produced an amplicon which was present only in Rio-de-Janeiro group. So the ISSR primers also could be utilized for identification of Maran and Rio-de-Janeiro cultivars.

5.3.2.4. Genetic distance between somaclones based on ISSR data

A binary data matrix was used for calculating the similarity coefficient using Jaccard's coefficient (Jaccard, 1908). The mean similarity coefficient was calculated and used for cluster analysis using the UPGMA method and a dendrogram generated using the software package NTSYS pc version 2.02i (Rohlf, 1993).

The dendrogram generated using NTSYS grouped the somaclones into two main clusters. Cluster I includes eleven groups of somaclones and two source parent cultivars. Rio-de-Janeiro regenerants from calli irradiated with 20 Gy and somatic embryo regenerants from embryogenic calli irradiated with 10 Gy formed the second main cluster.

Cluster I was divided into two major sub clusters. Somaclones of cultivar Maran were grouped in first sub cluster and somaclones of Rio-de-Janeiro were grouped in second sub cluster (Figure 2). This is in confirmation with the RAPD data.

A genetic similarity matrix of different groups of ginger somaclones based on the proportion of shared ISSR fragments was also generated (Table 16). The pairwise similarity coefficient values varied between 0.7603 and 1.0000 in the present investigation. Kizhakkayil and Sasikumar (2010) reported genetic similarity coefficients (Jaccard's) in RAPD and ISSR assay of ginger germplasm in the range of 0.76-0.97.

The extent of variability in somaclones from the source parent cultivar Maran was found more in MC20 Gy (5.8%) followed by MSe20 (4.1%), MC10 Gy (3.3%) and MSe10 Gy (2.5%). However, callus and somatic embryo regenerants showed less variability.

In Rio-de-Janeiro somaclones the highest variability was recorded in RC20 Gy (19%) followed by RSe10 Gy (18%). However, bud, callus and somatic embryo regenerants were found similar to source parent cultivar.

5.3.3. Variability analysis based on the two markers (RAPD and ISSR) data

A total of 251 markers were produced by the amplification of thirteen groups of ginger somaclones and their source parent cultivars by RAPD and ISSR assay with an average of 10.45 markers per each primer. The total polymorphic bands were 30.27 per cent, each primer detecting on an average 3.16 polymorphic bands per primer.

Using NTSYS, ginger somaclones were grouped into two main clusters. RAPD and ISSR markers successfully separated Maran and Rio-de-Janeiro groups of somaclones in two major sub clusters. Cluster I includes all groups of somaclones except RC20 Gy, RSe10 Gy, these two groups formed Cluster II (Figure 3). This is in conformity with the clustering pattern obtained by RAPD and ISSR assay separately.

A genetic similarity matrix of different groups of ginger somaclones showed that the pairwise similarity coefficient values ranged from 0.7092 and 0.9960 (Table 18). The extent of variability in somaclones from the source parent cultivar was found more in MC20 Gy (5.6%) followed by MSe20 Gy (4%), MC10 Gy (3%) and MSe10 Gy (2.5%). However, bud, callus and somatic embryo regenerants of the cultivar Maran showed less variability (2%) as compared to irradiated groups.

In the case of Rio-de-Janeiro somaclones, the highest variability was recorded by RC20 Gy (24%) followed by RSe10 Gy (21%). In Rio-de-Janeiro somaclones also, the irradiated group exhibited more variability as compared to non-irradiated groups (Figure 4).

5.3.4. Comparison of RAPD and ISSR marker systems

The average number of markers produced per primer in each marker system viz. RAPD and ISSR were 10.75 and 10.16 respectively. The number of polymorphic amplicons detected per primer in RAPD and ISSR marker system was 3.66 and 2.66 respectively. The extent of genetic variation was more in somaclones of cultivar Maran than clones of cultivar Rio-de-Janeiro. Genetic variation from the source parent cultivar was also more in somaclones of cultivar Maran. Paul *et al.* (2012) also reported similar result when she did molecular characterization of selected somaclones in ginger using RAPD markers.

On the basis of various modes of regeneration viz. bud culture, indirect organogenesis / embryogenesis and *in vitro* mutagenesis, irradiated callus and somatic embryo regenerants showed more variability. Araujo *et al.*, 2001 reported that polymorphism was detected in banding pattern in regenerants of upland rice cultivar IAC 47 regenerated through indirect organogenesis and somaclones showed morphological variants and exhibited differences in reaction to rice blast. Afrasiab and Iqbal (2012) analyzed somaclonal variants and gamma induced mutants of potato (*Solanum tuberosum* L.) cv. Diamant using RAPD-PCR technique. The study showed that RAPD markers were efficient in discriminating somaclonal variants and induced mutants of potato.

It is clear that RAPD marker system is better than the ISSR marker system with respect to total number of markers and polymorphic markers detected. This is due to the ability of RAPD primers to amplify random regions of the genome. So for genetic variability analysis, RAPD marker system gives better result than ISSR. Suitability of RAPD markers in diversity analysis of ginger was also reported by Rout *et al.*, 1998; Nayak *et al.*, 2005; Palai and Rout, 2007; Paul *et al.*, 2012.

The dendrogram as well as genetic similarity values calculated from the similarity matrix obtained by each marker system was found different. The dendrogram derived from combined molecular data revealed better representation of the relationships than individual markers. The dendrogram obtained by RAPD and ISSR systems showed some differences. This may be because RAPD and ISSR techniques use two different approaches to identify DNA sequence variations. RAPD identifies random regions of the genome, while ISSR identifies insertions and deletions in the DNA sequence.

5.3.5. Variability analysis in ginger somaclones of groups RC20 Gy and RSe10 Gy

Initially, amplification of bulked DNA samples from each of the thirteen groups of ginger somaclones and their source parent cultivars was carried out using selected primers of each marker system. Amplification of thirteen groups of ginger somaclones and their source parent cultivars could reveal noticeable variability in RC20 Gy and RSe10 Gy among all the groups of somaclones studied. This may be due to the mode of regeneration and effect of γ irradiation on *in vitro* multiplication.

The groupwise variability analysis using bulked DNA gave an indication of the extent of variability of the group from source parent cultivars. Hence RC20 Gy and RSe10 Gy groups were focused for further investigations. DNA from each of these twelve somaclones (one of RC20 Gy and eleven of RSe10 Gy groups), were used for further variability analysis.

Two RAPD (OPA 28 and S11) and one ISSR (ISSR 05) primers which recorded highest polymorphism were used to amplify the DNA of individual

somaclones in RC20 Gy and RSe10 Gy groups along with Rio-de-Janeiro source parent cultivar (Control R) (Plate 9).

The number of polymorphic amplicons detected by OPA 28, S 11 and ISSR 05 primer were 9, 9 and 6 respectively. Hence polymorphism percentage recorded by OPA 28, S 11 and ISSR 05 primer was 64.28, 69.23 and 60 per cent respectively (Table 19). Variability exhibited in plantwise analysis using the three selected primers was thus very high (39%) as compared to groupwise analysis (25%). Similar observations were reported by Fu *et al.* (2003) when they assessed effectiveness of several bulking strategies in detecting RAPD variations in flax (*Linum usitatissimum* L.). In the study they observed that about 30 per cent of the polymorphic RAPD loci observed in plant-by-plant analysis were undetected in the bulked samples of the same accession.

Using NTSYS, individual ginger somaclones were grouped into two main clusters (Figure 7). From individual somaclones analysis five somaclones exhibiting more variability from source parent cultivar could be isolated (37% to 59%). Two somaclones RC2Kr1031 of the callus regenerants and RSe1Kr1052 of the somatic embryo regenerants showed more variability exhibiting 59 and 53 per cent variability respectively from source parent cultivar Rio-de-Janeiro (Figure 8).

The mutants which exhibited more variability from source parent cultivar could be further evaluated for their desirable traits. The groups of somaclones which exhibited more variability in the present study were due to the absence of amplicons in the various molecular marker systems analysed. This may be due to the chromosome aberration and rearrangements, DNA methylation or histone modification as explained in somaclones and mutants by several workers (Larkin and Scowcroft, 1981; Skirvin and Janick, 1996; Bairu *et al.*, 2011).

The Polymorphic Information Content (0.88 to 0.91) and resolving power (14.92 to 19.69) of the selected primers was found high.

In the crops like ginger where natural variability is less, the present investigation could broaden the genetic base.

From the present investigations the following conclusions could be drawn:

- Molecular marker techniques could be employed for the assessing the variability in ginger somaclones.
- Of the two marker system studied, RAPD was more effective for bringing out variability. The variability observed in RAPD assay was 28 per cent while in ISSR it was 21 per cent and in the combined it was 25 per cent.
- The study could identify certain specific RAPD and ISSR markers for identification of Maran and Rio-de-Janeiro cultivars and also irradiated mutants from non-irradiated somaclones.
- The somaclone derived from cultivar Maran exhibited more variability than somaclones of Rio-de-Janeiro.
- Irradiated callus and somatic embryo regenerants showed more variability
- The groupwise variability analysis using bulked DNA gave an indication of the extent of variability of the group from source parent cultivars.
- The variability exhibited in plantwise analysis of the selected variable groups was found high and two plants with high variability could be selected.
- The somaclone RC2Kr1031 of the callus regenerants and RSe1Kr1052 of the somatic embryo regenerants showed higher variability exhibiting 59 and 53 per cent variability respectively from source parent cultivar Rio-de-Janeiro.
- In crops like ginger where the natural variability is very less, *in vitro* mutagenesis could be employed for widening the genetic base in ginger.

Molecular marker analysis of individual plants of the variable groups, use of advanced marker systems for assessment of somaclonal variation, use of more number of primers to bring out variability and more focus on *in vitro* mutagenesis for widening the genetic base in ginger are the future areas to be investigated.

Summary

6. SUMMARY

The investigations entitled "variability analysis in ginger (*Zingiber officinale* Rosc.) somaclones using molecular markers" were carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Kerala Agricultural University, Thrissur during the period February 2012 to May 2013, to assess somaclonal variation in ginger at molecular level, to study the influence of genotype and mode of regeneration on somaclonal variation to assess the extent of variability in somaclones from the original source parent cultivars and to select out the variants.

Ginger somaclones (180 Nos.) regenerated through various modes of regeneration viz. bud culture, indirect organogenesis / embryogenesis and *in vitro* mutagenesis, along with two source parent cultivars (Maran and Rio-de-Janeiro) maintained as potted plants in net house of CPBMB, College of Horticulture, were used for the present study.

The salient findings of the study are summarised below:

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

2. DNA isolated using Sigma's GenElute^{TM} Plant Genomic DNA Miniprep kit was found pure without RNA contamination.

3. The quality and quantity of DNA was analysed by NanoDrop^{R} ND-1000 spectrophometer. The absorbance ratio ranged from 1.8 to 2.0 for OD_{260}/OD_{280} which indicated good quality of DNA. The recovery of DNA was sufficient for RAPD and ISSR analyses.

4. Bulking of DNA samples was attempted as per the procedure reported by Dulson *et al.* (1997).

5. Bulked DNA samples from thirteen groups of ginger somaclones along with their source parent cultivars were subjected to the molecular marker analysis. Two marker systems viz. Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeats (ISSR) were utilized for the analyses.

6. A total of 35 RAPD primers and 30 ISSR primers were screened for their ability to amplify DNA fragments. Out of these, twelve RAPD and twelve ISSR primers were selected for the analysis based on the banding pattern.

7. Random Amplified Polymorphic DNA (RAPD) analysis with twelve selected primers produced 129 amplicons, 44 were polymorphic with an average of 3.66 polymorphic bands / primer and a polymorphism percentage of 34.10. The highest polymorphism percentage was recorded by the primer OPA 28 (54.54). The Polymorphism Information Content (PIC) of selected primers ranged from 0.87 (OPU 03) to 0.92 (OPA 28 and OPAH 03) with a mean of 0.90. The Resolving power (Rp) for the twelve random primers ranged from 15.5 (OPA 04) to 23.3 (OPAH 03) with an average of 19.98.

8. In ISSR assay, twelve selected primers produced total of 122 amplicons, 32 were polymorphic with an average of 2.66 polymorphic bands / primer and a polymorphism percentage of 26.23. The highest polymorphism percentage was recorded by the primer ISSR 05 (40). Polymorphic Information Content ranged from 0.69 (ISSR 06) to 0.92 (ISSR 04 and UBC 835) with an average of 0.87. Resolving power ranged from 11.7 (UBC 844) to 25.5 (ISSR 04) with an average of 19.13.

9. The study could identify certain specific RAPD and ISSR primers for identification of Maran and Rio-de-Janeiro cultivars and also irradiated mutants from non-irradiated somaclones. The RAPD primers OPA 12, OPA 27, OPU 03, S11 and OPAH 03 could be utilized for identification of Maran and Rio-de-

Janeiro cultivars. To separate the irradiated group from non-irradiated group, primer OPA 12 could be employed as it produced unique amplicons only in irradiated groups of Maran and Rio-de-Janeiro. The primer OPU 03 could be employed to separate Maran irradiated mutants from Rio-de-Janeiro mutants.

10. The ISSR primers (UBC 835, SPS 03, ISSR 05 and ISSR 06) produced unique amplicon which only in the Maran somaclones. The primer UBC 840 produced an amplicon which was present only in Rio-de-Janeiro group. So the ISSR primers also could be utilized for identification of Maran and Rio-de-Janeiro cultivars.

11. Dendrogram generated based on the RAPD and ISSR profiles grouped the somaclones into two separate clusters, with somaclones of Maran in first subcluster of cluster I and somaclones of Rio-de-Janeiro in second subcluster of cluster I. The regenerants from Rio-de-Janeiro calli irradiated with 20 Gy and somatic embryogenic calli irradiated with 10 Gy formed the second main cluster.

12. The RAPD marker system was more effective for bringing out variability. In RAPD assay variability observed was 28 per cent while in ISSR assay variability was 21 per cent and in the combined system it was 25 per cent.

13. Somaclones derived from cultivar Maran exhibited more variability than somaclones of cultivar Rio-de-Janeiro.

14. Of the various modes of regeneration studied, irradiated callus and somatic embryogenic regenerants showed more variability.

15. In both marker systems, the groups RC20 Gy and RSe10 Gy recorded higher variability from source parent cultivar.

16. The groupwise variability analysis using bulked DNA gave an indication of the overall variability of the group from source parent cultivar.

17. The individual plant analysis of the variable groups gave a clear picture of the extent of variability from source parent cultivar. The variability exhibited in plantwise analysis using selected three primers (OPA 28, S11 and ISSR 05) was found very high (39%) as compared to groupwise analysis (25%).

18. The somaclone RC2Kr1031 of callus regenerants and RSe1Kr1052 of the somatic embryo regenerants showed more variability exhibiting 59 and 53 per cent variability respectively from the source parent cultivar Rio-de-Janeiro.

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Annexures

ANNEXURE I

Reagents required for DNA isolation

Reagents:

1. **2x CTAB extraction buffer (100 ml)**

CTAB	:	2 g
(Cetyl trimethyl ammonium bromide)		
Tris HCl	:	1.21 g
EDTA	:	0.745 g
NaCl	:	8.18 g
PVP	:	1.0 g

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

2. **CTAB (10 per cent, 100 ml)**

CTAB	:	10 g
NaCl	:	4.09 g

Dissolved in autoclaved distilled water

3. **Chloroform- isoamylalcohol (24:1)**

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

4. **Chilled isopropanol**

Isopropanol was stored in refrigerator at 0°C and was used for the study.

5. **Ethanol (70 per cent)**

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

6. **TE buffer (pH8, 100 ml)**

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

7. **TAE buffer (50 X)**

Tris base	:	242 g
Glacial acetic acid	:	57.1 ml
0.5M EDTA (pH 8)	:	100 ml

The solution was prepared, autoclaved and stored at room temperature.

ANNEXURE II

Composition of buffers and dyes used for gel electrophoresis

1. TAE Buffer 50X

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

2. Loading Dye (6X)

0.25% bromophenol blue

0.25% xylene cyanol

30% glycerol in water

3. Ethidium bromide

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

VARIABILITY ANALYSIS IN GINGER (*ZINGIBER OFFICINALE* ROSC.) SOMACLONES USING MOLECULAR MARKERS

By

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ABSTRACT OF THE THESIS

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ABSTRACT

Ginger (*Zingiber officinale* Rosc.), an important spice crop grown in India, is much valued for its flavour and medicinal properties. As natural variability available in the crop is limited, somaclonal variation is being utilized for crop improvement programmes. Currently, molecular marker techniques are widely employed to detect and assess somaclonal variation in several crop species as they are stable, detectable in all tissues and are not confounded by environment, pleiotropic and epistatic effects.

The present investigations on "variability analysis in ginger (*Zingiber officinale* Rosc.) somaclones using molecular markers" were carried out at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Kerala Agricultural University during February 2012 to May 2013. The objectives of the investigations were to assess somaclonal variation in ginger at molecular level, to study the influence of genotype and mode of regeneration on somaclonal variation, to assess the extent of variability in somaclones from the original source parent cultivars and to select the variants. Two molecular marker systems viz. Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeats (ISSR) were utilized for the analyses.

Ginger somaclones (180 Nos.) regenerated through various modes of regeneration viz. bud culture, indirect organogenesis / embryogenesis and *in vitro* mutagenesis, along with two source parent cultivars (Maran and Rio-de-Janeiro) were used for the present study. The genomic DNA was extracted from somaclones using CTAB method (Rogers and Bendich, 1994) and Sigma's GenElute^{TM} Plant Genomic DNA Miniprep kit.

The somaclones were grouped as per genotype and mode of regeneration. DNA extracted from individual somaclones was bulked as per the procedure reported by Dulson (1998). Bulked DNA samples of the thirteen groups of somaclones along with two source parent cultivars were subjected to RAPD and ISSR analyses with selected primers.

Of the 35 RAPD primers screened, twelve gave good amplification. RAPD analysis using selected primers produced 129 amplicons, 44 were polymorphic with an average of 3.66 polymorphic bands / primer and a polymorphism percentage of 34.10. In ISSR assay, twelve selected primers produced 122 amplicons, 32 were polymorphic with an average of 2.66 polymorphic bands / primer and a polymorphism percentage of 26.23. The study could identify certain specific RAPD and ISSR primers for identification of Maran and Rio-de-Janeiro cultivars and irradiated mutants from non-irradiated somaclones.

The dendrograms generated based on RAPD and ISSR profiles grouped the somaclones into two separate clusters, with somaclones of Maran in first subcluster of cluster I and somaclones of Rio-de-Janeiro in second subcluster of cluster I. The regenerants from Rio-de-Janeiro calli irradiated with 20 Gy and somatic embryogenic calli irradiated with 10 Gy formed the second cluster.

RAPD and ISSR marker systems showed that somaclones derived from cultivar Maran exhibited more variability than Rio-de-Janeiro. RAPD marker system was more effective for bringing out variability. The variability observed in RAPD assay was 28 per cent while in ISSR assay it was 21 per cent and in the combined it was 25 per cent. In groupwise variability analysis using bulked DNA, the groups RC20 Gy and RSe10 Gy recorded higher variability from source parent cultivar. The variability exhibited in plantwise analysis using three selected primers (OPA 28, S11 and ISSR 05) was found very high (39%) as compared to groupwise analysis (25%). The somaclone RC2Kr1031 of the callus regenerants and RSe1Kr1052 of the somatic embryo regenerants showed more variability exhibiting 59 and 53 per cent variability respectively from the source parent cultivar Rio-de-Janeiro.

The extent of variability in ginger somaclones could be assessed using molecular markers and *in vitro* mutagenesis could be employed to widen the genetic base in ginger.