IDENTIFICATION OF MOLECULAR MARKERS LINKED TO ANTHRACNOSE RESISTANCE IN GREATER YAM (*Dioscorea alata* L.)

By

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THESIS

Submitted in partial fulfilment of the

requirement for the degree of

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2018

DECLARATION

I hereby declare that the thesis entitled **"Identification of molecular markers linked to anthracnose resistance in greater yam (*Dioscorea alata* L.)"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title of any other University or Society.

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LIST OF ABBREVIATIONS

^{o}C	Degree Celsius
%	Percentage
µg	Microgram
µl	Microlitre
µ*M*	Micromolar
A_{260}	Absorbance at 260 nm wavelength
A_{280}	Absorbance at 260 nm wavelength
bp	Base pair
CAD	Cassava anthracnose disease
CTAB	Cetyl trimethyl ammonium bromide
CTCRI	Central Tuber Crops Research Institute
Da	*Dioscorea alata*
DNA	Deoxyribo nucleic acid
dNTPs	Deoxy nucleotide tri phosphates
EDTA	Ethylene diamine tetra acetic acid
EST	Expressed Sequence Tags
FAO	Food and Agriculture Organization
F_1	Filial 1
G	Gram
ICAR	Indian Council of Agricultural Research
IITA	International Institute of Tropical Agriculture
ISSR	Inter simple sequence repeat
kbp	Kilo base pair
kg	Kilogram
kcal	Kilocalories
LOD	Logarithm of the odds
M	Molar
mg	Milligram
$MgCl_2$	Magnesium Chloride

min	Minute
ml	Millilitre
m*M*	Millimolar
NaCl	Sodium chloride
NBS-LRR	Nucleotide-binding site- leucine-rich repeat
ng	Nanogram
OD	Optical Density
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PIC	Polymorphism Information Content
PVP	Polyvinyl pyrrolidone
QTL	Quantity Trait Loci
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
RGAs	Resistance gene analogues
RILs	Recombinant inbred lines
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolution per minute
s	Second
SNP	Single Nucleotide Polymorphism
SSR	Simple sequence repeat
SCAR	Sequence Characterized Amplified Regions
Taq	*Thermus aquaticus*
TBE	Tris-borate EDTA buffer
TE	Tris- EDTA buffer
T_m	Melting temperature
Tris HCl	Tris (Hydroxy Methyl) aminomethane hydrochloride
U	Enzyme unit
UPGMA	Unweighted Pair-group Method with Arithmetic Average

UV	Ultraviolet
V	Volt
v/v	volume/volume
W	Watt
w/v	Weight/volume

1. INTRODUCTION

Tropical root and tuber crops are important food crops that serve a pivotal role in human diet as sustenance food in various parts of the tropical zone. They contain large quantities of starch in addition to many vitamins, minerals, etc. They form a considerable part of the world's food supply and various processed products of tuber crops are used for human consumption and industrial use and are also an important source of animal feed.

Yams (*Dioscorea* spp.) constitute a multi-species, polyploid crop that is vegetatively propagated. Yam is generally consumed as cooked yam, soup, and powder or flour in food preparations. Yam tubers are a good source of many bioactive components, including, diosgenin, dioscin, allantoin, mucin, choline, dioscorin, polyphenols, and vitamins such as carotenoids and tocopherols (Bhandari *et al.*, 2003). Several studies have shown medically significant activities of yam extracts such as antimicrobial, antioxidant and hypoglycemic activities (Kelmanson *et al., 2000*).

According to a survey by International Institute of Tropical Agriculture in 2010, about 5 million hectares of land covers yam cultivation in about 47 countries in tropical and subtropical regions of the world.

Yams is a large genus comprising more than 600 species under the family Dioscoreaceae. The most common cultivated species of yams include *D. alata* (Greater yam/ water yam), *Dioscorea rotundata* (white yam), *D. bulbifera* (air potato), *D. esculenta* (lesser yam), *D. opposita* (Chinese yam) and *D. dumetorum* (Bitter yam). Harvest of tubers is done at about 8–12 months after planting. Some of the species produce aerial tubers also.

D. alata or greater yam is the most widely distributed yam species in the world and is the second most cultivated yam species after the *D. rotundata/cayennensis* complex. It is a perennial climber, but grown as an annual crop. The plant produces large tubers and also aerial tubers which are rich in carbohydrate.

The major limitation of greater yam in the field is that many popular cultivars are susceptible to anthracnose, and the disease that exerts an overwhelming impact

on productivity. Anthracnose is caused by an ubiquitous fungi *Colletotrichum gloeosporioides,* that belongs to the order Melanconiales. Anthracnose causes leaf necrosis and dieback of yam vines, which results in the reduction of the total photosynthetic surface area of the crop which in turn causes a concomitant reduction in the amount of food reserves stored in the tuber. No effective measures are available at present for the sustainable control of anthracnose and the disease is still a major worry among yam farmers (Abang *et al.*, 2003).

Genetic improvement can contribute significantly in addressing this challenge. Now, a large number of DNA marker techniques have been evolved as prominent tools for genetic studies in plant breeding and germplasm management (Mba *et al.,* 2001). Different markers can be used for the molecular characterization and profiling of agronomically significant traits such as disease resistance, high yield, shelflife etc. within the *D. alata* germplasm, and it will be useful in the molecular breeding programmes. RAPD markers have found extensive application in breeding for major genes that confer resistance to plant pathogens (Melchinger, 1990; Kelly, 1995). Since their introduction, RAPD markers have been used to identify genes linked to resistance to *Colletotrichum* diseases of various crops such as coffee (Agwanda *et al.*, 1997) and common bean (Young *et al.*, 1998; Arruda *et al.*, 2000). ISSR is a simple and quick co-dominant marker used for the detection of polymorphisms in inter-microsatellite loci, using a primer synthesized from di- or trinucleotide repeats that flanks the gene of interest. The use of ISSR markers are advantageous in terms of stability and reproducibility, reliability, high polymorphism, much larger numbers of fragments per primer and relatively low cost (Gupta *et al.,* 1994). SSR markers are locus-specific, co-dominant markers, randomly tandem repeats of short nucleotide motifs and the copy number of these repeats varies among individuals and is a source of polymorphism in plants. SSR markers serve as powerful tools for monitoring anthracnose resistance and help to increase efficiency of breeding yam for anthracnose resistance. Kaduvakkayan, a popular variety grown in Kerala is highly susceptible to anthracnose disease causing heavy income loss to farmers.

This study attempts in the identification of molecular markers linked to

anthracnose (*C. gloeosporioides*) resistance in water yam (*D. alata*). The identified markers can be used in breeding programmes and the identification of resistant lines and incorporation of resistant genes in susceptible genotypes would allow a direct increase of the resistance level in the improved genotypes. The present project aims at screening of greater yam germplasm for anthracnose resistance and identification of molecular markers associated with anthracnose resistance in greater yam based on RAPD, ISSR and SSR markers.

2. REVIEW OF LITERATURE

2.1 ROOT AND TUBER CROPS

Root and tuber crops are plants that store food reserves as starch in subterranean stems, rhizomes roots, and corms and hence these tubers are important, food sources, after cereals, generally consumed in tropical regions of the world. Yams, cassava, Potatoes, sweet potatoes, and aroids belonging to different botanical families are grouped together under root and tuber crops since all these types produce underground food. Tuber crops are more adaptable to tropics since they can easily adapt to diverse soil and environmental conditions and a variety of farming systems with minimum agricultural inputs. However, they are bulky in nature with high moisture content of 60–90% causing limitations such as reduced short shelf life, high transportation cost and low market margin even in developing countries, where they are mainly cultivated (Chandrasekara and Kumar, 2016).

2.2 YAMS

Yam (*Dioscorea* spp) is a multi-species crop comprising about 600 species of which only 6 are edible. Yam is also highly polyploidy in which the ploidy level varies between the species and within the same species. *Dioscorea rotundata* and *D. cayenensis* (known as white and yellow Guinea yams, respectively) are the most favored and economically influential yams eventhough *D. alata* (water yam or greater yam) is the most widely distributed species globally. *Dioscorea esculenta* (known as the lesser yam), native to Southeast Asia, was one of the first yam species cultivated. *Dioscorea polystachya*, commonly called 'Chinese yam' tubers are smaller than the African yams, and their vines are about 3 m in length, and are harvested 6 months of planting. *Dioscorea bulbifera*, the 'air potato', found in Africa and Asia, is not much favoured commercially since the flavour of other yams is preferred (Tan and Zaharah, 2015).

Dioscorea spp. or yam is believed to have originated in South East Asia and have been carried across the savannah to West Africa by Malaysians in the second century AD. In the late 1450s yam was brought from West Africa across the Atlantic to the Caribbean during the slave trade (Johnson, 2003). Different yam species were believed to be originated in three independent areas of the tropics:

Asia, West Africa and tropical America. *Dioscorea alata* L. and *D. esculenta* (Lour) Burk originated from Asia while *D. rotundata* Poir, *D. cayenensis* Lam., and *D. dumetorum* Pax. originated from West Africa. In general, there was an eastward movement of yam species during the domestication process (Hahn *et al.*, 1987).

Yam belongs to the order Liliflorae, family Dioscoreaceae, and genus *Dioscorea*. Yam tubers, which are enlarged storage organs that store food reserves in the form of starch and carbohydrate, vary greatly in size ranging from small potato size to more than 2.5 m in length, and may weigh up to 2.5 kg. They are round, cylindrical, oval or flattened (Toyohara *et al.*, 2000) and are usually produced underground. The tuber flesh may be of various shades of off-white, yellow, purple, or pink, and the skin can be off-white to dark brown depending on the variety. Some species of yams, such as *D. bulbifera,* produce aerial tubers (Coursey, 1967).

Yam is important in many countries of South-East Asia, West Africa, the Pacific and Caribbean islands and parts of Brazil as a source of food, income and for socio-cultural activities (Nweke *et al.*, 1991). Yam peels are used as traditional animal feed which is a rich source of carbohydrate and wild species make up part of the diet of omnivorous and herbivorous burrowing animals in the humid tropics (Degras, 1993). Some wild species of yam such as *D. villosa* contain steroidal saponins and sapogenins, precursors for cortisone used for the treatment of menstrual disorders and arthritis and for the management of menopausal symptoms (Komesaroff *et al.*, 2001). A female hormone precursor, Diosgenin, used in the manufacture of birth control pills and sex hormones have been extracted from some other wild species (Ulbricht *et al.*, 2003). Another species, the cinnamon vine or *D. batatas* Deene, is usually cultivated as a decorative plant and other wild species are also of ornamental value (Komesaroff *et al.*, 2001; Hou *et al.*, 2002).

2.3 Dioscorea Alata

Dioscorea alata L. or water yam is one among the top 10 important yam species with wide adaptation and cultivation used for food in tropical and subtropical regions (Tay, 2013; Lebot, 2009). Ease of vegetative propagation (Petro *et al.*, 2011) and high nutritional content of underground tubers (Siqueira *et al.*,

2012) showed the relatively better agronomic flexibility of water yam when compared to other cultivated yam species.

Dioscorea alata generally has a very sheen appearance with a smooth vine (2-30 m length) with round, winged or ridged shoots and climbs twining towards the right. The leaves are often large, but may be smaller in better varieties. They are in opposite arrangement on the stem, heart shaped, ovate to oblong and glabrous. The yam plant is dioecious and flowers during the last third of the growing season, when tubers are forming. The male flowers are small and are produced in large numbers in crowded panicles originating from the axils of the leaves and the tips of branches. The female flowers are borne on long racemes from the axils of the leaves. Yam fruits are dry capsules, 1-2 cm long and usually broader with flattened and hard seeds encircled by a wing. The tubers of *D. alata* are round, cylindrical, oval or flattened (Toyohara *et al.*, 2000) and are usually produced underground. Depending on the variety, the tuber flesh may be of various shades of off-white, yellow, pink, or purple and the peel can be off-white to dark brown, which is characterized by the presence or absence of anthocyanins (Hasan *et al.*, 2008).

2.4 HEALTH BENEFITS

The tubers are traditionally used mainly as food and serves as a relatively cheaper source of calories. Yam tubers are found to have high health and medicinal properties, but most species of the genus remain underutilized and are yet to be exploited. As the main component of yam are the complex carbohydrates, yams are taken as a tonic food and are excellent in an athlete's diet. Most remarkable is its high fiber content and low fat content (Coursey, 1977). Since most of the *D. alata* varieties contain high amylose content, it is not easily digestible and hence its absorbption in the digestive tract is slow, which could be useful in diets of diabetic patients and other health conscious individuals (Riley *et al.*, 2006). Intake of *D. alata* will significantly helps the populace to meet their recommended dietary allowance (RDA) for Dietary Fiber (DF) (Dufie *et al.*, 2013). Other medicinal uses of greater yam include;

- Used as a vermifuge and laxative in various healing practices.
- Helps to solve a number of digestive problems

- For curing burned hemorrhoids, tumors, fever,gonorrhea, leprosy and many more.
- Controls high blood pressure impacts
- Provides relief in muscle tension, nerve tension, neuralgia and cramps.
- Anthocyanin content in purple varieties offers antioxidant properties and it is protective to sight.

Table 1: Nutritional value of 100g of raw Yam (*D. alata*)

NUTRIENT	AMOUNT
Dietary fiber	4.1g
Carbohydrates	27.9g
Vitamin B6	0.3mg (23%)
Thiamine	0.112mg (10%)
Fat	0.17g
Riboflavin	0.032mg (3%)
Vitamin A equiv.	7µg (1%)
Sugar	0.5g
Niacin	0.552mg (4%)
Magnesium	21mg (6%)
Vitamin C	17.1mg (21%)
Potassium	816mg (17%)
Calcium	17mg (2%)
Iron	0.5mg (4%)
Vitamin K	2.3µg (2%)
Phosphorous	55mg (8%)
Protein	1.5g
Zinc	0.24mg (3%)
Energy	494kJ (118 kcal)

2.5 ANTHRACNOSE IN GREATRER YAM

Anthracnose is a serious disease caused by the fungal pathogen *Colletotrichum gleosporioides* in greater yam. *C. gloeosporioides* causes anthracnose to a variety of subtropical fruit crops such as mango, papaya, guava, custard apple and pomegranate. Anthracnose is favored by wet, humid, warm conditions and spread to healthy plants through infected seeds, rain splash and moist winds. The disease can be affected to any plant part at any growth stage. Leaves and ripe fruits showed visible symptoms. Anthracnose symptoms are generally found on leaves as small and irregular yellow, brown, or black spots and then gradually become dark, grow and coalesce to cover the whole affected area. The disease also produce lesions on petioles and on stems and causes severe defoliation and rotting of fruits and roots. Small, water soaked, sunken, irregular spots that may expand up to 1.2 cm in diameter are seen on infected fruits. (Prasad and Singh, 1960).

The major limitation to the sustainable production of *D. alata* is its susceptibility to anthracnose disease and it can cause yield reduction of up to 80% (Nwankiti and Ene, 1984). Water yam (*D. alata*) was thought to be more susceptible to anthracnose than other yam types (Amusa, 1997). *Gloeosporium pestis* Massee was the first to be identified as the pathogen causing yam anthracnose from yam in Fiji (Winch *et al.*, 1984). Later it was identified in *D. alata* and classified as *C. gloeosporioides* in India (Prasad and Singh, 1960; Singh *et al.*, 1966).

Four forms of *Colletotrichum* associated with yam anthracnose were reported in Nigeria: the slow-growing grey (SGG), the fast-growing salmon (FGS), the fast-growing grey (FGG), and the fast-growing olive (FGO) forms (Abang *et al.*, 2002).

A detailed characterization of the different symptoms of *Colletotrichum* diseases and how these establish on *D. alata* was provided by Winch *et al.* (Winch *et al.*, 1984). They reported that during the initial stage of infection on young leaves or the final stage of invasion of mature and fully expanded leaves, pinpoint lesions (<2 mm diameter) appear more frequently on the adaxial leaf surface. Dark brown to black, irregular or roughly circular lesions with about 2-20 mm diameter appear on leaves as the plant gets infected. Lesions may expand and coalesce to form large

blotches, and as necrotic tissue falls away, 'shot-holes' may develop in expanded lesions. Early infection may leads to premature death of the plant. Melanin-like compounds were found to be linked with appressoria of *C. gloeosporioides*, but there was little indication of fungal penetration into host cells (Winch *et al.,* 1993).

Plant characters such as thickness of cuticle, position, and length of stomata and polyphenol content of leaf tissues were used to explain the disease resistance in *D. alata*. The cuticle thickness was observed to be more in resistant cultivars than the susceptible ones. Also the stomata of the resistant cultivar was sunken below the epidermal layers while that of the susceptible clones are even with or slightly raised above other epidermal cells (Nvankiti, 1982). Leaf extracts showed higher polyphenol activity against the organism in the resistant clone than in the susceptible clone; as was also shown by the presence of a chemical substance which accumulated around infected areas in the resistant clones, which has to be further investigated.

2.6 MOLECULAR MARKERS

A genetic marker is a gene or a DNA sequence with a known location on a chromosome and associated with a particular gene or trait. It can be a variation in the genomic loci, which may arise due to mutation or any other alterations that can be observed (Al-Samarai and Al-Kazaz, 2015).

A vast array of molecular markers has been discovered since 1980 and new marker types are developed every year. DNA markers are classified into two:

(1) Those based on DNA-DNA hybridization (eg: RFLP)

(2) Those based on amplification of DNA sequences using the polymerase chain reaction (PCR) (eg; RAPD, SSR, AFLP, ISSR).

Following are the criteria that an ideal marker should meet for efficient use in marker-assisted breeding.

- High level of polymorphism
- Even distribution across the whole genome
- Co-dominance in expression
- Single copy and no pleiotropic effect
- Low cost

- Easy detection and automation
- High availability (un-restricted use) and suitability to be duplicated/multiplexed
- Genome-specific in nature (especially in the case of polyploids such as yams)
- Should not cause any detrimental effect on phenotype

Botstein *et al.* (1980) was the first to use DNA restriction fragment length polymorphism (RFLP) in human linkage mapping and then significant progress has been made in development and advancement of molecular techniques that help to easily identify markers of interest on a large scale (Botstein *et al.,* 1980). Some of the extensively used marker types for application in genetics and breeding include restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD), microsatellites or simple sequence repeats (SSR), inter simple sequence repeats (ISSR) and single nucleotide polymorphism (SNP).

2.6.1 Inter-simple sequence repeats (ISSRs)

Inter-simple sequence repeats (ISSRs) are regions in the genome flanked by microsatellite sequences. PCR amplification of these regions using a single primer yields multiple amplification products and thus can be used as a dominant multilocus marker system for the study of genetic variation. The advantages of ISSR markers include low-cost, easy to use, and methodologically less demanding compared to other dominant markers, making it a preferable marker system for beginners and for organisms whose genetic information is unavailable (Ng and Tan, 2015). According to Anne, a good genetic marker, which can be used for genetic variation studies, is defined by high genetic variability and the ability to generate multilocus data from the genome under study, which is mostly met by ISSR markers (Anne, 2006). The ISSR markers makes are generated using the microsatellite sequences that are highly variable and frequently distributed across the genome, and also it should achieve higher reproducibility compared to RAPDs and cost effective in terms of time and money compared to AFLPs. All these advantages make ISSR an ideal genetic marker for genetic variation or diversity studies (Wang *et al.*, 2012; Shafiei-Astani *et al.*, 2015), DNA fingerprinting (Shen *et al.*, 2006), and phylogenetics (Iruela *et al.*, 2002).

An ISSR primer is usually 16–25bp in length, comprised mainly, or solely, of repeated DNA motifs (2–4 bp each) that are complementary to microsatellite regions in the genome. ISSR-PCR involves single-primer PCR amplification since this single primer can acts as both the 'forward' and 'reverse' primers which are essential for an amplification to take place (Ng and Tan, 2015).

2.6.2 Random Amplified Polymorphic DNA (RAPD)

RAPD is a PCR-based marker system in which a single, short (usually decamers) and random primer is used to amplify the total genomic DNA of an individual. A specific segment of the template gets amplified when the RAPD primer binds to its complementary sequence in the template genome and then again at a second nearby location, but in the opposite orientation from the first priming site. The PCR products are then separated by agarose gel electrophoresis and visualized by ethidium bromide (EtBr) staining. A single RAPD primer can reveal multiple loci in the genome. Polymorphisms are detected by the presence or absence of a particular RAPD band resulted from mutations or rearrangements either at or between the primer-binding sites (Kumari and Thakur. 2014).

RAPD yields high levels of polymorphism and is simple and easy to be conducted and also efficient technique since the procedure does not involve blotting or hybridization steps. Neither DNA probes nor sequence information is required for the design of specific primers. Another advantage of these markers is that the RAPD products of interest can be cloned, sequenced and then used to develop advanced types of PCR-based markers, such as sequence characterized amplified region (SCAR), single nucleotide polymorphism (SNP), etc. Still, RAPD has some limitations, such as low reproducibility and is dominant, hence unable to detect allelic differences in heterozygotes (Kumar and Gurusubramanian, 2011). RAPD is generally not considered as a good marker system for mapping purposes since the level of polymorphism detected in mapping populations was low. But they are adequate for genetic diversity studies.

2.6.3 Simple Sequence Repeats (SSRs)

SSRs, also called microsatellites or short tandem repeats (STRs), are another class of PCR-based markers. They are random, tandem repeats of short nucleotide

motifs (2-6 bp long). Di-, tri- and tetra-nucleotide repeats, e.g. (GT) n, (AAT) n and (GATA) n, are widely distributed throughout plant and animal genomes and the copy number of these repeats varies among individuals and is a good source of polymorphism in plants (Selkoe and Toonen, 2006).

Microsatellite show high level of allelic variation, which makes them valuable genetic markers. Pairs of oligonucleotide primers designed based on the SSR motifs that flanks unique DNA sequences are used to amplify SSR loci via PCR. The PCR products can be separated in high-resolution electrophoresis systems (e.g. AGE and PAGE) and the bands can be visualized by fluorescent labelling or silver-staining.

SSR markers are considered to be the best marker system for the detection of intervarietal polymorphisms (Stepien *et al.*, 2007). The advantages of SSR markers are co-dominance, hyper-variability, locus-specificity, reproducibility, and random genome-wide distribution in most cases. Also the SSR markers can be multiplexed, have high throughput genotyping and can be automated. However, SSR technique requires prior information on nucleotide sequence for primer design, labour-intensive marker development process and high start-up costs for automated detections. Since 1990s, SSR markers have been widely used in many applications such as constructing genetic linkage maps, QTL mapping, marker-assisted selection and germplasm analysis in plants.

Table 2: Comparison of six widely used markers (Kordrostami and Rahimi, 2015)

	RFLP	RAPD	AFLP	SSR	SNP	ISSR
Genomic coverage	Low copy Coding region	Whole genome	Whole genome	Whole genome	Whole genome	Whole genome
Amount of DNA required	5-50 µg	0.01-0.1 µg	0.5-1.0 µg	0.05-0.12µg	≥ 0.05 µg	≥ 0.05 µg
Quality of DNA required	High	Low	High	Medium high	High	Medium
Type of polymorphism	Single base Changes Indels	Single base Changes, Indels	Single base Changes, Indels	Changes in Length of Repeats	Single base Changes Indels	Occurence of simple repeats in amplicable distance

Level of polymorphism	Medium	High	High	High	High	Medium
Inheritance	Co-dominant	Dominant	Dominant/ Co-dominant	Co-dominant	Co-dominant	Dominant
Type of primers	Low copy DNA cDNA clone	Usually 10 bp random Nucleotides	Specific Sequence	Specific Sequence	Allele-specific PCR primers	Microsatellite repeats
Reproducibility	High	Low to medium	High	High	High	Low to medium
Automation	Low	Medium	High	High	High	High
Cost per analysis	High	Low	Moderate	Low	Low	Moderate
Primary application	Genetics	Diversity	Diversity and genetics	All purposes	All purposes	Diversity and genetics

2.7 MOLECULAR STUDIES IN *Dioscorea alata*

Jyothi *et al.* (2017) characterized 45 accessions of greater yam maintained in the National repository of tuber crops germplasm at ICAR-CTCRI, TVM based on 25 qualitative and 13 quantitative traits including the major yield components biochemical characteristics. They found that the traits like tuber shape, tuber skin texture, tuber flesh color, tuber cortex color, leaf shape and young leaf color are crucial in distinguishing the different accessions of greater yam. These traits were considered for Principal Component Analysis (PCA) with a measure of Sample Adequacy greater than 0.5. The cluster dendrogram based on morphological data revealed that the *D. alata* accessions used for the study exhibited greater degree of genetic variation for the 42 different morphological traits evaluated (Jyothi *et al.*, 2017).

Saranya *et al.* (2016) have reported the isolation and characterization of RGAs from *Dioscorea alata* for the first time. Nucleotide-binding site- leucine-rich repeat (NBS- LRR) gene family holds the vast majority of resistance genes identified in plants. NBS type sequences in tolerant and susceptible greater yam (*Dioscorea alata*) cultivars were isolated using primers based on the conserved motifs. The identified NBS-type sequences called resistance gene analogues (RGAs) were cloned and sequenced and the results showed similarity to other

cloned RGA sequences available in the public database. The deduced amino acid sequences of the RGAs showed the presence of conserved domains, viz. P-loop, RNBS-B, RNBS-C, Kinase-2 and GLPL, categorizing them with the NBS–LRR class gene family. Amino acid sequence alignment of the *Dioscorea* RGAs with RGAs of other plant species grouped them with the non-toll interleukin receptor subclasses of the NBS sequences. The sequences of RGAs in susceptible and tolerant variety were compared and they found very little difference between them (Saranya *et al.,* 2016).

Arnau *et al.* (2016) contributed to the understanding of *D. alata* genetic diversity by genotyping 384 accessions from different geographical regions (South Pacific, Asia, Africa and the Caribbean), using 24 SSR markers. Diversity analysis was done using Principal Coordinate Analysis, UPGMA analysis and the Bayesian approach implemented in STRUCTURE. They revealed the existence of a wide genetic diversity among the studied genotypes and a significant structuring associated with geographic origin, ploidy levels and morpho-agronomic characteristics in greater yam. UPGMA analysis helped to identify seventeen major groups of genetically close cultivars, including 11 groups of diploid cultivars, 4 groups of triploids and 2 groups of tetraploids. STRUCTURE revealed the existence of six populations in the diploid genetic pool and a few admixed cultivars (Arnau *et al.,* 2016).

Genetic improvement programmes at International Institute of Tropical Agriculture (IITA, Nigeria) and at the Central Tuber Crops Research Institute (CTCRI, India) have been developing high yielding varieties of *D. alata* and *D.rotundata* with pest and disease resistance to meet farmers' requirements. More than 3000 germplasm accessions of yams have been collected, maintained and are being used for yam improvement in these institutions and other yam improvement programmes in different countries. Yam genome mapping was initiated since mid-1990s, however, the lack of mapping populations and suitable polymorphic molecular markers hindered rapid progress. By the end of 2001, significant progress had been made in developing advanced molecular tools and mapping populations for developing linkage maps of the two most important yam species, *D. alata* and

D. rotundata. RAPD and AFLP markers showed higher levels of polymorphism among diverse yam species, in which AFLP revealed the highest. Mignouna *et al.* (1998) tested 64 AFLP primer combinations for their potential use in assessment of genetic diversity in white Guinea yam (Mignouna *et al.,* 1998). The high level of polymorphism coupled with robustness revealed by AFLP markers made them a more reliable and reproducible marker-system for yam genome analysis, mapping purposes and other genetic studies in yam. Later, as progress was being made, microsatellite markers were used in yam genomics. Thirty-six microsatellite primers were developed from a genomic library and tested for their ability to detect polymorphism in genetic diversity studies of white Guinea yam. High levels of polymorphism, codominant inheritance, high abundance and an even distribution across the genome are the major highlights of SSR markers. Microsatellites were not treated as a good marker-system for mapping purposes because of the low level of polymorphism detected in mapping populations and relatively small number of markers developed.

Obidiegwu *et al.* (2010) have been successfully used flow cytometry to determine ploidy level of *D. alata* collected mainly from West African countries. They studied 74 *D. alata* genotypes, of these 63 were found to be tetraploid, one was hexaploid and 10 were octoploid. A high percentage of tetraploids together with a small percentage of hexaploid individuals and no diploid individuals were found in the studied germplasm collection. Ploidy level and place of cultivation was not found to be associated each other for the tested material. The resulted knowledge on ploidy level can be applied for enhancing the breeding methodologies and optimize germplasm management of *Dioscorea* species. It also offers further understandings on the phylogeny and origin of Dioscorea species.

2.8 MOLECULAR STUDIES LINKED TO ANTHRACNOSE

Several studies have been done on anthracnose, a group of fungal diseases that affect a variety of plants in warm, humid areas.

Anthracnose, caused by *Colletotrichum gloeosporioides* (Abang *et al.,* 2002), is a major constraint to the effective cultivation and production of yam (Winch *et al.,* 1984; McDonald *et al.,* 1998). *D. alata*, compared to other cultivated species of

yams, is more susceptible to the disease. Identification of disease resistant yam cultivars could form the base of sustainable management strategies for anthracnose. Genetic inheritance studies in yams showed that anthracnose resistance in *D. alata* is dominantly but quantitatively inherited (Mignouna *et al.,* 2001).

Mignouna *et al.* (2002) reported the identification of RAPD markers tightly linked to the broadly effective resistance gene present in *D. alata* breeding line, TDa 95/00328. Although the variation for this trait is continuous, the working hypothesis assumed the existence of a single major gene, tentatively designated as *Dcg-1* (Geffroy *et al.*, 2000; Mignouna *et al.*, 2001). Using bulked segregant analysis, they identified two RAPD markers (OPI 71700 and OPE 6950) that flanked the *Dcg- 1* locus and were found to be closely linked to the anthracnose resistance gene (2.3 and 6.8 cM, respectively).

Mignouna *et al.* (2002) constructed a genetic linkage map of the tetraploid water yam genome using 469 AFLP markers segregating in an intraspecific F1 cross. Two improved breeding lines, TDa 95/00328 and TDa 87/01091 selected as female and male parent respectively were crossed to obtain the F_1 generation. The combined markers identified from both the parents were used to construct a linkage map. The linkage map was of length 1,233 cM with a mean marker spacing of 2.62 cM, positioning the 469 markers on 20 linkage groups. The markers segregated like a diploid cross-pollinator population suggesting that the water yam genome is allo-tetraploid ($2n = 4x = 40$). One AFLP marker E-14/M52-307 was found to be linked to anthracnose resistance and located on linkage group 2 by QTL mapping and thus explained 10% of the total phenotypic variance. This was the first linkage map reported for *D. alata* and it covers 65% of the yam genome. The map serves as an aid for further genetic analysis of traits of agronomic importance and for using marker-assisted selection in *D. alata* breeding programmes (Mignouna *et al.*, 2002).

Maricilia *et al.* (2000) identified RAPD markers linked to anthracnose resistance in the common bean cultivar TO (carrying the *Co-4*gene). They inoculated F_1, F_2, and $F_{2:3}$, BC1s, and BC1r populations from the cross Rudá × TO with race 65 of *Colletotrichum lindemuthianum*, causative agent of anthracnose in bean. The resistant and susceptible genotypes were obtained in the ratio 3:1 in the

F_2 population, 1:1 in the BC1s, and 1:0 in the BC1r, confirming that resistance to anthracnose in the cultivar TO be monogenic and also dominant. Six RAPD markers linked to the *Co-4* gene were identified, 4 in the coupling phase: OPY20830C (0.0 cM), OPC08900C (9.7 cM), OPI16850C (14.3 cM), and OPJ011, 380C (18.1 cM); and 2 in the repulsion phase: OPB031 (3.7 cM) and OPA18830 (17.4 cM). OPY20830C and OPB031, 800T, when used in association as a codominant pair, 3 genotypic classes with a high degree of confidence were identified. Marker OPY20830C is found to be tightly linked to *Co-4*, and is being used in anthracnose resistance breeding programmes (Maricilia *et al.*, 2000).

Beshir *et al.* (2016) selected 126 $F_{8:9}$ sorghum recombinant inbred lines, obtained from a cross between MUC007/009 and *Epuripuri* for the mapping of resistance to anthracnose in sorghum. The F8:9 RILs were evaluated for field resistance to anthracnose in Uganda. There were significant differences among locations ($P<0.001$), indicating a strong influence of environments on reaction to the disease. SSRs Xtxp25, Xtxp201 and Xtxp303 were found to be associated with anthracnose resistance, consistent with dominant epistasis gene action. They obtained high polymorphic information content (0.44 to 0.59) and gene diversity values (0.54 to 0.66) (Beshir *et al.*, 2016).

Upadhyaya *et al.* (2013), using 14,739 SNP markers, have mapped eight loci linked to anthracnose resistance in sorghum through association analysis of a sorghum mini core collection consisting of 242 different accessions evaluated for 2 years in the field. Genes linked to anthracnose resistance were found in all loci except loci 8 based on their physical distance from linked SNP markers.

Singh *et al.* (2006) identified a RAPD marker OPA 12383, which is closely linked to a gene for resistance to anthracnose in Sorghum. A total of 29 resistant and 20 susceptible recombinant inbred lines (RILs) derived from a cross between HC 136 and G 73 was used for bulked segregant analysis. 84 RAPD primers were used for initial screening from which 24 were selected based on the polymorphism generated. On BSA, primer OPA 12 amplified a unique band of 383 bp size in G 73 (resistant parent) and resistant bulk, but not in HC 136, the susceptible parent and in the susceptible bulk. They found that the marker OPA 12_{383} was 6.03 cM

from the locus governing resistance to anthracnose. Then they cloned the marker OPA 12_{383} into suitable host, sequenced and based on this sequence, a pair of SCAR markers SCA 12-1 and SCA 12-2 was designed using the MacVector program. These newly synthesized SCAR markers could specifically amplify this RAPD fragment in resistant parent G 73, resistant bulk and respective RILs. Therefore, it was confirmed that SCAR marker SCA 12 is at the same locus as OPA 12_{383} and hence, is linked to the gene for resistance to anthracnose (Singh *et al.,* 2006).

Cassava anthracnose disease (CAD) is one of the major stem diseases of cassava (*Manihot esculenta* Crantz). To identify markers associated with resistance to CAD, Akinbol *et al.* (2007) produced F_1 progenies from a cross between resistant genotype TME 117, and susceptible genotype TMS 92/0326, as the experimental population. An expected ratio of 1:1 for resistant to susceptible genotypes was obtained from the chi-square test, suggesting a monogenic dominant inheritance. They screened 200 decamer primers using both resistance and susceptible parents to CAD. Bulk segregant analysis was quickly used to search for Random amplified polymorphic DNA (RAPDs) linked to anthracnose resistance in F_1. The fragment linked to the gene conferring resistance was observed to be flanked on both sides by primers OPAF2 and OPF06 at 13.1 and 22.2 cM. This is the first report on cassava anthracnose and of molecular markers that tag CAD resistance in cassava. The identified markers-linked to CAD1 can be used for marker assisted cassava breeding (Akinbol *et al.,* 2007).

Petro *et al.* (2011), constructed an intraspecific genetic map of water yam based on AFLP markers and QTL analysis for anthracnose resistance. An F_1 diploid population was developed by crossing 'Boutou' a female clone (with field resistance to anthracnose) with 'Pyramide' (susceptible). Then they generated a linkage map with 523 polymorphic markers from 26 AFLP primer combinations. The resulting map covered a total length of 1538 cM and included 20 linkage groups and they reported it as the most saturated of all genetic linkage maps of yam to the date with over 80% genome coverage. QTL analysis of anthracnose resistance was performed based on response to two isolates of *C. gloeosporioides* and the resistance to anthracnose appeared to be inherited quantitatively. Using a logarithm

of the odds (LOD) significance threshold of 2.6 they identified a total of nine QTLs for anthracnose resistance (Petro *et al.,* 2011).

The inheritance of resistance to a moderately virulent (FGS) strain of *Colletotrichum gloeosporioides* in water yam was investigated by Mignouna *et al.* (2001). They produced F_1 from crosses between tetraploid *D. alata* genotypes: TDa 95/00328 (resistant) ×TDa 95–310 (susceptible) (cross A), and TDa 85/00257 (resistant) ×TDa 92–2 (susceptible) (cross B). Segregation of F_1 progeny fitted genetic ratios of 3:1, 5:1 (crosses A and B) and 7:1 (cross A) resistant: susceptible when inoculated with the FGS strain. These ratios indicate that resistance to yam anthracnose is dominantly inherited and more than one gene controls the inheritance of resistance to this strain in the accessions studied. They also showed strain-specific resistance in TDa 95/00328, which is resistant to FGS strain but highly susceptible to an aggressive SGG strain of the pathogen. TDa 85/00257 and TDa 87/01091 which were highly resistant to the SGG strain, can be used both as sources of resistance and in the development of a host differential series for *D. alata* (Mignouna *et al.,* 2001).

MATERIALS AND METHODS

The Study entitled "Identification of molecular markers linked to Anthracnose resistance in greater yam (*Dioscorea alata* L.)" was carried out at the Division of Crop Improvement, ICAR-Central Tuber Crops Research Institute (CTCRI), Sreekariyam, Thiruvananthapuram during 2017-2018. In this chapter, details pertaining to the experimental material and procedures used in the study are elaborated.

3.1 MATERIALS

Forty accessions of greater yam maintained in the germplasm collection of ICAR-CTCRI were collected and used for the study. The list of accessions used is furnished in table 3.

Table 3: Details of the greater yam accessions used for the study

Sl. No.	GENOTYPE	Place of Collection	Sl. No.	GENOTYPE	Place of Collection
1	Da 110	Dandakaranya	21	TCR 64	NBPGR,Thrissur
2	Da 198	Thiruvananthapuram	22	Da 12	Kottayam
3	Da 200	NBPGR,Thrissur	23	Da 377	Kollam
4	Da 209	NBPGR,Thrissur	24	Da 489	Kottayam
5	Da 210	Coimbatore	25	TCR 113	NBPGR,Thrissur
6	Da 293	Pathanamthitta	26	Da 820	Idukky
7	Da 374	Pathanamthitta	27	Da H 17-5	Hybrid,ICAR-CTCRI
8	DaH 9-196	Hybrid,ICAR-CTCRI	28	Da 810	Thiruvananthapuram
9	DaH22-2-3	Hybrid,ICAR-CTCRI	29	Orissa Elite	Released variety,ICAR-CTCRI
10	DaH 58FG	Hybrid,ICAR-CTCRI	30	Sree Neelima	Released variety,ICAR-CTCRI
11	TCR 308	NBPGR,Thrissur	31	TCR 208	NBPGR,Thrissur
12	TCR 319	NBPGR,Thrissur	32	TCR 282	NBPGR,Thrissur
13	Sree Karthika	Released variety,ICAR-CTCRI	33	Da 508	Assam
14	Sree Keerthi	Released variety,ICAR-CTCRI	34	TCR 102	NBPGR,Thrissur
15	Sree Swathy	Released variety,ICAR-CTCRI	35	TCR 342	NBPGR,Thrissur
16	Da 264	Thiruvananthapuram	36	Da 503	Pre-breeding line,ICAR-CTCRI
17	Da 340	Thiruvananthapuram	37	DaH 24-6-3	Hybrid,ICAR-CTCRI

18	Da 817 V	Pathanamthitta	38	TCR 142	NBPGR,Thrissur
19	JAS 2	Pre-breeding line,ICAR-CTCRI	39	Da 509	Thiruvananthapuram
20	TCR 226	NBPGR,Thrissur	40	TCR 43	NBPGR,Thrissur

3.2 FIELD SCREENING

The 40 accessions were screened in the field for anthracnose resistance. Whole plant area scoring method was done in which the lesions were rated visually on a 0-5 scale based on the percentage of lesion appeared on leaves and vines, where 0= no infection, 1= 1-10%, 2= 10-25%, 3= 25-50%, 4= 50-75% and 5= >75%.

3.3 LABORATORY SCREENING

Excised leaf assay was carried out using 40 yam genotypes with varying resistance levels to anthracnose, collected from germplasm collections maintained in the field gene bank at ICAR-CTCRI, Thiruvananthapuram. Resistance screening was performed with pure isolates of *Colletotrichum gleosporioides*, which were isolated from infected leaves. Leaves of similar age and size (leaves at nodes 3 to 4, counted from the top) were collected from the field. The leaves were washed with running tap water. Monoconidial cultures of *Colletotrichum gloeosporioides* grown on potato dextrose agar (PDA) were scraped with a sterilized inoculation loop and transferred to a vial containing 9ml sterile distilled water. It is then agitated using a rotary shaker to obtain a uniform, homogenized spore suspension. A drop of the suspension was observed under 10X objective lens of light microscope to ensure the presence of adequate count of live spores. 25µl of spore suspension was inoculated onto the test leaves arranged in petriplates. 25µl sterile water was used for inoculating the control leaves. The petioles of the leaves were kept in contact with the folded wet tissue paper arranged inside the petriplates to prevent wilting. Wet tissue paper was also placed on the lid of petriplates for maintaining humidity. The plates were incubated at room temperature. Lesions formed were rated visually at 3 days after inoculation on a 0-5 scale based on the percentage of lesion per inoculated droplet, where 0= no infection, 1= 1-10%, 2= 10-25%, 3= 25-50%, 4= 50-75% and 5= >75%.

Thirty accessions with better result were selected for molecular marker analysis.

3.4 MOLECULAR MARKER ANALYSIS

In order to identify molecular markers linked to Anthracnose resistance in *D. alata*, the molecular characterization using ISSR, RAPD and SSR markers were carried out among the thirty greater yam genotypes as listed in table 4.

Table 4: List of greater yam accessions used for molecular marker analysis

Sl. No.	Genotype	Sl. No.	Genotype
1	Da 110	16	Da 264
2	Da 198	17	Da 340
3	Da 200	18	Da 817 V
4	Da 209	19	JAS 2
5	Da 210	20	TCR 226
6	Da 293	21	TCR 64
7	Da 374	22	Da 12
8	DaH 9-196	23	Da 377
9	DaH22-2-3	24	Da 489
10	DaH 58FG	25	TCR 113
11	TCR 308	26	Da 820
12	TCR 319	27	Da H 17-5
13	Sree Karthika	28	Da 810
14	Sree Keerthi	29	Orissa Elite
15	Sree Swathy	30	Sree Neelima

3.4.1 Glasswares and Instruments in Molecular Biology Lab

Eppendorf tubes, mortar and Pestle, micropipette tips for 1ml, 200µl and 10µl, and PCR tubes were autoclaved and used. Eppendorf tube stand, PCR tube holders, micropipettes, water bath, icebags, sample covers, scissors, tags, labels, wipes, bottles, conical flasks, spatula and weigh boats are other materials needed for molecular work.

3.4.2 Instruments

Ice machine, weighing balance, Water bath, Vortexer, Centifuge, Electrophoresis apparatus, Hot air oven, Autoclave, PCR machine, Gel documentation system and-20 °C and 4°C refrigerator were used for the work.

3.4.3 DNA Extraction

The pre requisite for any molecular characterization work is isolation of good quality DNA from the plant tissue. DNA was isolated from young leaf tissues of 30 accessions of *D.alata* using a modified CTAB method.

3.4.3.1 Sample collection for DNA extraction

Fresh, young leaves of 30 *Dioscorea alata* accessions were collected from germplasm collection of ICAR-CTCRI, Thiruvananthapuram. Young leaf tissues were collected in plastic sample collection bags from the field and brought to lab in an ice box.

3.4.3.2 Procedure

DNA was extracted from fresh and young leaves of collected samples using modified protocol of Raj *et al.* (2014).

Leaf tissues (200–250 mg) were ground to a fine powder in liquid nitrogen using mortar and pestle. Prewarmed extraction buffer (1 ml) was added to the samples and it was ground once more. The fine samples were transferred to 2.0 ml Eppendorf tubes and 10 µl Proteinase K (10 mg/ml) was added. The tube was incubated in waterbath at 37 °C for 30 mins and then at 65 °C for another 30 mins with frequent mixing. After incubation, the samples were centrifuged at 12,000 rpm for 15 min at RT and supernatant was transferred to fresh eppendorf tubes. Equal volume of Chloroform: isoamyl alcohol (24:1) were added and mixed by gentle inversion for 30–40 times. The samples were centrifuged at 12,000 g for 10 min at RT and the supernatant was transferred to fresh tubes. The Chloroform: isoamyl alcohol wash was repeated to remove further proteins, if present. To the supernatant collected in a fresh tube, 150 µL of 2M NaCl solutions containing 4% PEG was added. The samples were centrifuged at 12,000 g for 10 min at RT. The supernatant was transferred to fresh tubes and precipitated with 200 µl of ethanol. The nucleic acids was precipitated and collected by centrifuging at 12,000 rpm for 10 min. The nucleic acid pellet was washed twice with 400 µl wash solution (15 mM Ammonium acetate in 75% ethanol), air-dried until the ethanol was completely removed and then dissolved in 200 µl of TE buffer. The nucleic acid dissolved in TE buffer were treated with 5 µl ribonuclease (RNase, 10 mg/ml), incubated at 37

°C for 30 min and stored at -20 °C until use. All samples were checked in 1% agarose gel and confirmed.

3.4.4 Quantification of DNA

Using Nanospectrophotometer (DENOVIX), the isolated DNA was quantified. It was used to determine the yield and purity of the isolated DNA. TE buffer in which the DNA was dissolved was used to calibrate the machine. To measure the quantity and purity of DNA, 1µl of DNA sample was placed in the sensor probe of the machine. It's the benefit of Nanospectrophotometer that no other dye is required. The quantity and quality of the DNA was displayed as the concentration of the DNA in ng/µL, the absorbance ratio $OD_{260/280}$ and $OD_{260/230}$. At OD_{260}, the quantity of DNA was determined and the purity was determined by calculating the $OD_{260/280}$ ratio. Quantification values of the DNA are given in table 13. The samples were selected based on the better absorbance value or OD value.

3.4.5 Dilution of DNA Samples

Samples were diluted to a concentration of 10ng/µl using nuclease free water, irrespective of the varying concentrations calculated spectrophotometrically.

3.4.6 Primers

Primers for ISSR, SSR and RAPD were selected from literatures (Mignouna *et al.,* 2002; Singh *et al.,* 2006; Akinbol *et al.,* 2007; Maricilia *et al.*, 2000; Obidiegwu *et al.*, 2009) based on their ability to show high polymorphism. Twenty Two SSR primers were newly designed using bioinformatics tools. They were ordered and shipped in lyophilized form. Sometimes this dry DNA becomes dislodged from the bottom of the tube during shipping. This loose DNA can easily fly out of the tube when first opened, particularly as electrostatic attraction is present if the user is wearing latex gloves. Therefore oligos, before opening for first time, was briefly centrifuged to avoid loss of DNA pellet .The oligos were dissolved in TE buffer or nuclease free water. Initially freezer stock was made at 100 µM concentrations by adding a volume of TE buffer or DNAase/RNAase free water equal to ten times the number of nanomoles of DNA present in the tube and stored as main stock.

3.4.7 Primer dilution

Working stock of 10 µM concentration were made out of the 100µM stock and stored in -20°C refrigerator.

3.4.8 PCR amplification

The diluted samples were amplified in thermal cycler using different primers of ISSR, SSR and RAPD markers at different conditions. All the primers were screened and the best primers and optimum amplifying conditions were selected and used for validating the primers of ISSR, SSR and RAPD.

3.4.8.1 PCR using *EMERALD Amp GT PCR master mix*

EMERALD Amp GT PCR Master Mix by TAKARA BIO INC is a 2x premix composed of a DNA polymerase, optimized reaction buffer, dNTPs, and a density reagent. The premix also contains a vivid green dye that will separate into blue and yellow dye fronts when run on an agarose gel. The premix simplifies PCR assembly; simply add primers, template, and water and start the reaction. After PCR, the reaction mixture can be applied directly to a gel for analysis. For the sake of convenience and for saving time during the PCR mix preparation, Emerald Amp GT PCR master mix was used for the present study.

3.4.9 Molecular characterization using ISSR markers

3.4.9.1 Source of primers

The primers selected from collected literatures showing high polymorphic values in *Dioscorea* species, were ordered and shipped from IDT in lyophilized form. Out of a total of fifteen primers (Table 4) screened, seven primers namely UBC 807, UBC 809, UBC 825, UBC 836, $(GA)_9AC$ and $(GA)_9AT$ were found to be highly polymorphic and hence were selected for further validation.

Table 5: Details of ISSR primers selected for initial screening

Sl. No	Primer name	Sequence (5' ⟶ 3')
1	UBC 14	CGCGCGCGCGCGCGTG
2	UBC 807	AGAGAGAGAGAGAGAGT
3	UBC 808	AGAGAGAGAGAGAGAGC
4	UBC 809	AGAGAGAGAGAGAGACG
5	UBC 810	GAGAGAGAGAGAGAGAT
6	UBC 811	GAGAGAGAGAGAGAGAC

7	UBC 817	CACACACACACACACAA
8	UBC 818	CACACACACACACACAG
9	UBC 825	ACACACACACACACACT
10	UBC 827	ACACACACACACACACG
11	UBC 836	AGAGAGAGAGAGAGAGYA
12	UBC 864	ATGATGATGATGATGATG
13	$(GA)_9AC$	GAGAGAGAGAGAGAGAGAAC
14	$(GA)_9AT$	GAGAGAGAGAGAGAGAGAAT
15	$(ACC)_6Y$	ACCACCACCACCACCACCY

Table 6: ISSR REACTION MIXTURE

Components	Stock Concentration	Required Concentration	Volume For One Reaction (20µl)
EMERALD Amp GT PCR Master Mix	2X	1X	10µL
Primer	10µM	0.25µM	0.5µL
$MgCl_2$	50Mm	1Mm	0.4µL
DNA	10ng/µL	40ng	4.0µL
Sterile Distilled Water	.....	.....	5.1µL
TOTAL			**20µL**

3.4.9.2 ISSR PCR Conditions

PCR was carried out in ProFlex Thermal cycler. The PCR programme used comprised of an initial denaturation at 94 °C for 5 minutes followed by 30 cycles of denaturation at 94°C for 30 seconds, primer annealing at 56.3 °C for 1 minute and extension at 72 °C for 1 minute. The final extension was performed at 72 °C for 10 minutes followed by hold at 4 °C. The lid temperature of the machine was maintained at 105 °C throughout the run.

The amplicons resolved in an ethidium bromide stained 2 % agarose gel along with 100 bp and 1 kb DNA ladders were visualized under UV light in a gel documentation system (G: Box using GeneSyS softwere ; M/s. Syngene). The gel images were documented for further scoring.

3.4.10 Molecular characterization using RAPD markers

Eleven RAPD primers were used for initial screening and are listed in table 7.

Table 7: RAPD Primers used

Sl. No	Primer name	Sequence (5'⟶3')
1	OPI-07	CAGCGACAAG
2	OPB-19	ACCCCCGAAG
3	OPE-06	AAGACCCCTC
4	OPF-06	GGGAATTCGG
5	OPG-09	CTGACGTCAC
6	OPI-16	TCTCCGCCCT
7	OPJ-10	AAGCCCGAGG
8	OPAC-14	GTCGGTTGTC
9	OPAF-02	CAGCCGAGAA
10	OPAF-17	TGAACCGAGG
11	OPAG-03	TGCGGGAGTG

On screening the above primers, OPI-07, OPE-06, OPF-06, OPI-16 and OPAF-17 were found to be highly polymorphic and hence were selected for further validation.

Table 8: RAPD REACTION MIXTURE

Components	Stock Concentration	Required Concentration	Volume For One Reaction (15µl)
EMERALD Amp GT PCR Master Mix	2X	1X	7.5µL
Primer	10µM	0.6µM	0.9µL
$MgCl_2$	50Mm	1Mm	0.3µL
DNA	10ng/µL	50ng	5.0µL
Sterile Distilled Water	.....	.....	1.3µL
TOTAL			**15µL**

3.4.10.1 RAPD PCR Conditions

Amplification was done in a programmable thermocycler that was programmed as follows:

An initial denaturation at 94°C for 3 minutes followed by 45 cycles of denaturation at 94°C for 1 minute, annealing at 40°C for 1 minute and extension at 72°C for 2 minutes. The synthesis step of final cycle was extended further by 7 minutes. Finally the products of amplification were cooled to 4°C. The lid temperature was maintained at 105°C.

Table 9: Details of SSR Primers used

Sl. No	Primer	Forward Primer sequence (5' to 3')	Reverse Primer sequence (5' to 3')	Ta (°C)	Expected band size (bp)
1	Da1F08	AATGCTTCGTAATCCAAC	CTATAAGGAATTGGTGCC	47	132-197
2	Da3G04	CACGGCTTGACCTATC	TTATTCAGGGCTGGTG	60.1	278-318
3	Dab2D08	ACAAGAGAACCGACATAGT	GATTTGCTTTGAGTCCTT	49.1	316-349
4	Dab2E07	TTGAACCTTGACTTTGGT	GAGTTCCTGTCCTTGGT	47	131-190
5	YamSSR 1	AATCATTTGCTCAGGGAGGAT	TTGGTATAACCGCACAAAACC	49.7	181
6	YamSSR 2	TCCGTTTCCTATTTCCCAAAA	CAAGGAGGAGGAGGAGAAGAA	48.3	134
7	YamSSR 3	CCGTTTCCTATTTCCCAAAA	TTGTATCAAGGAGGAGGAGGA	46.8	139
8	YamSSR 4	TGGTCCTCCAACTATCTTCCA	TGGCTATTGTTGACAGAGGAGA	51.5	203
9	YamSSR 5	CTCCTCCTTCATTCCTCTTCC	AGGCTGTGCAGTTCAAGTCAT	51.5	165
10	YamSSR 6	GTCTGGTGGATGGAAATGAAA	CGACTGCGTGAAAAACCAAT	49.1	174
11	YamSSR 7	TTGCTCTTCTTCCCTGATGAC	GACGAACACCGACTCTCAATC	51.2	141
12	YamSSR 8	ATTACAAGCCTCGTTCGGATT	AGCGAGGTGAAGTGCTAGGAG	50.4	232
13	YamSSR 9	AGAGAAGAGGGGAACAGCTTG	CCTACTCCAGAAAGAGAGCATCA	52.4	182
14	YamSSR 10	CCCTTCCAATGATGATGAAGA	GTTGAGGAAGTTCATGGTGGA	48.4	147
15	YamSSR 11	GGAAGTACCGAGTCCTGATCC	TCATCACTGAAAAGCAGCAAA	48.8	104
16	YamSSR 12	TATCACATTGCACCCCTGCTA	CGCATTTCGATACATACTCACC	49.9	173
17	YamSSR 13	CATCATTCGTGTGTTGTTTCG	TTGCCAACGAGGTCACTTTAC	48.4	153
18	YamSSR 14	GACAAGGAGGACATGGTGAAC	TGAGCCTTAACACCCTTTGAC	51.5	170
19	YamSSR 15	CAGAGGAGCAGGACCAGATG	CCTCTCCGGTTACCTCAAGAT	52.6	158
20	YamSSR 16	AACTTTTGACCGGATGGAGAT	CCGTTGATTTTTGATTGGATG	46.1	145
21	YamSSR 17	CTTCCCCAAGTCACCTTCTTC	GGGGAGAGAGTGAGGGGTATT	52.2	130
22	YamSSR 18	AGAAGGTGACGACGGAGTTG	CTTCCCCCACTACTCCCACTA	53.4	166
23	YamSSR 19	AGATGGAGGGGAGGAGGAG	CCCCCAACTAATAAATCCAAAA	46.7	163
24	YamSSR 20	CTCAATGGCAGCAAAGAGTGT	AACCATGAGAATGAGGGGAAT	49.7	148
25	YamSSR 21	TTGGGAACTTGACAAATCTTGA	CTCACTTGGGATTGTTCCTGA	48.3	162
26	YamSSR 22	TCCACTTTGAGAGATGAAATGC	TCCATTTTGATTTGCATTGGT	46.7	162

3.4.11 Molecular characterization using SSR markers

Twentysix SSR primers as listed in table 9 were screened for the study. Of these 4 were already reported (Obidiegwu *et al.*, 2009). The rest 22 were designed by Dr. K. M. Senthilkumar, Scientist of ICAR- CTCRI, based on the yam genome sequence information available at NCBI. The protein sequence information of the functionally validated anthracnose resistance genes from *Phaseolus vulgaris L* were used as a query in the NCBI database to identify the orthologs of *Dioscorea alata* (Ferreira *et al.*, 2013). A set of 22 SSRs were designed using BlastPrimer 3 gene

sequence information of *Dioscorea alata*.

On screening the listed (table 9) primers, YamSSR1, YamSSR2, YamSSR3, YamSSR4, YamSSR5, YamSSR6, YamSSR8, YamSSR9, YamSSR12, YamSSR15, YamSSR16, YamSSR18, YamSSR19, Dab2E07, Da3G04 and Dab2D08 were found to be highly polymorphic and hence were selected for further validation.

Table 10: SSR REACTION MIXTURE

Components	Stock Concentration	Required Concentration	Volume For One Reaction (15µl)
Emerald Master Mix	2X	1X	7.5µL
Forward Primer	10µM	0.25µM	0.4µL
Reverse Primer	10µM	0.25µM	0.4µL
DNA	10ng /µL	30ng	3.0µL
Sterile Distilled Water	.....	.....	5.1µL
TOTAL			**15µL**

3.4.11.1 SSR PCR Conditions

PCR was carried out in Proflex Thermocycler programmed for an initial denaturation at 95 °C for 3 minutes followed by 30 cycles of denaturation at 95 °C for 45 seconds, primer annealing (Ta) for 45 seconds and extension at 72 °C for 1 minute. The final extension was performed at 72 °C for 10 minutes followed by hold at 4 °C.

The amplified products were separated on 4% agarose gel along with 100bp ladder to compare the molecular weight of obtained bands and for polymorphism studies.

3.4.12 Agarose gel electrophoresis

Agarose gel electrophoresis is widely used to separate biomolecules such as DNA, RNA and proteins based upon charge, size and shape. Agarose gel electrophoresis posses great resolving power, yet it is simple to perform. The samples are mixed with loading buffer containing glycerol or sucrose and tracking dyes are loaded into wells in agarose gel. A direct current supply is connected to

the electrophoresis apparatus. Molecules having a net negative charge (DNA) migrate towards the positive electrode (anode) while net positively charged molecules migrate to towards the negative electrode (cathode). The 1X TBE or TAE buffer in the gel tank serves as a conductor of electricity and control pH. pH is important to the charge and stability of biomolecules. Smaller molecules move faster through the pores in the gel than larger ones. Molecules can have similar molecular weight and charge but different shape. Molecules that have a very compact shape can move faster through the pores.

Weighed 1.7g of agarose in 250ml conical flask, added 85ml 1X TBE buffer (for 2% gel) and gently boiled the solution in a microwave oven with occasional mixing until the agarose gets completely dissolved in buffer. For making 4% gel for the resolution of SSR PCR products, 3g agarose was dissolved in 75mL 1X TBE buffer. Allowed it to cool for 40°C and added 0.4µl ethidium bromide carefully without spilling. Get ready the gel plates and kept combs in position. Poured the warm gel to plate and cooled for 20 minutes. Once the gel gets solidified, removed the comb and placed the plate with gel in to the tank containing 1X TBE buffer (Appendix IV). 8uL of samples, which already contain the Emerald dye, were loaded into the wells and 3uL 1Kb or 100bp ladders were also loaded as reference. Run the gel at 85V and 220mA for 1 to 1.5 hour. Visualized and documented the gel on a gel documentation system. The images could be finally scored to detect polymorphism or to identify specific bands that can be linked to a particular trait.

3.5 STATISTICAL ANALYSIS OF MOLECULAR DATA

Clear and reproducible bands were only selected for scoring. Binary scoring was carried out by assigning "1" for presence of band and "0" for absence of band. A binary matrix of presence/absence was obtained from gels for each marker. The data matrix created in excel format was used as the input for cluster analysis. Jaccard's similarity coefficient was calculated for use in clustering analysis by Unweighted Pair-group Method with Arithmetic Average (UPGMA). Codes written in the R statistical language (http://www.rproject. org) used for analysis and the GLIMMIX procedure from SAS (2007) and DARwin5.5 (Perrier and Jacquemoud- Collet, 2006) were also used. The R statistical package was used for

hierarchal cluster analysis based on Euclidean distance. Dendrogram grouping the 30 accessions based on ISSR, SSR and RAPD marker data was constructed based on complete linkage method using Jaccard's distance as well as Dice coefficient on employing bootstraps using DarWin6.0 package. Correlation between SSR, ISSR and RAPD markers were assessed using mantel test. Expected heterozygosity and Polymorphism Information Content (PIC) were calculated using PowerMarker V3.0 software.

Gene diversity, often referred to as expected heterozygosity, is defined as the probability that two randomly chosen alleles from the population are different. An unbiased estimator of gene diversity at the *l*th locus is

$$\widehat{D_l} = (1 - \sum_{u=1}^{k} \tilde{p}_{lu}^2).$$

A closely related diversity measure is the polymorphism information content (PIC) (Botstein *et al.* 1980). It is estimated as

$$\widehat{PIC_l} = 1 - \sum_{u=1}^{k} \tilde{p}_{lu}{}^2 - \sum_{u=1}^{k-1} \sum_{v=u+1}^{k} 2\tilde{p}_{lu}^2 \tilde{p}_{lv}^2$$

4. RESULTS

The results of study entitled "Identification of molecular markers linked to Anthracnose resistance in Greater yam (*Dioscorea alata* L.)" carried out at the Division of Crop improvement, CTCRI, Sreekariyam, Trivandrum are explained in this chapter.

4.1 FIELD SCREENING

Forty accessions of greater yam were screened in the field for anthracnose resistance incidence. Field screening was done by whole plant area scoring method in which, the lesions appeared on leaves and vines were rated visually on a 0-5 scale based on the percentage of lesion, where 0= no infection, 1= 1-10%, 2= 10-25%, 3= 25-50%, 4= 50-75% and 5= >75%. The scores obtained were given in Table 10. Plate 1 shows the field screening images of Orissa Elite, a major susceptible genotype and two major resistant accessions Sree Karthika and Sree keerthi.

Table 11: Scores obtained by field screening

Sl. No.	GENOTYPE	FIELD SCORE	Sl. No.	GENOTYPE	FIELD SCORE
1	Da 110	1	21	TCR 64	1
2	Da 198	2	22	Da 12	1
3	Da 200	2	23	Da 377	3
4	Da 209	3	24	Da 489	2
5	Da 210	3	25	TCR 113	1
6	Da 293	1	26	Da 820	4
7	Da 374	2	27	Da H 17-5	3
8	DaH 9-196	1	28	Da 810	3
9	DaH22-2-3	2	29	Orissa Elite	4
10	DaH 58FG	1	30	SreeNeelima	5
11	TCR 308	2	31	TCR 208	3
12	TCR 319	2	32	TCR 282	3
13	SreeKarthika	3	33	Da 508	2
14	SreeKeerthi	1	34	TCR 102	1
15	SreeSwathy	1	35	TCR 342	3
16	Da 264	2	36	Da 503	2
17	Da 340	0	37	DaH 24-6-3	5
18	Da 817 V	1	38	TCR 142	0
19	JAS 2	1	39	Da 509	3
20	TCR 226	1	40	TCR 43	3

Orissa Elite

Die back in Orissa Elite

Sree Karthika

Sree Keerthi

Plate 1: Field screening of major greater yam genotypes

4.2 LABORATORY SCREENING

Laboratory screening was done by excised leaf assay using 40 greater yam genotypes and the lesions formed were rated visually at 3 days after inoculation on a 0-5 scale based on the percentage of lesion per inoculated droplet, where 0= no infection, 1= 1-10%, 2= 10-25%, 3= 25-50%, 4= 50-75% and 5= >75%. The scores obtained were tabulated in Table 11. Plate 2 depicts the response of greater yam genotypes to *Colletotrichum gleosporioides* during laboratory screening.

Table 12: Scores obtained by Lab screening

Sl. No.	GENOTYPE	LAB SCORE	Sl. No.	GENOTYPE	LAB SCORE
1	Da 110	0	21	TCR 64	3
2	Da 198	3	22	Da 12	4
3	Da 200	1	23	Da 377	2
4	Da 209	1	24	Da 489	2
5	Da 210	1	25	TCR 113	1
6	Da 293	2	26	Da 820	5
7	Da 374	2	27	Da H 17-5	3
8	DaH 9-196	1	28	Da 810	3
9	DaH22-2-3	2	29	Orissa Elite	3
10	DaH 58FG	1	30	Sree Neelima	4
11	TCR 308	2	31	TCR 208	1
12	TCR 319	2	32	TCR 282	5
13	Sree Karthika	1	33	Da 508	4
14	Sree Keerthi	1	34	TCR 102	4
15	Sree Swathy	2	35	TCR 342	5
16	Da 264	2	36	Da 503	5
17	Da 340	3	37	DaH 24-6-3	5
18	Da 817 V	4	38	TCR 142	1
19	JAS 2	0	39	Da 509	5
20	TCR 226	4	40	TCR 43	5

Thirty accessions were selected based on the above results for further study. Table 13 shows the response of selected 30 greater yam accessions to anthracnose disease in field and laboratory conditions and were used for genetic diversity study and molecular marker identification. Plates 3 and 4 are the tubers of major resistant and susceptible yam genotypes identified.

Da 340

Da 817 V

TCR 64

TCR 226

Da 12

Da 820

Sree Neelima

Da Assam

TCR 342

Da 110

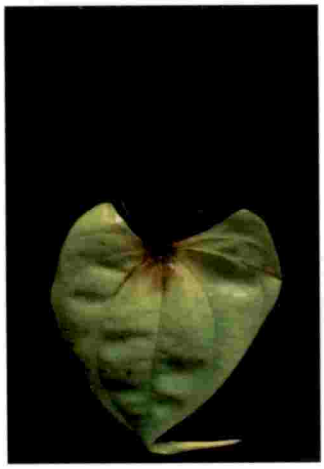
Da 200

Da 210

Da 209

Da 293

DaH 22-2-3



DaH 58FG

TCR 308

Sree Karthika

Da 503-O

TCR 102

TCR 208

TCR 282

Da 198

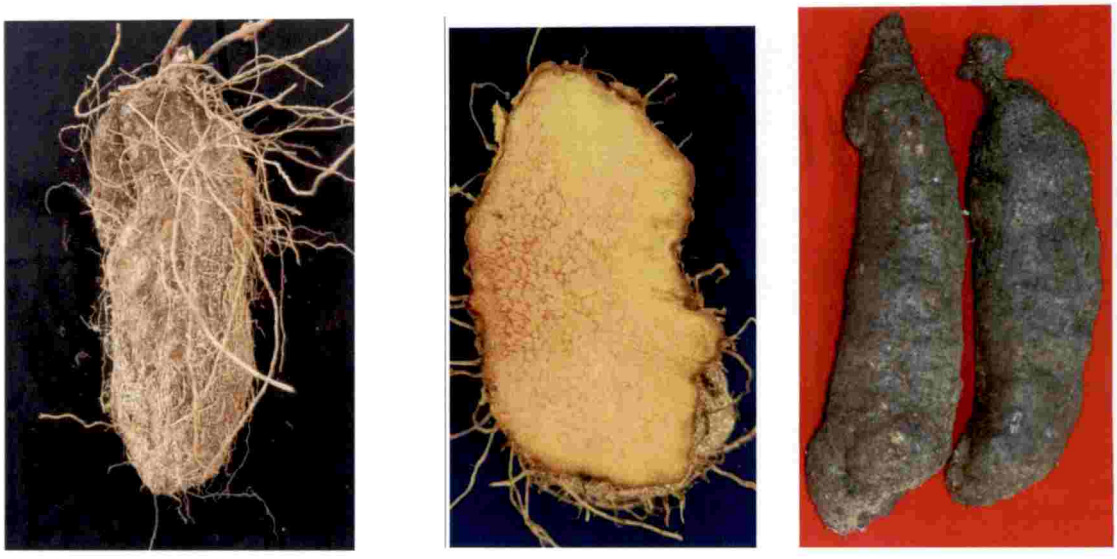
Sree Swathv

Jas 2

Da 377

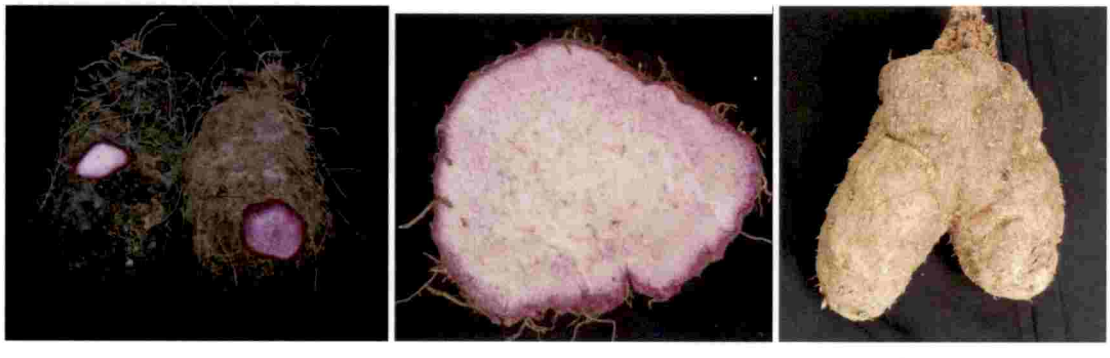
Da 489

Plate 2: Lab Screening images of major greater yam genotypes



Sree Swathy Da 110 Sree Nidhi

Plate 3: Tubers of major resistant genotypes identified



Sree Neelima Da 817 Da 810

Plate 4: Tubers of major susceptible genotypes identified

Table 13: Selected 30 accessions of greater yam with their lab screening and field screening scores.

Sl. No.	GENOTYPE	FIELD SCORE	LAB SCORE	R/S
1	Da 110	1	0	R
2	Da 198	2	3	S
3	Da 200	2	1	R
4	Da 209	3	1	S
5	Da 210	3	1	S
6	Da 293	1	2	R
7	Da 374	2	2	R
8	DaH 9-196	1	1	R
9	DaH22-2-3	2	2	R
10	DaH 58FG	1	1	R
11	TCR 308	2	2	R
12	TCR 319	2	2	R
13	Sree Karthika	3	1	R
14	Sree Keerthi	1	1	R
15	Sree Swathy	1	2	R
16	Da 264	2	2	R
17	Da 340	0	3	R
18	Da 817 V	1	4	S
19	JAS 2	1	0	R
20	TCR 226	1	4	S
21	TCR 64	1	3	S
22	Da 12	1	4	S
23	Da 377	3	2	S
24	Da 489	2	2	R
25	TCR 113	1	1	R
26	Da 820	4	5	S
27	Da H 17-5	3	3	S
28	Da 810	3	3	S
29	Orissa Elite	4	3	S
30	Sree Neelima	5	4	S

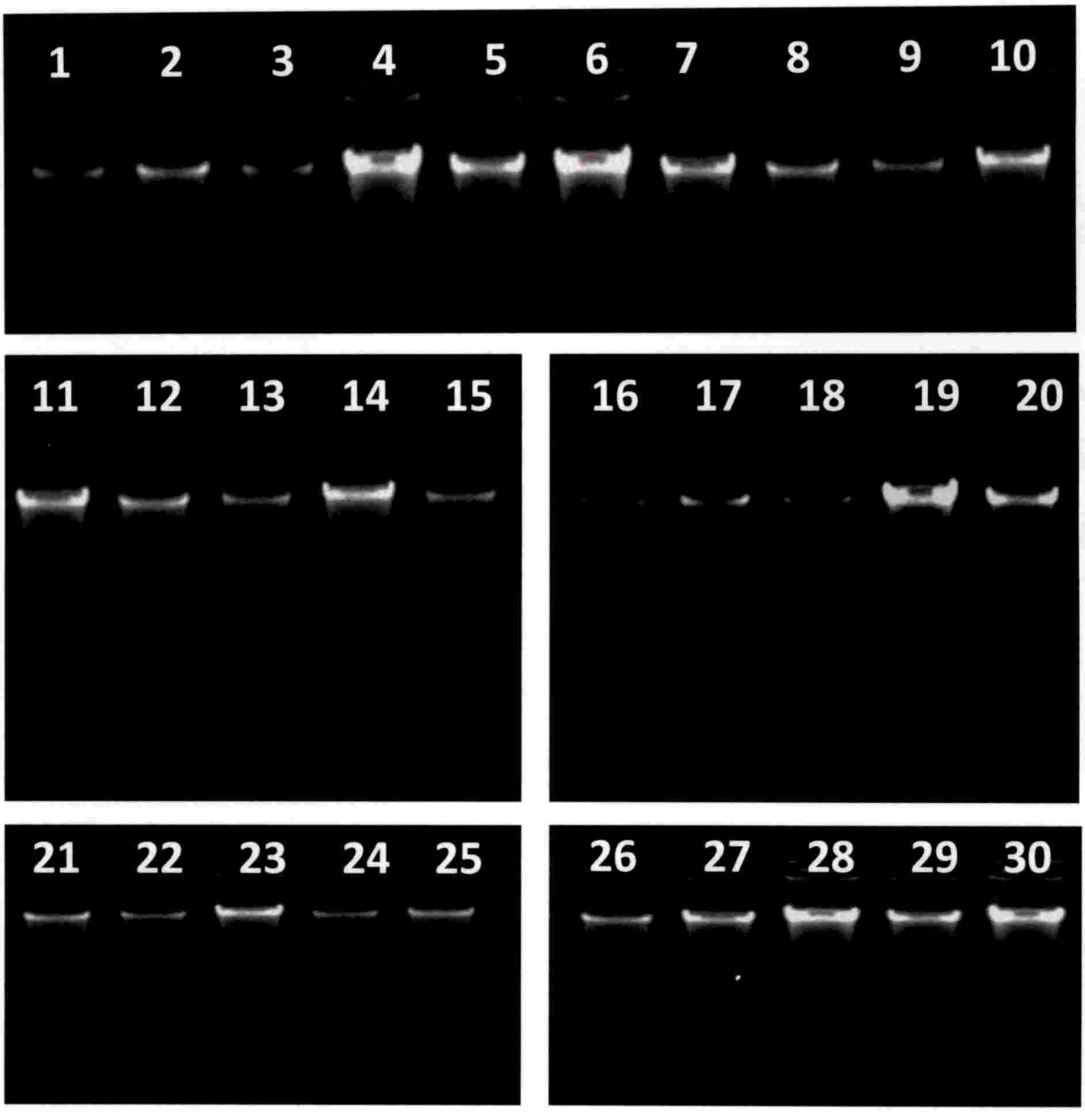
4.3 MOLECULAR DATA ANALYSIS

4.3.1 DNA Isolation

Isolation of DNA from collected samples of *Dioscorea alata* was done using modified protocol of Raj *et al.* (2014). Concentration of DNA in ng/µL, the absorbance ratio OD $_{260/280}$ and OD $_{260/230}$ were measured using Nano spectrophotometer (DENOVIX). The quantity as well as purity of the DNA obtained from spectrophotometric readings are included in Table 14. Concentration of isolated DNA ranged from 95.5 to 633.9. Good quality DNA were obtained with less RNA contamination. DNA gel check using 1% agarose is shown in plate 5.

Table14: Spectrophotometric readings of DNA isolated

Sl. NO.	Genotype	Concentration	A$_{260/280}$
1	Da 110	182.011	2.02
2	Da 198	215.805	1.38
3	Da 200	184.249	1.31
4	Da 209	633.855	1.26
5	Da 210	458.524	0.68
6	Da 293	929.4	2.16
7	Da 374	615.038	1.12
8	DaH 9-196	119.267	2.15
9	DaH22-2-3	228.253	1.26
10	DaH 58FG	270.099	1.58
11	TCR 308	299.087	0.95
12	TCR 319	507.556	0.98
13	Sree Karthika	138.877	2.16
14	Sree Keerthi	196.604	2.18
15	Sree Swathy	295.449	2.08
16	Da 264	95.437	2.17
17	Da 340	134.625	2.1
18	Da 817 V	522.127	1.07
19	JAS 2	246.654	1.04
20	TCR 226	238.481	1.75
21	TCR 64	118.469	1.33
22	Da 12	415.086	0.62
23	Da 377	536.554	1.05
24	Da 489	148.375	1.56
25	TCR 113	180.478	1.45
26	Da 820	208.722	2.01
27	Da H 17-5	261.206	1.82



1	2	3	4	5	6	7	8	9	10
Da 110	Da 198	Da 200	Da 209	Da 210	Da 293	Da 374	DaH 9-196	DaH 22-2-3	DaH 58 FG
11	12	13	14	15	16	17	18	19	20
TCR 308	TCR 319	Sree Keerthi	Sree karthika	Sree Swathy	Da 264	Da 340	Da 817 V	JAS 2	TCR 226
1	2	3	4	5	6	7	8	9	10
TCR 64	Da 12	Da 377	Da 489	TCR 113	Da 820	DaH 17-5	Da 810	Orissa Elite	Sree neelima

Plate 3: Agarose gel profile of DNA (0.8%) extracted from greater yam accessions

28	Da 810	209.364	1.85
29	Orissa Elite	473.075	2.17
30	SreeNeelima	389.127	2.01

4.3.2 PCR STANDARDIZATION

PCR conditions for newly designed SSR primers were standardized by gradient PCR to obtain clear and consistent amplicons under reduced cycle duration.

4.3.3 PRIMER SCREENING

For preliminary screening of ISSR, 15 ISSR primers were used of which, only 6 were selected based on their polymorphism and repeatability. Primers namely UBC 807, UBC 809, UBC 825, UBC 836, (GA)9AC and $(GA)_9AT$ were found to be highly polymorphic and hence were selected for further validation. Plates 6-11 shows the gel profiles of the ISSR primers used at annealing temperature of 56.3°C.

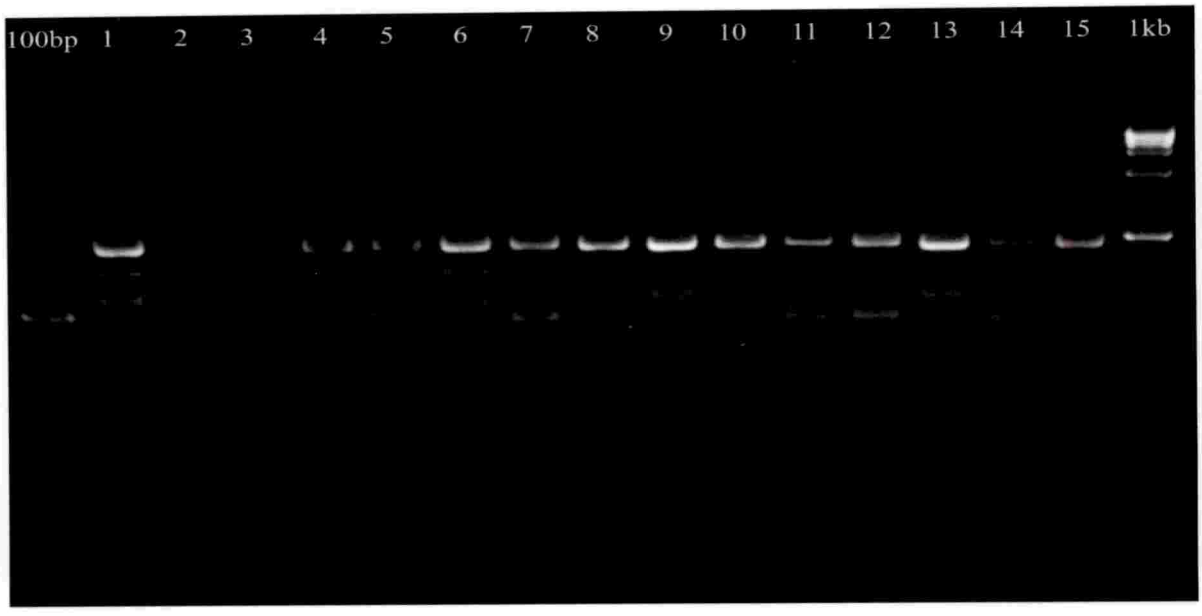
In the case of RAPD, 11 primers were used for initial screening of which, only 5 primers namely OPI-07, OPE-06, OPF-06, OPI-16 and OPAF-17 were selected for validation. Plates 12 – 16 shows the gel profiles of the RAPD primers used at annealing temperature of 36 °C.

Twentysix SSR primers were screened in which, four were already reported and the rest were newly designed. On screening these primers, YamSSR1, YamSSR2, YamSSR3, YamSSR4, YamSSR5, YamSSR6, YamSSR8, YamSSR9, YamSSR12, YamSSR15, YamSSR16, YamSSR18, YamSSR19, Dab2E07, Da3G04 and Dab2D08 were found to be highly polymorphic and hence were selected for further validation. The annealing temperature of newly synthesized primers were selected by online Tm calculator provided by Thermo fisher Scientific and some of the annealing temperatures were optimized by running gradient PCR and it ranges from 46.1°C to 60.1°C. Plates 17- 32 shows the gel profiles of the SSR primers used at annealing temperature of 51 °C.

4.3.4 Molecular characterization using ISSR markers

The amplicons obtained after the ISSR analysis of 30 accessions using the selected primers were resolved in two per cent agarose gel (Plates 6-11). Total number of bands per ISSR primer ranged from 13 (UBC 807 & UBC 836) to 26 (UBC 809). The primer (GA)9AT recorded 21 bands followed by (GA)9AC with

Molecular characterisation using ISSR markers



1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
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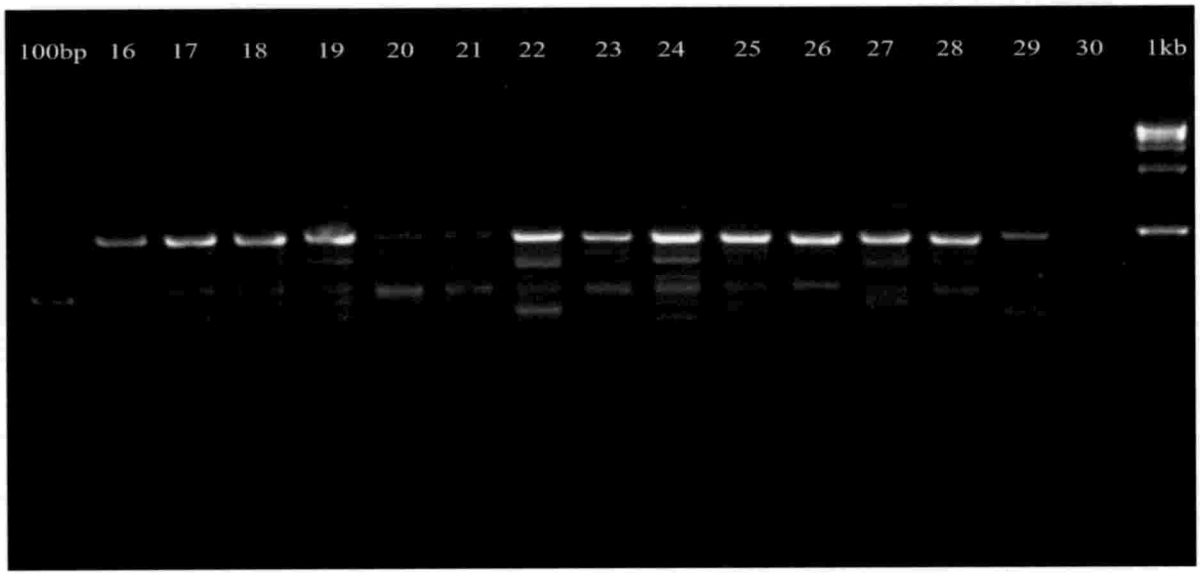
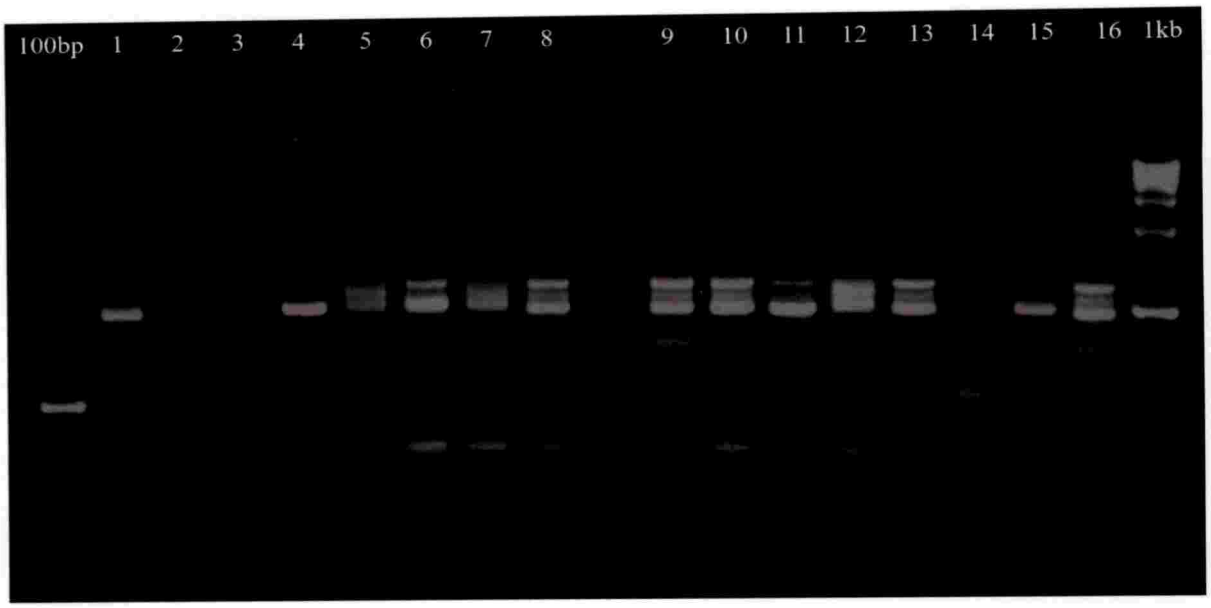


16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
DaH 22-2-3	DaH 58 FG	TCR 308	TCR 319	Sree karthika	Sree keerthi	Sree swathy	Da 377	Da 489	TCR 113	Da 820	DaH 17-5	Da 810	Orissa elite	Sree neelima

Plate 6: Agarose gel profile of the ISSR primer UBC 807 for 30 accessions



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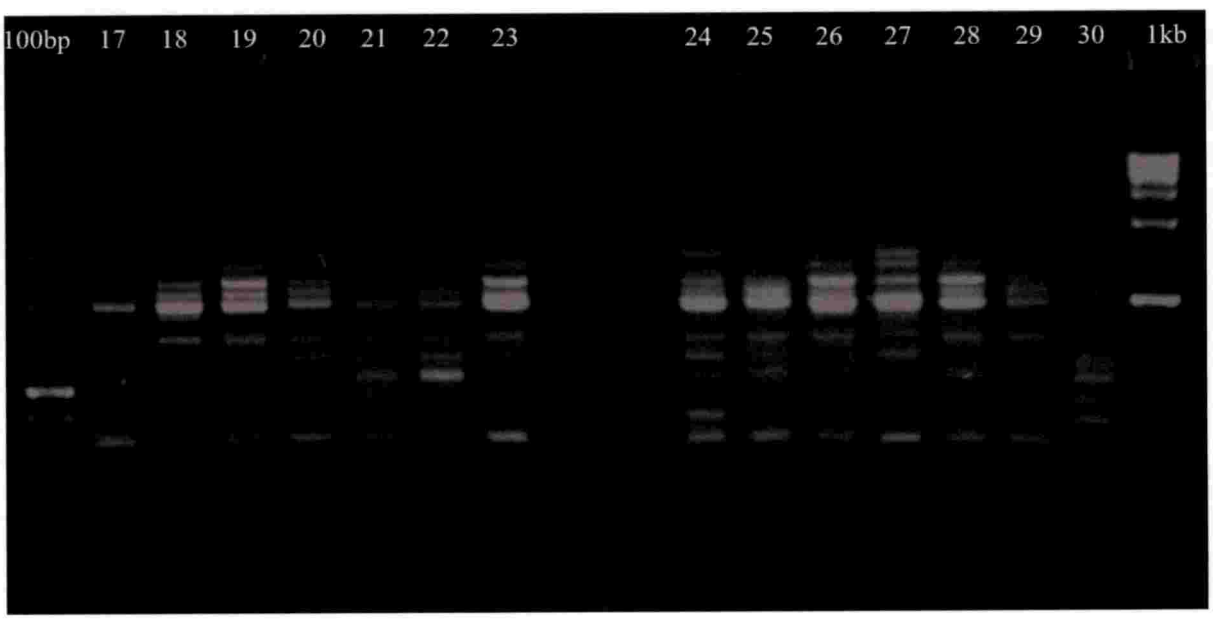

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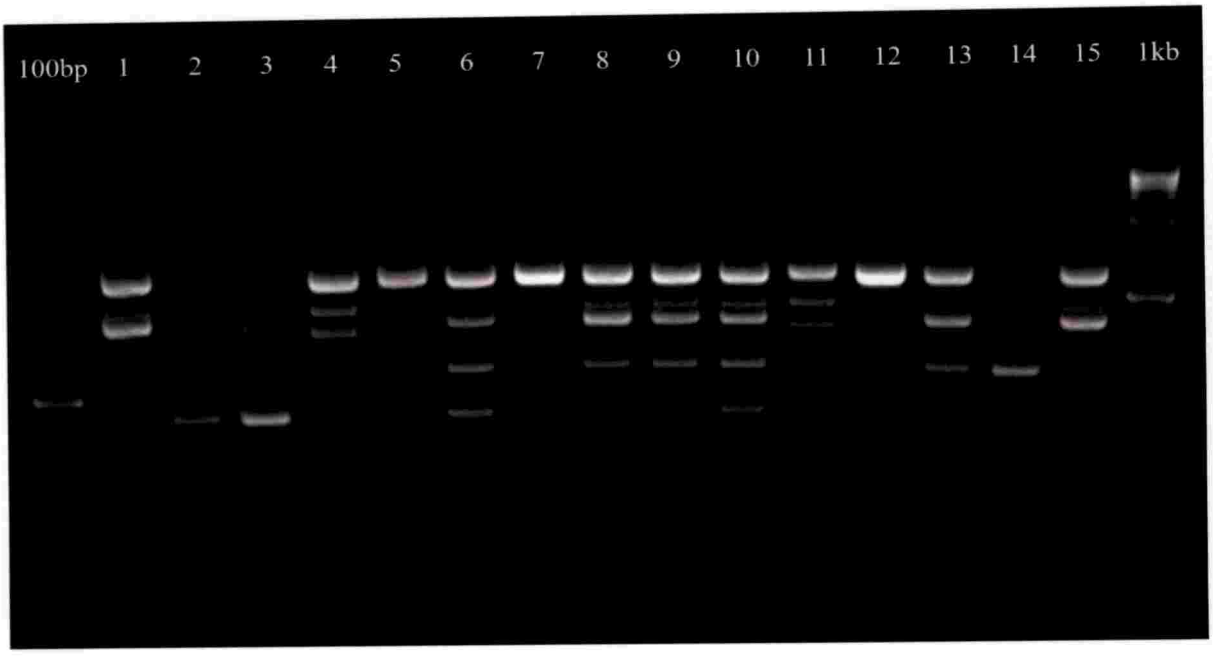
DaH 22-2-3	DaH 58 FG	TCR 308	TCR 319	Sree karthika	Sree keerthi	Sree swathy	Da 489	TCR 113	Da 820	DaH 17-5	Da 810	Orissa elite	Sree neelima

Plate 7: Agarose gel profile of the ISSR primer UBC 809 for 30 accessions



1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
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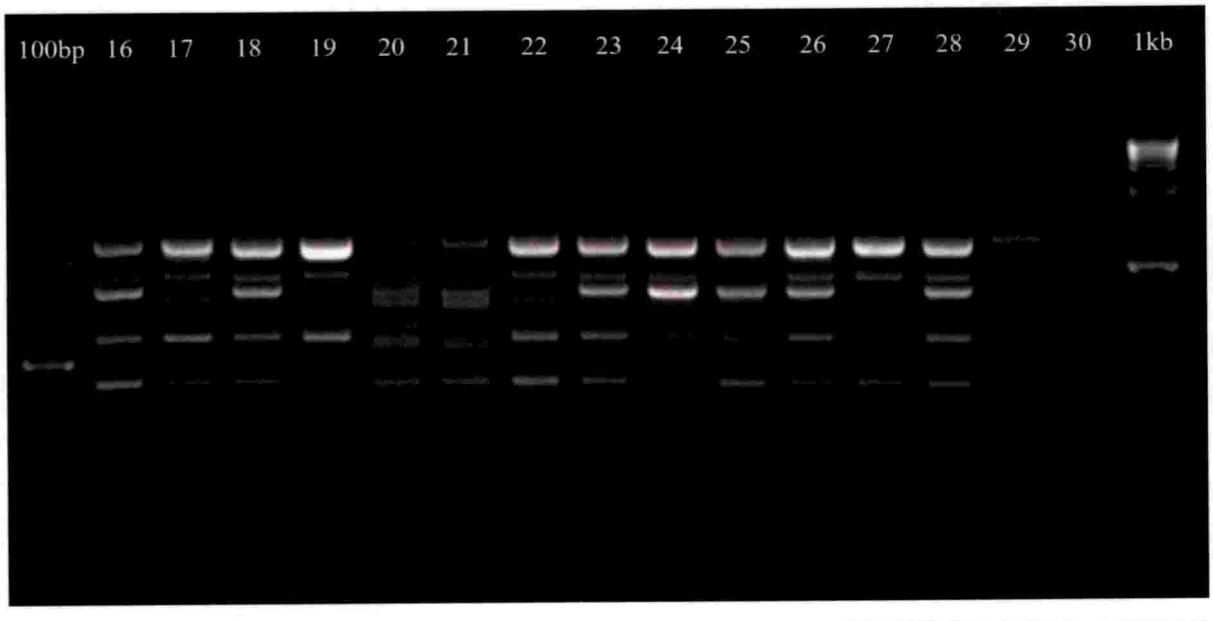
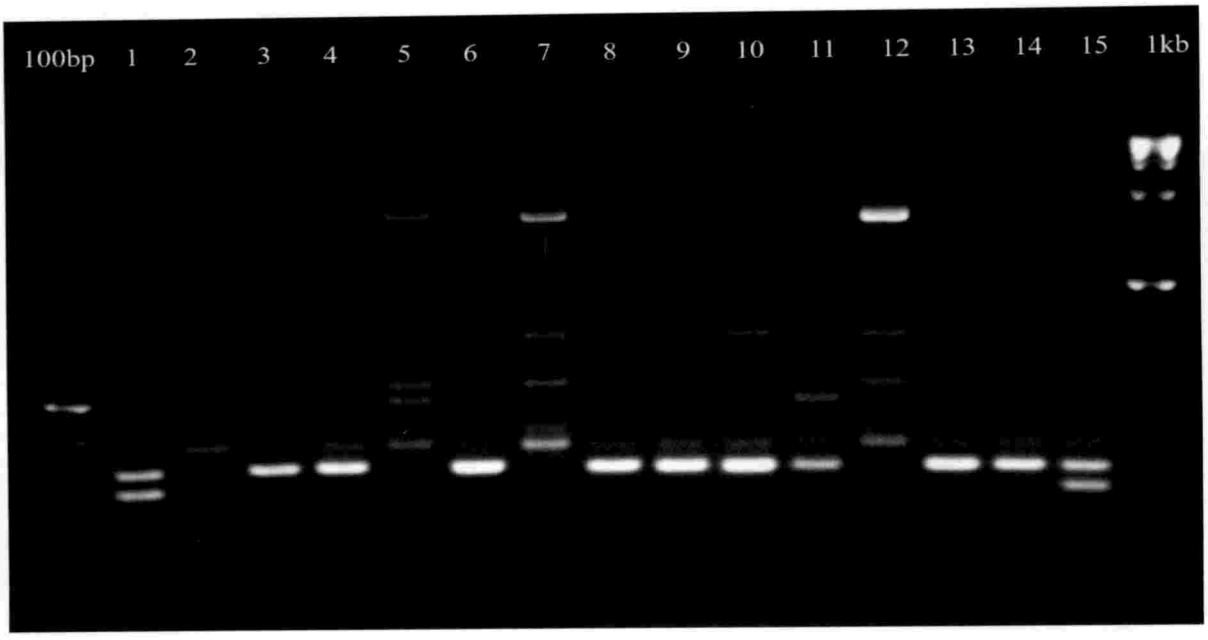


16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
DaH 22-2-3	DaH 58 FG	TCR 308	TCR 319	Sree karthika	Sree keerthi	Sree swathy	Da 377	Da 489	TCR 113	Da 820	DaH 17-5	Da 810	Orissa elite	Sree neelima

Plate 8: Agarose gel profile of the ISSR primer UBC 825 for 30 accessions



1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
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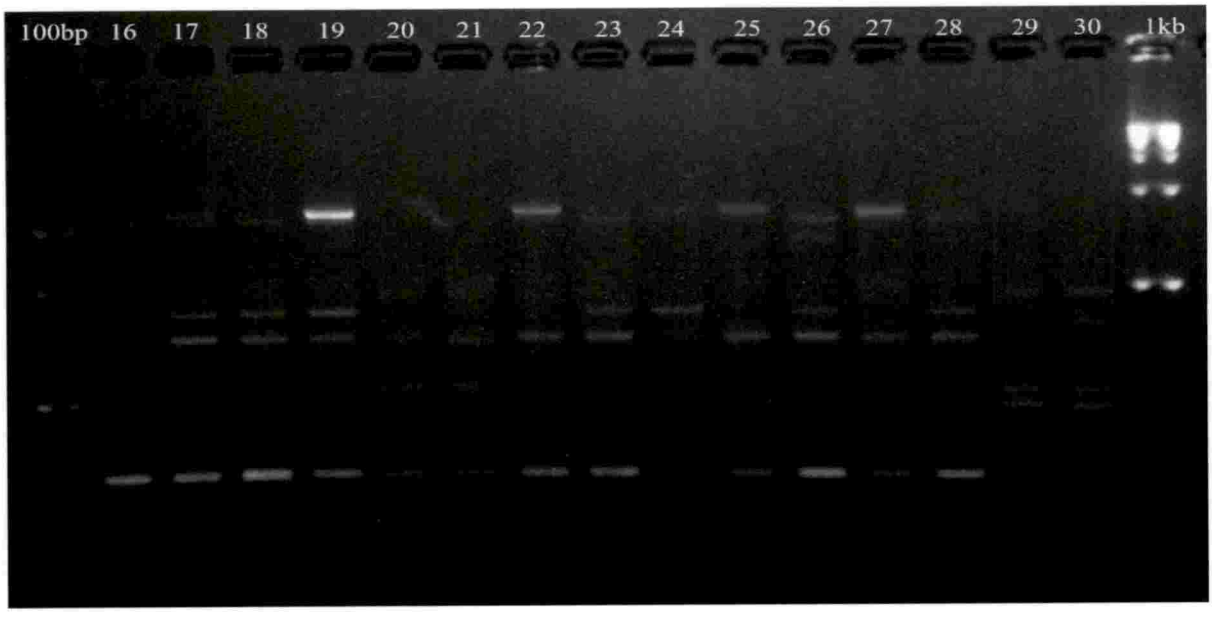
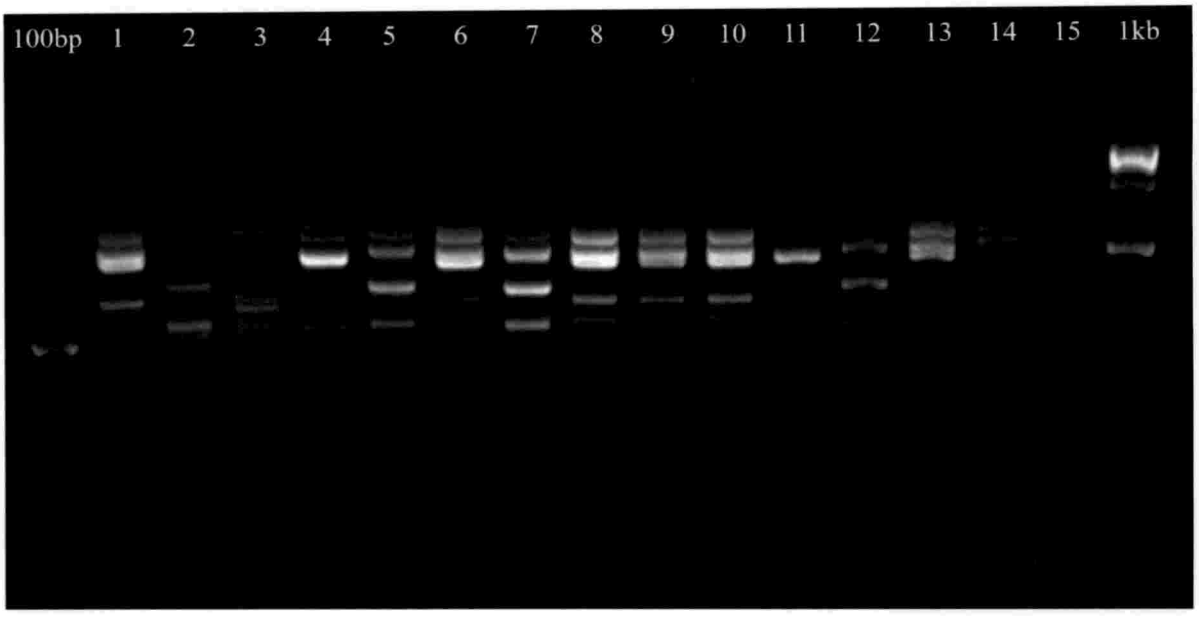
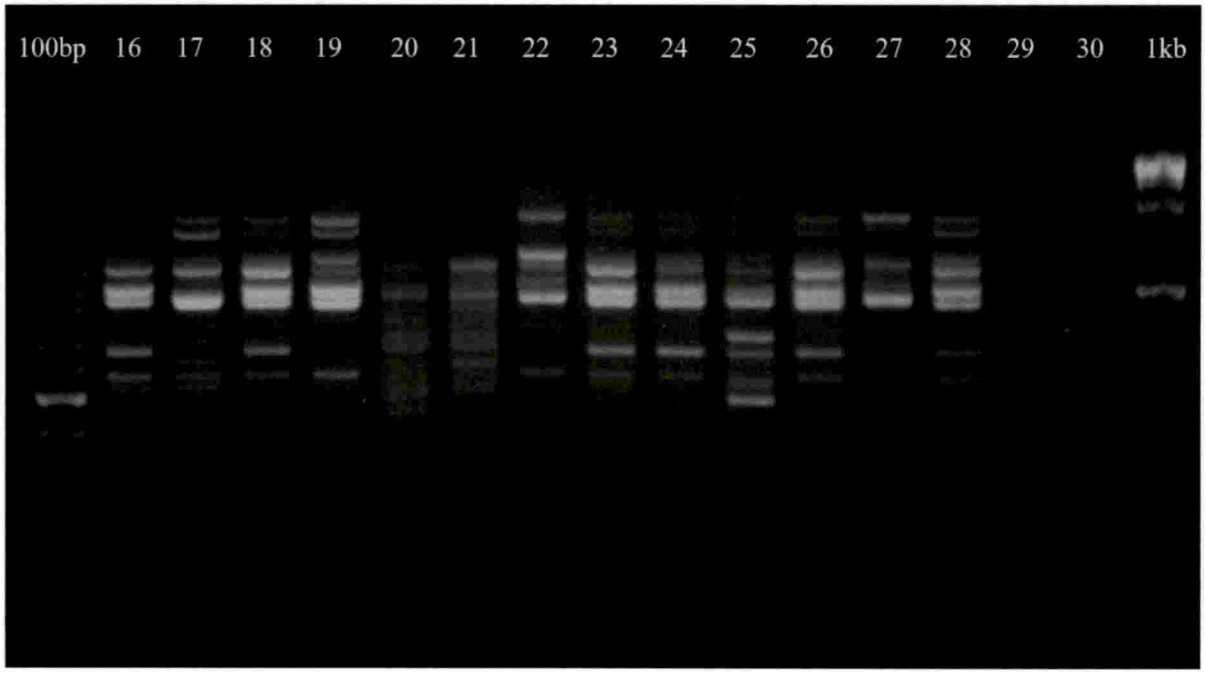


16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
DaH 22-2-3	DaH 58 FG	TCR 308	TCR 319	Sree karthika	Sree keerthi	Sree swathy	Da 377	Da 489	TCR 113	Da 820	DaH 17-5	Da 810	Orissa elite	Sree neelima

Plate 9: Agarose gel profile of the ISSR primer UBC 836 for 30 accessions

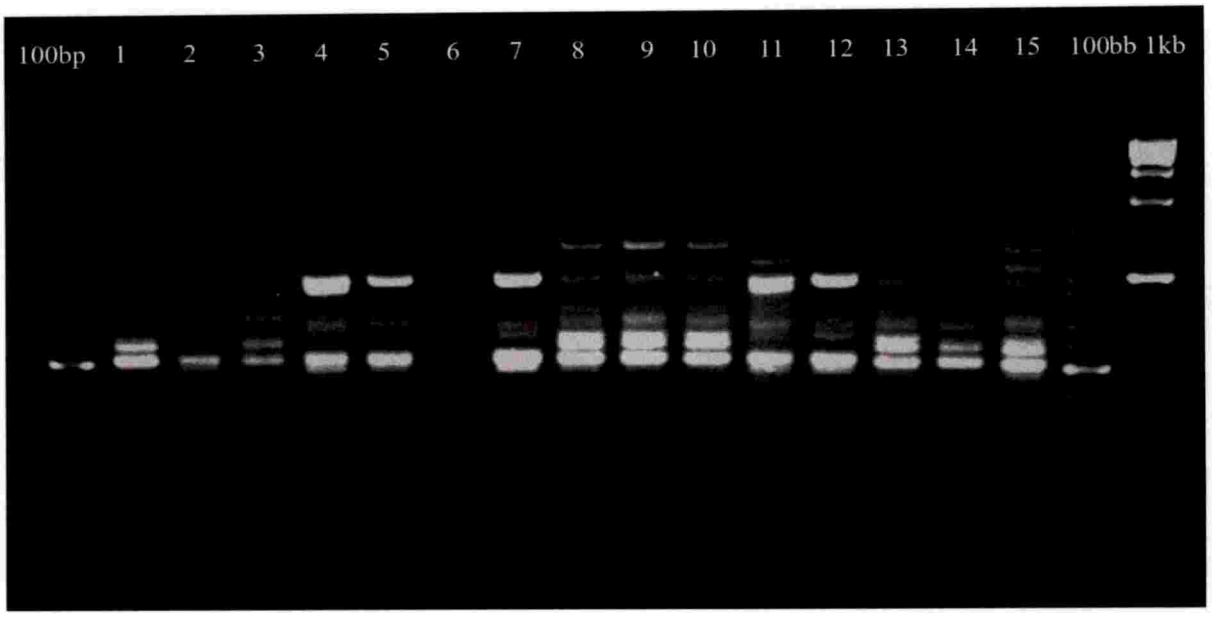


1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Da 110	Da 198	Da 200	Da 209	Da 210	Da 293	Da 374	DaH 9-196	Da 264	Da 340	Da 817 V	JAS 2	TCR 226	TCR 64	Da 12



16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
DaH 22-2-3	DaH 58 FG	TCR 308	TCR 319	Sree karthika	Sree keerthi	Sree swathy	Da 377	Da 489	TCR 113	Da 820	DaH 17-5	Da 810	Orissa elite	Sree neelima

Plate 10: Agarose gel profile of the ISSR primer (GA)9AC for 30 accessions



1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
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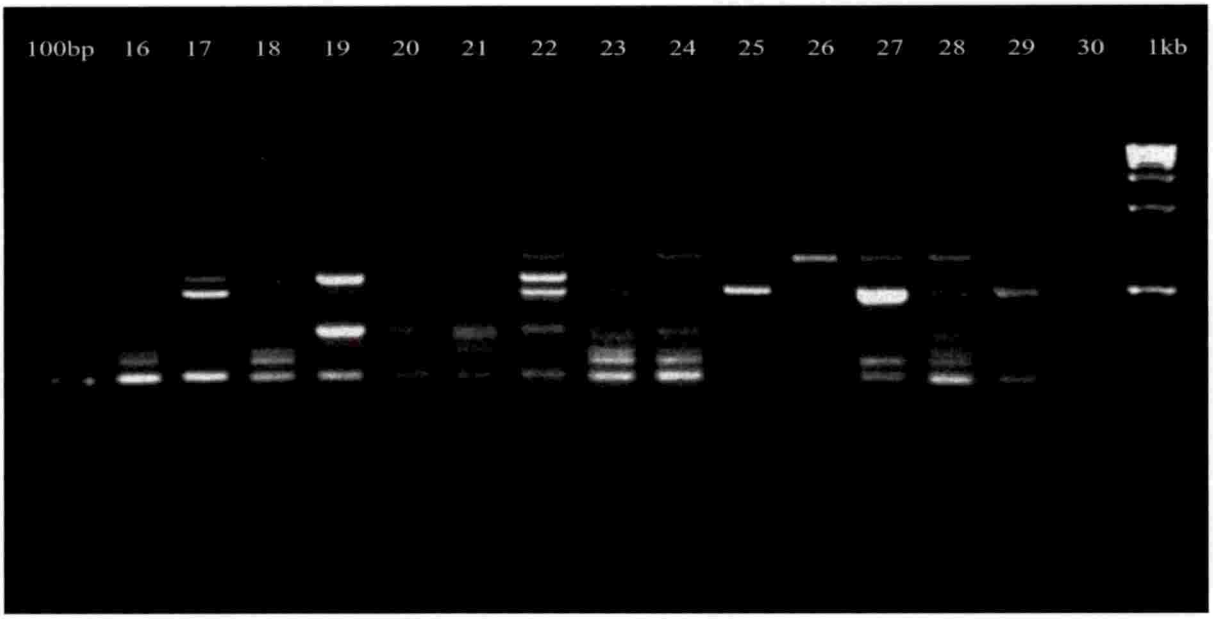


16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
DaH 22-2-3	DaH 58 FG	TCR 308	TCR 319	Sree karthika	Sree keerthi	Sree swathy	Da 377	Da 489	TCR 113	Da 820	DaH 17-5	Da 810	Orissa elite	Sree neelima

Plate 11: Agarose gel profile of the ISSR primer (GA)9AT for 30 accessions

19 bands. Eighteen bands were recorded in the primer UBC 825. All the amplicons of UBC 825, (GA)9AT and (GA)9AC were found to be polymorphic. UBC 809 that recorded the highest number of bands (26) also recorded the highest number of polymorphic bands (25) followed by UBC 827 with nine amplicons. UBC 836 showed the lowest number of polymorphic bands (11) and the highest number of monomorphic bands (2). The polymorphism of the ISSR primers studied ranged from 84.61% to 100%. UBC 807 recorded the lowest polymorphism (84.61%) among the primers followed by UBC 836 (92.31%).

The expected heterozygosity value (He) of the ISSR primers ranged between 0.53 (UBC 836) to 0.81 ((GA)9AC). The polymorphism Information content (PIC) of the primers ranged from 0.5164 (UBC 836) to 0.7929 ((GA)9AC). The primers, UBC 807, UBC 809, UBC 825 and (GA)9AT recorded PIC values 0.7074, 0.7046, 0.6808 and 0.6722 respectively. All the primers recorded high PIC value of >0.6. The gene diversity values ranged from 0.1680 to 0.2104. The primers UBC 836, (GA)9AC and (GA)9AT recorded the same gene diversity value of 0.1680.

Table 15: Genetic characteristics of the ISSR markers used

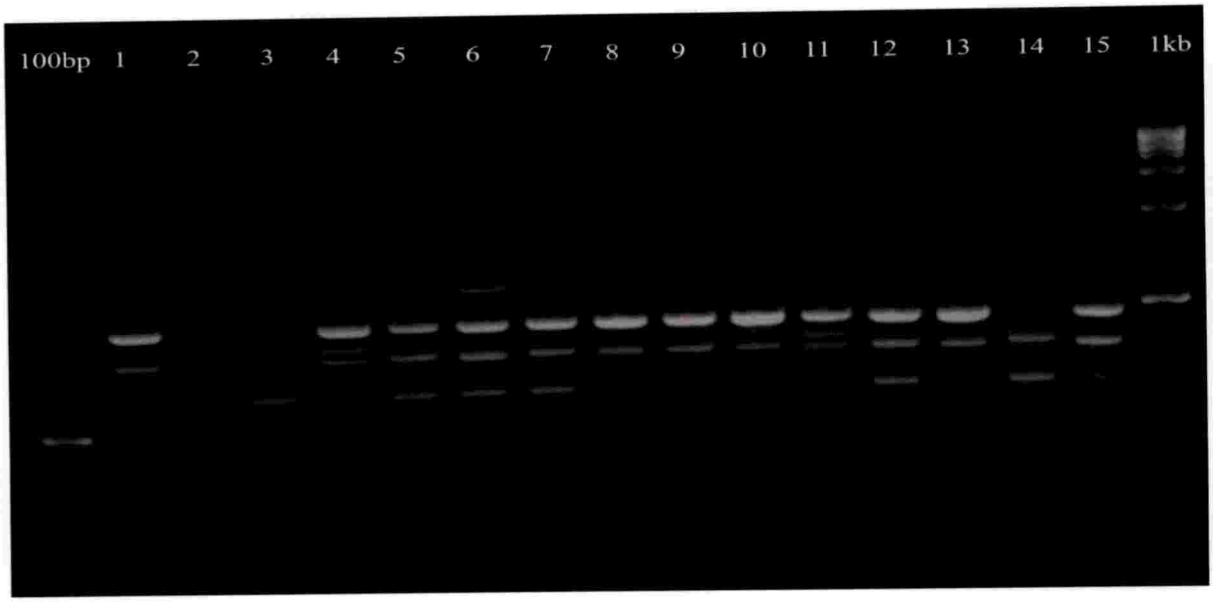
Marker	Total No. of alleles	No. of polymorphic alleles	% polymorphism	He	PIC	Gene Diversity
UBC807	13	11	84.61	0.7477	0.7074	0.2046
UBC809	18	18	100.00	0.7332	0.7046	0.2104
UBC825	26	25	96.15	0.7258	0.6802	0.1673
UBC836	13	12	92.31	0.5334	0.5164	0.1680
(GA)9AC	21	21	100.00	0.8169	0.7929	0.1680
(GA)9AT	19	19	100.00	0.7146	0.6722	0.1680

4.3.5 Molecular characterization using RAPD markers

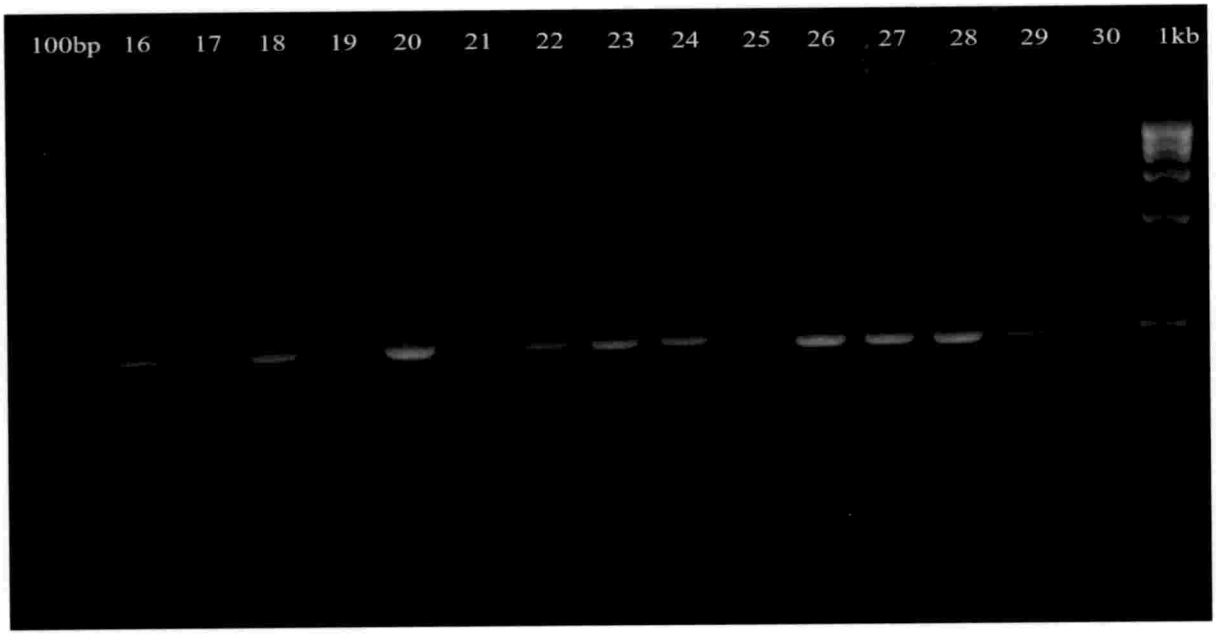
The amplicons obtained after the RAPD analysis of 30 accessions using the selected primers were resolved in two percent agarose gel (Plates 12- 16).

Total number of bands per RAPD primer ranged from 14 to 24. All the primers except OPE 06 showed 100% polymorphism. Out of 18 bands of OPE 06, two were monomorphic and the rest 16 were polymorphic. The primer OPI 16 couldn't amplify many of the DNA samples and hence no bands were formed in

Molecular characterisation using RAPD markers

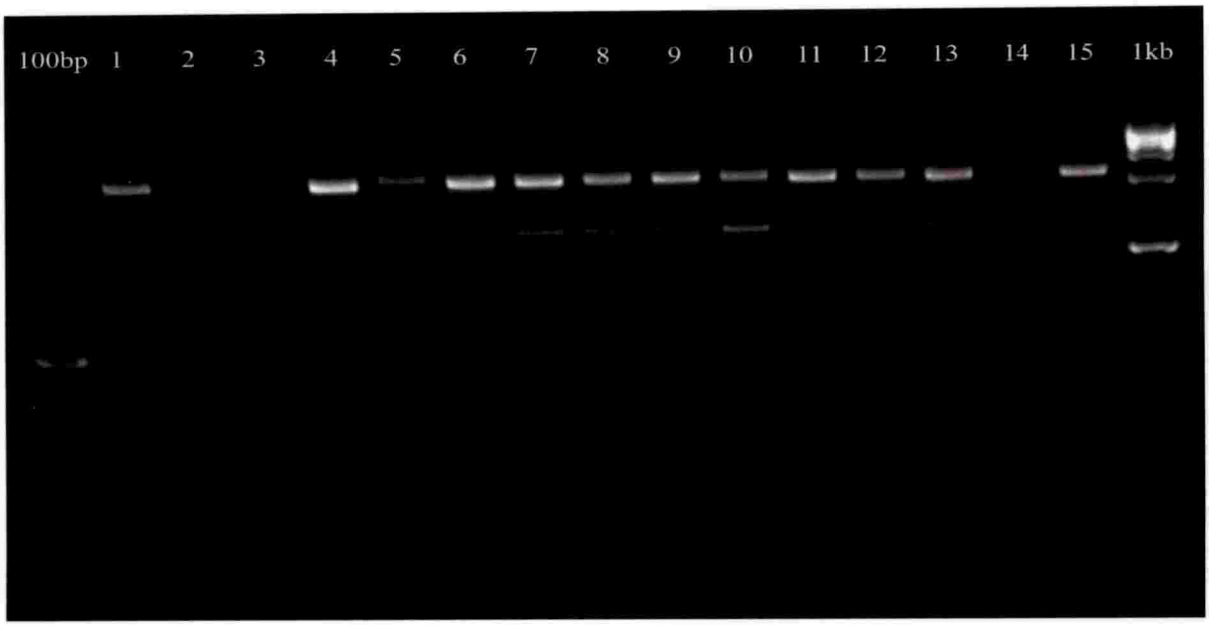


1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Da 110	Da 198	Da 200	Da 209	Da 210	Da 293	Da 374	DaH 9-196	Da 264	Da 340	Da 817 V	JAS 2	TCR 226	TCR 64	Da 12

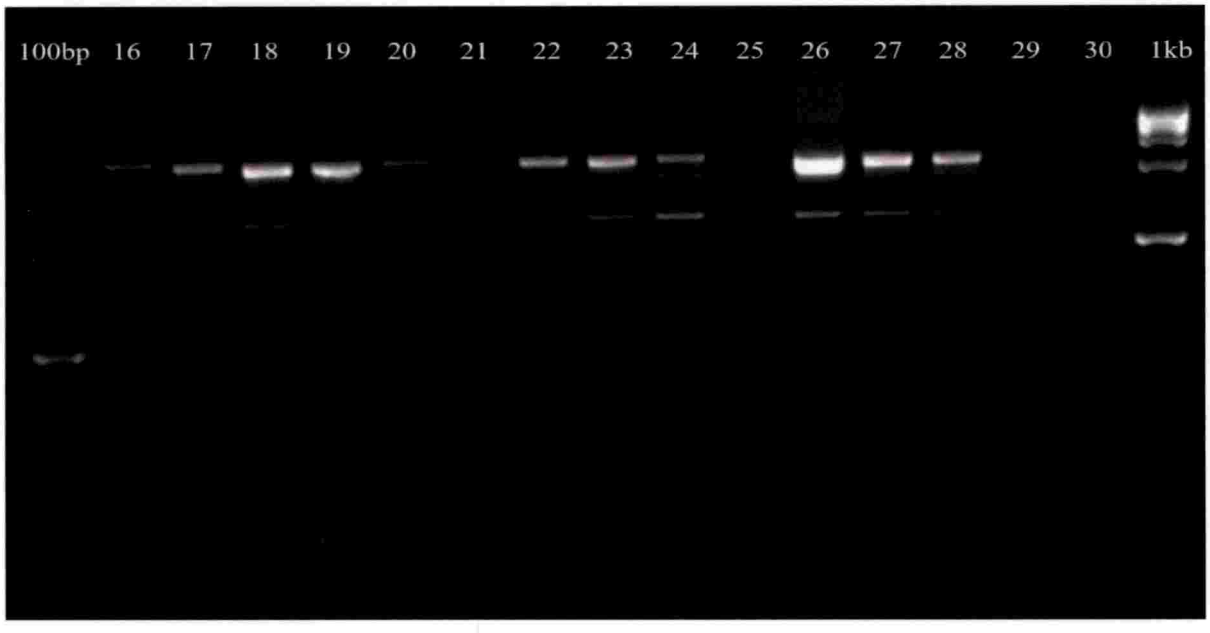


16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
DaH 22-2-3	DaH 58 FG	TCR 308	TCR 319	Sree karthika	Sree keerthi	Sree swathy	Da 377	Da 489	TCR 113	Da 820	DaH 17-5	Da 810	Orissa elite	Sree neelima

Plate 12: Agarose gel profile of the RAPD primer OPE 06 for 30 accessions

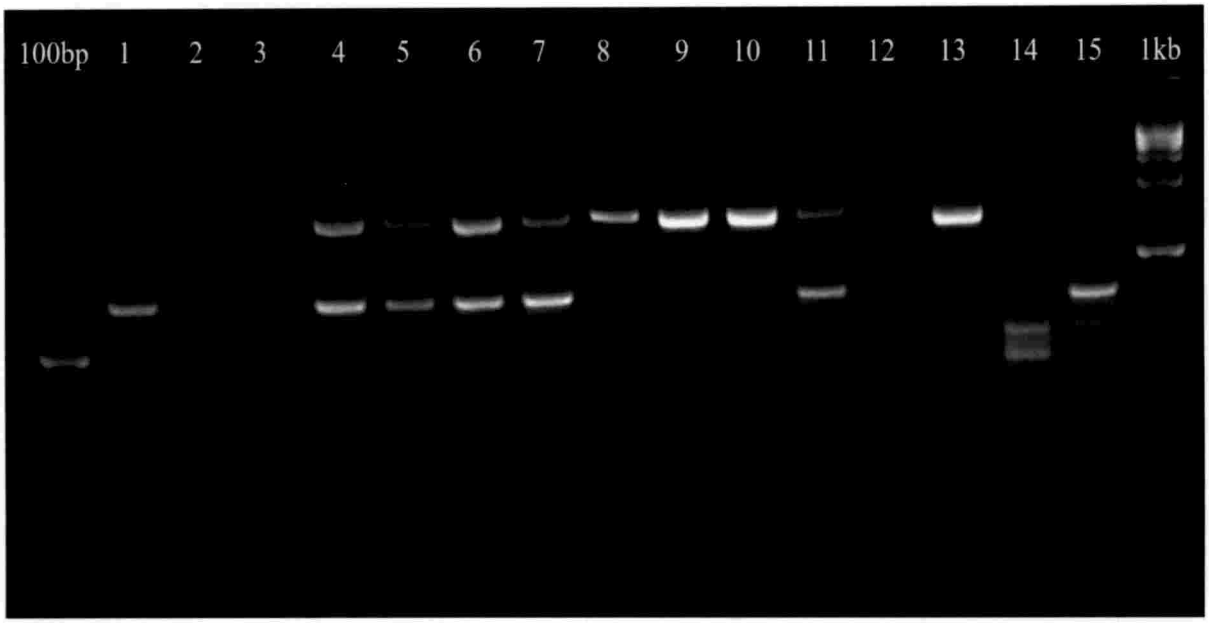


1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Da 110	Da 198	Da 200	Da 209	Da 210	Da 293	Da 374	DaH 9-196	Da 264	Da 340	Da 817 V	JAS 2	TCR 226	TCR 64	Da 12

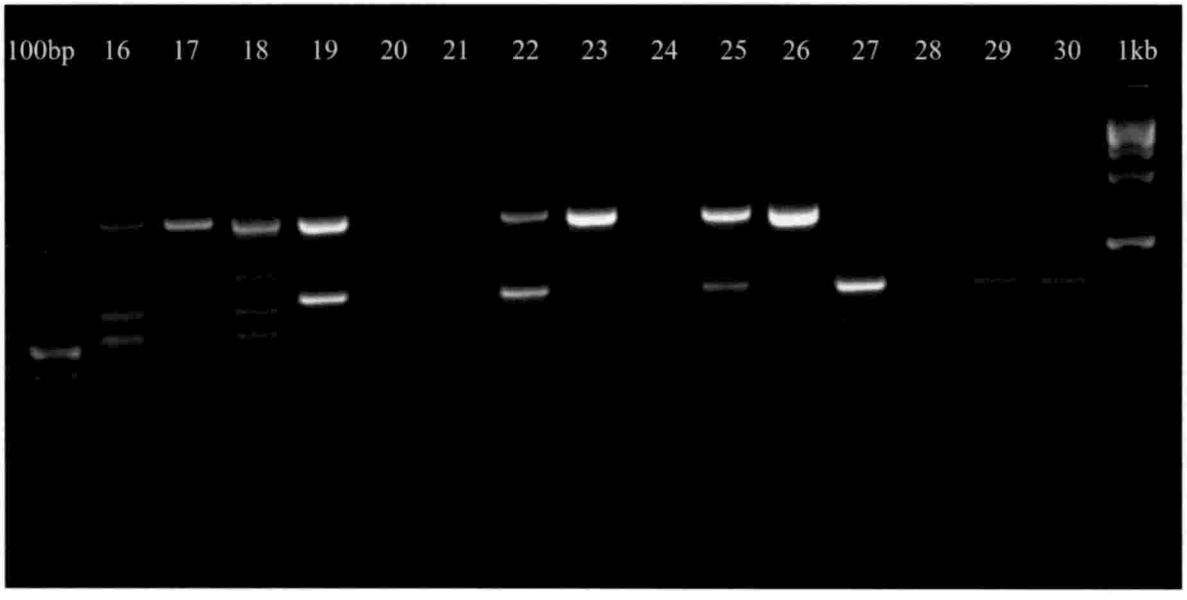


16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
DaH 22-2-3	DaH 58 FG	TCR 308	TCR 319	Sree karthika	Sree keerthi	Sree swathy	Da 377	Da 489	TCR 113	Da 820	DaH 17-5	Da 810	Orissa elite	Sree neelima

Plate 13: Agarose gel profile of the RAPD primer OPF 06 for 30 accessions

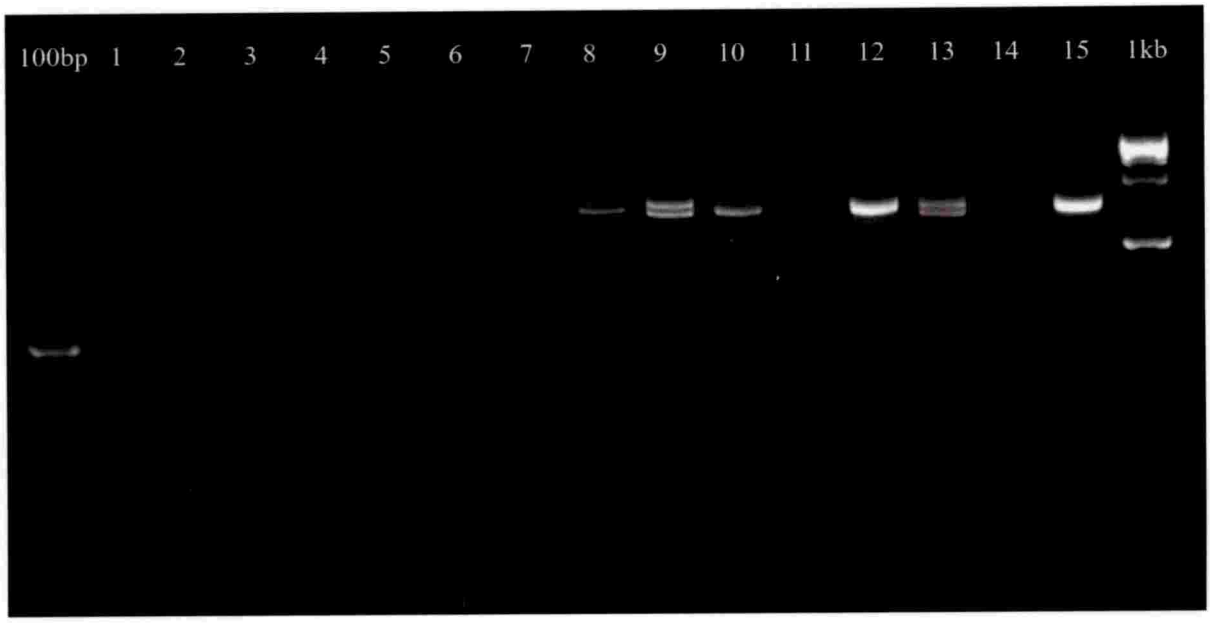


1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Da 110	Da 198	Da 200	Da 209	Da 210	Da 293	Da 374	DaH 9-196	Da 264	Da 340	Da 817 V	JAS 2	TCR 226	TCR 64	Da 12

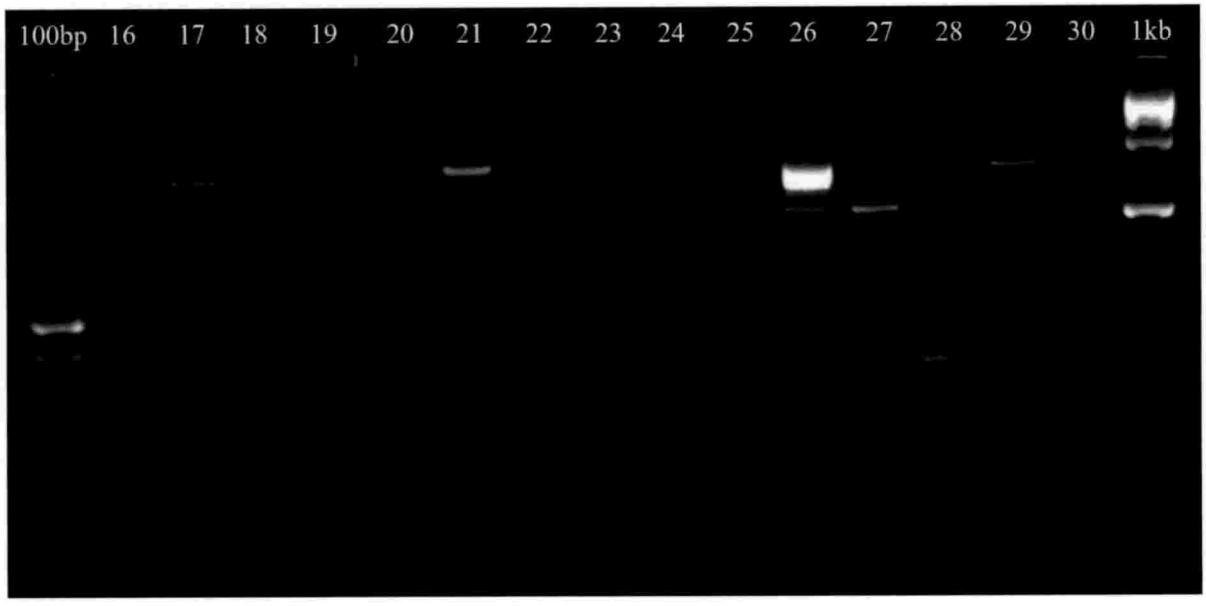


16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
DaH 22-2-3	DaH 58 FG	TCR 308	TCR 319	Sree karthika	Sree keerthi	Sree swathy	Da 377	Da 489	TCR 113	Da 820	DaH 17-5	Da 810	Orissa elite	Sree neelima

Plate 14: Agarose gel profile of the RAPD primer OPI 07 for 30 accessions

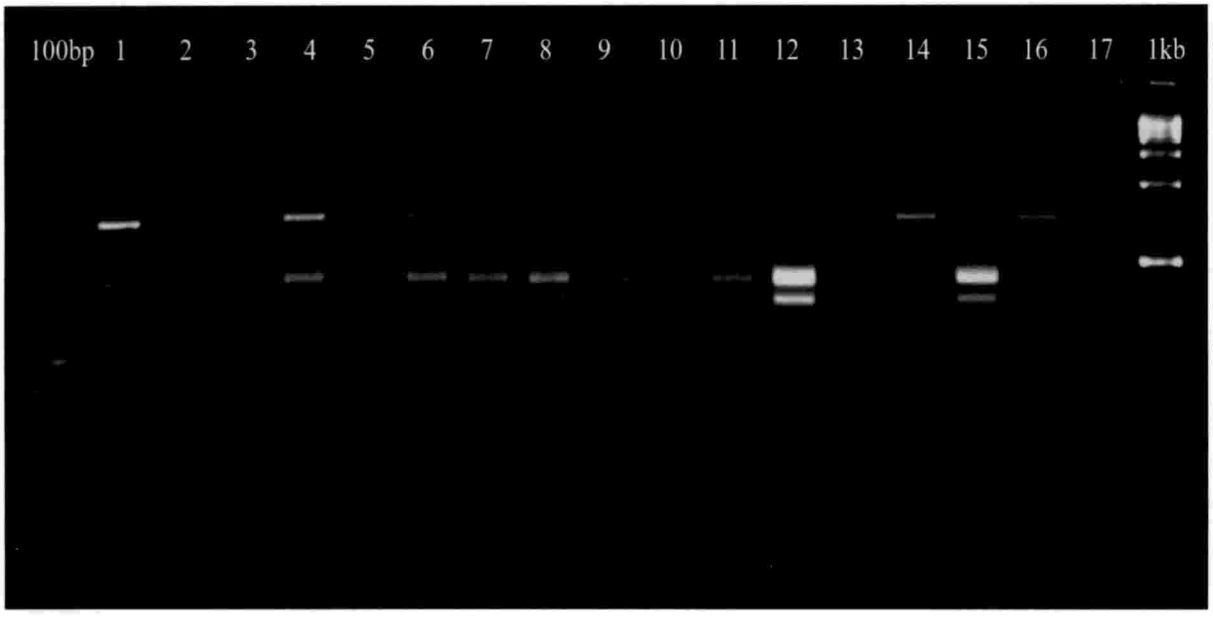


1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Da 110	Da 198	Da 200	Da 209	Da 210	Da 293	Da 374	DaH 9-196	Da 264	Da 340	Da 817 V	JAS 2	TCR 226	TCR 64	Da 12

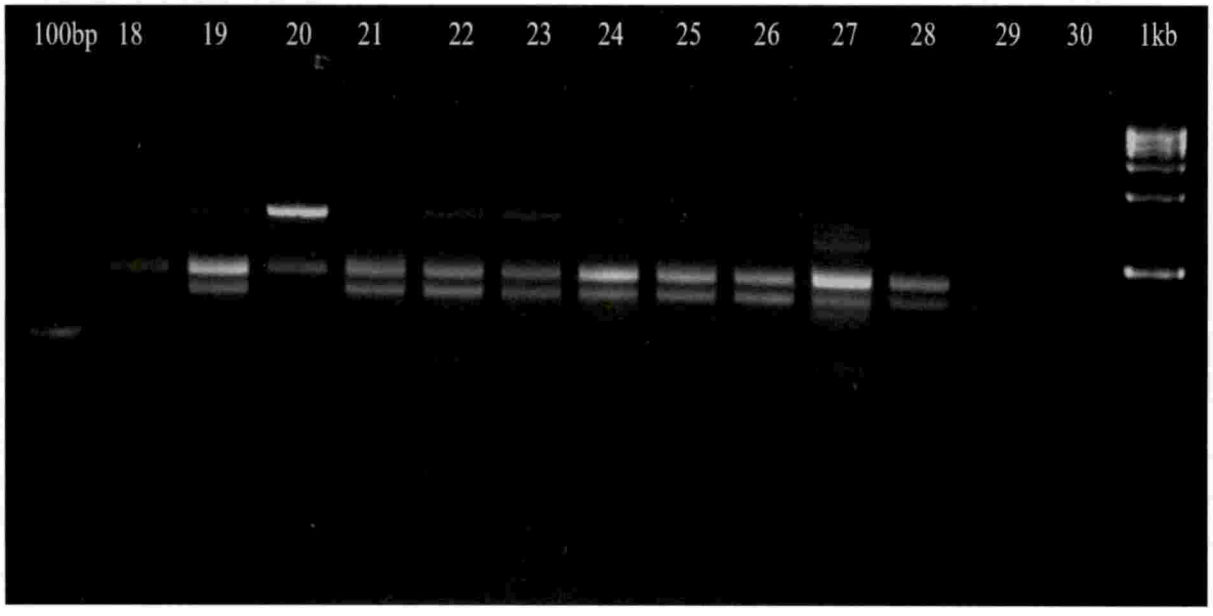


16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
DaH 22-2-3	DaH 58 FG	TCR 308	TCR 319	Sree karthika	Sree keerthi	Sree swathy	Da 377	Da 489	TCR 113	Da 820	DaH 17-5	Da 810	Orissa elite	Sree neelima

Plate 15: Agarose gel profile of the RAPD primer OPI 16 for 30 accessions



1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Da 110	Da 198	Da 200	Da 209	Da 210	Da 293	Da 374	DaH 9-196	Da 264	Da 340	Da 817 V	JAS 2	TCR 226	TCR 64	Da 12



16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
DaH 22-2-3	DaH 58 FG	TCR 308	TCR 319	Sree karthika	Sree keerthi	Sree swathy	Da 377	Da 489	TCR 113	Da 820	DaH 17-5	Da 810	Orissa elite	Sree neelima

Plate 16: Agarose gel profile of the RAPD primer OPAC 17 for 30 accessions

these tracks. The primer OPI 07 recorded the highest number of bands (24) whereas the primer OPF 06 produced the lowest number of bands (14). OPE 06 is the only RAPD primer that produced monomorphic bands. The primer OPE 06 exhibited 88.89% polymorphism and 100% polymorphism was observed in the rest four primers.

The expected heterozygosity values ranged from 0.5120 (OPF 06) to 0.7476 (OPE 06). OPF 06 exhibited the lowest PIC value 0.4870. The highest PIC value of 0.7117 was recorded by OPE 06 followed by OPI 16 (0.6287), OPAF 17 (0.5515) and OPI 07 (0.5048). All the primers showed gene diversity values <0.2. The highest gene diversity value was expressed by the primer OPE 06 (0.1754) and the lowest value is 0.1314 expressed by OPAF 17.

Table 16: Genetic characteristics of the RAPD markers used

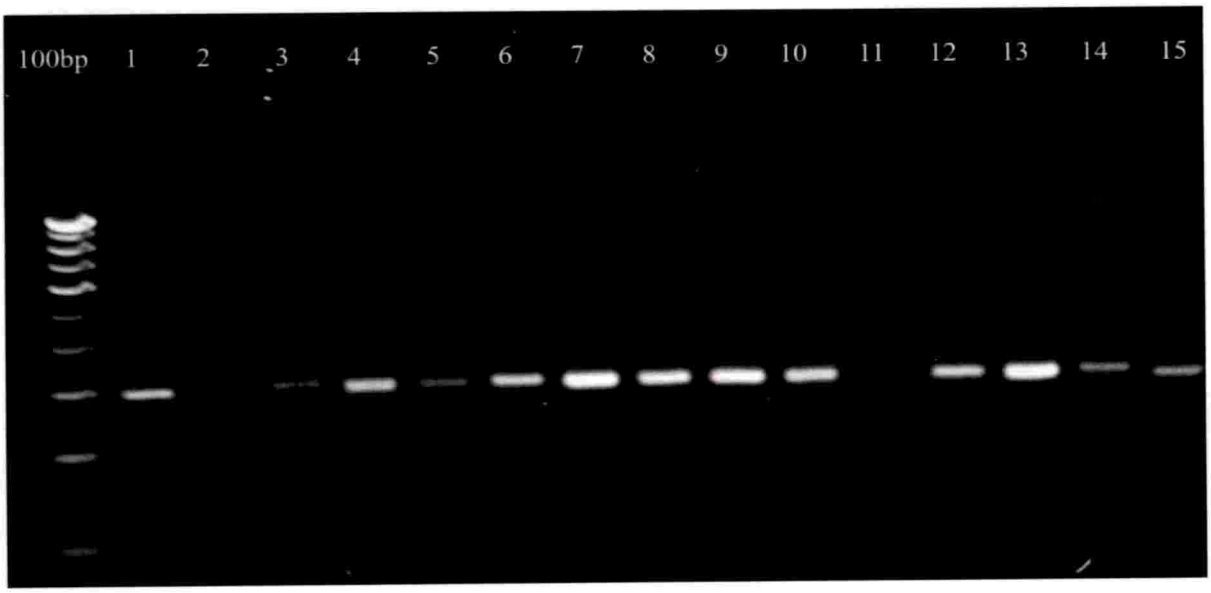
Marker	Total No. of alleles	No. of polymorphic alleles	% polymorphism	He	PIC	Gene Diversity
OPI07	24	24	100.00	0.5453	0.5048	0.1611
OPF06	14	14	100.00	0.5120	0.4870	0.1584
OPE06	18	16	88.89	0.7476	0.7117	0.1754
OPI16	22	22	100.00	0.6812	0.6287	0.1370
OPAF17	17	17	100.00	0.6011	0.5515	0.1314

4.3.5 Molecular characterization using SSR markers

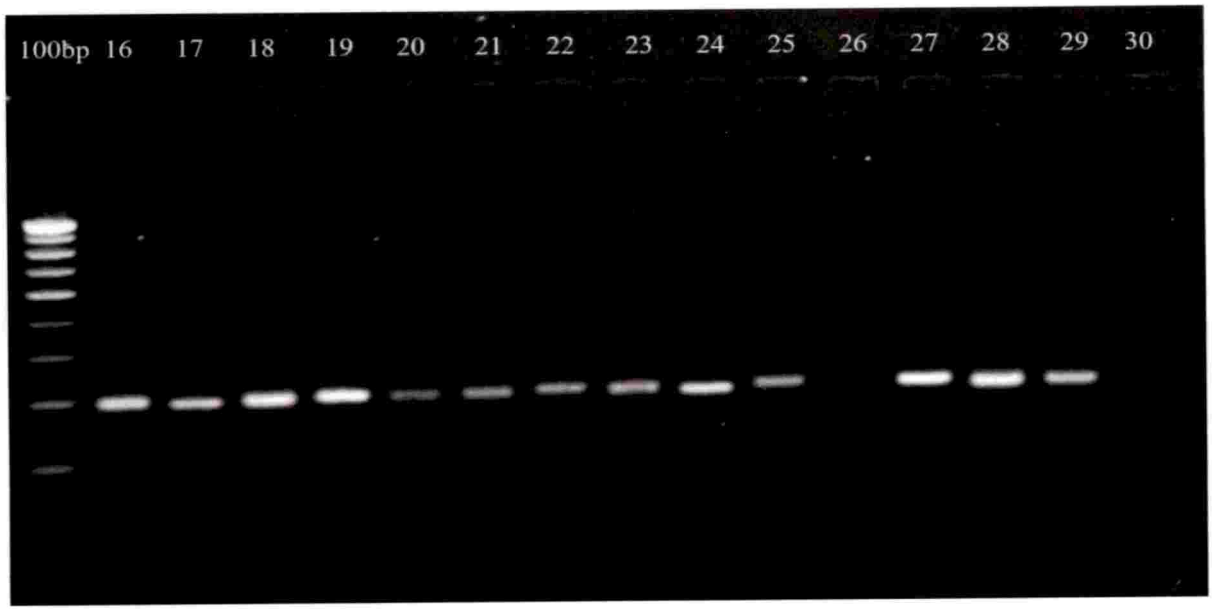
The amplicons obtained after the SSR analysis of 30 greater yam genotypes using the selected primers were initially resolved in four percent agarose gel (Plates 17- 32).

Among the sixteen SSR markers studied, both the number of alleles per marker and the number of polymorphic alleles ranged from 1 to 3. Yam SSR 06, Yam SSR 09, and Yam SSR 16 showed highest number of bands (3) in which, all the three bands of Yam SSR 09 were found to be polymorphic. But the bands produced by Yam SSR 02, Yam SSR 03, Yam SSR 15,Yam SSR 18 and Yam SSR 19 were highly monomorphic. The primers Yam SSR 8 and Yam SSR 19 were found to be of poor quality since they produced more unwanted bands. The primer DabD08 produced two unique bands with the accessions Da 209 and Da 817V at a

Molecular characterisation using SSR markers

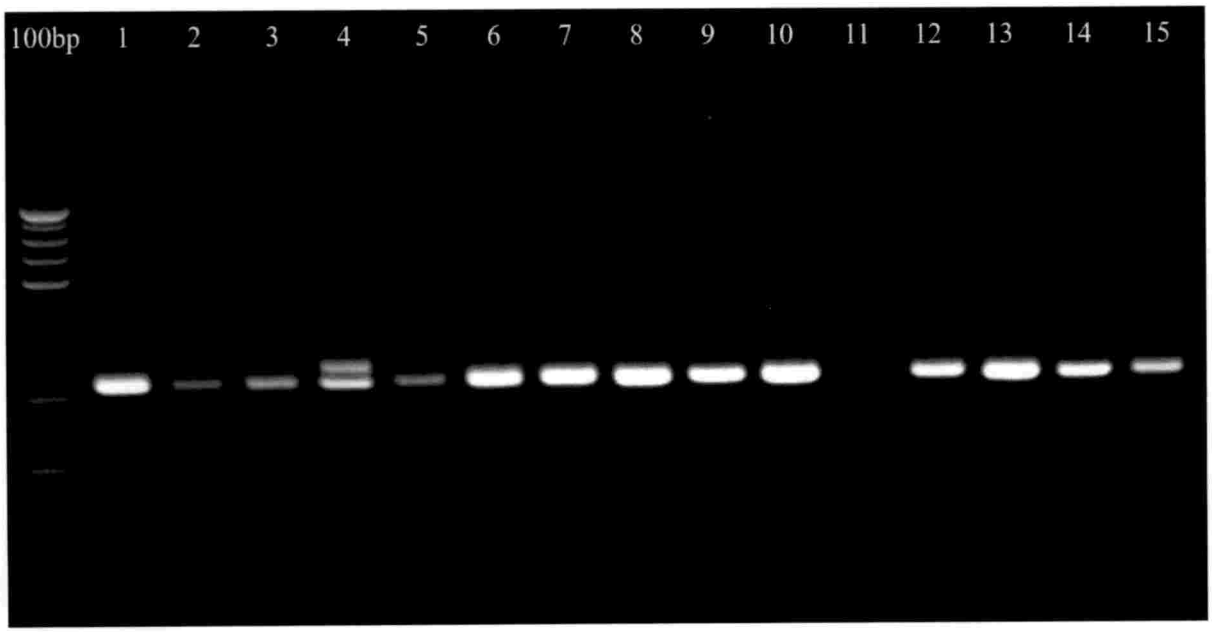


1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Da 110	Da 198	Da 200	Da 209	Da 210	Da 293	Da 374	DaH 9-196	Da 264	Da 340	Da 817 V	JAS 2	TCR 226	TCR 64	Da 12

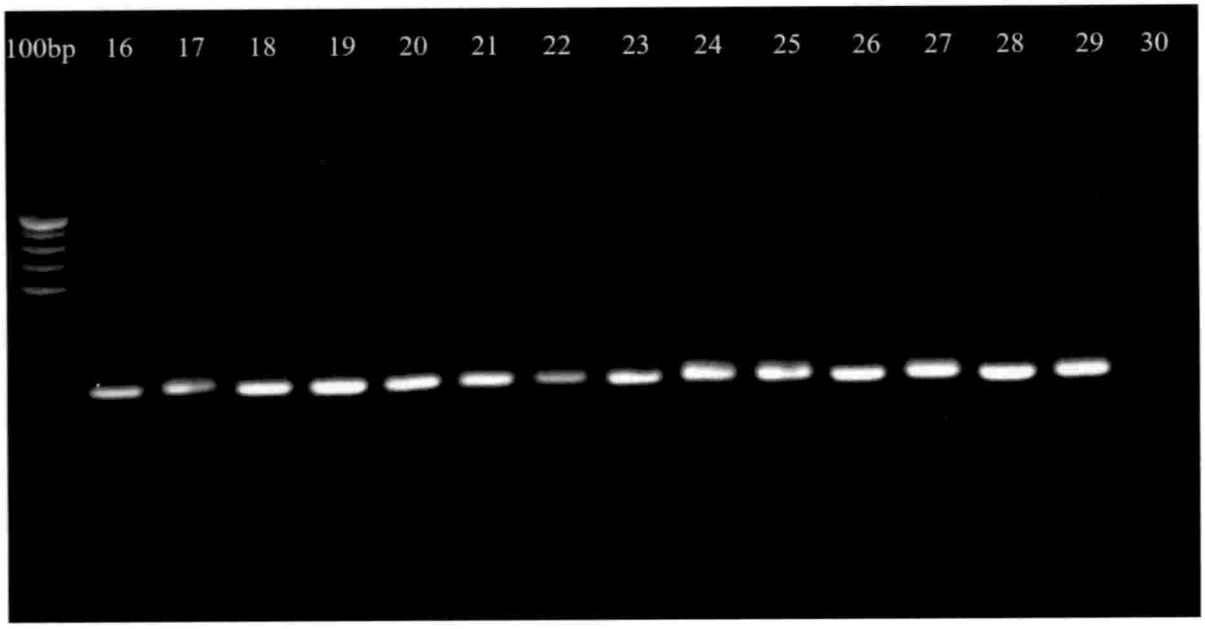


16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
DaH 22-2-3	DaH 58 FG	TCR 308	TCR 319	Sree karthika	Sree keerthi	Sree swathy	Da 377	Da 489	TCR 113	Da 820	DaH 17-5	Da 810	Orissa elite	Sree neelima

Plate 17: Agarose gel profile of the SSR primer Da3G04 for 30 accessions

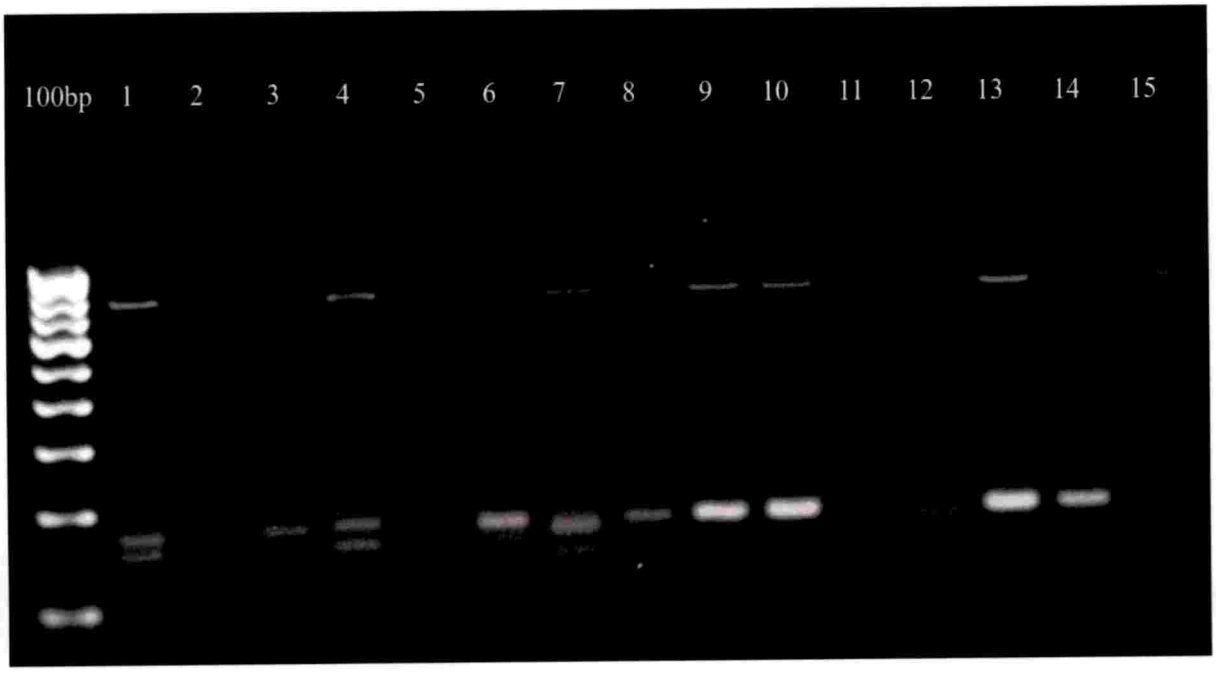


1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Da 110	Da 198	Da 200	Da 209	Da 210	Da 293	Da 374	DaH 9-196	Da 264	Da 340	Da 817 V	JAS 2	TCR 226	TCR 64	Da 12

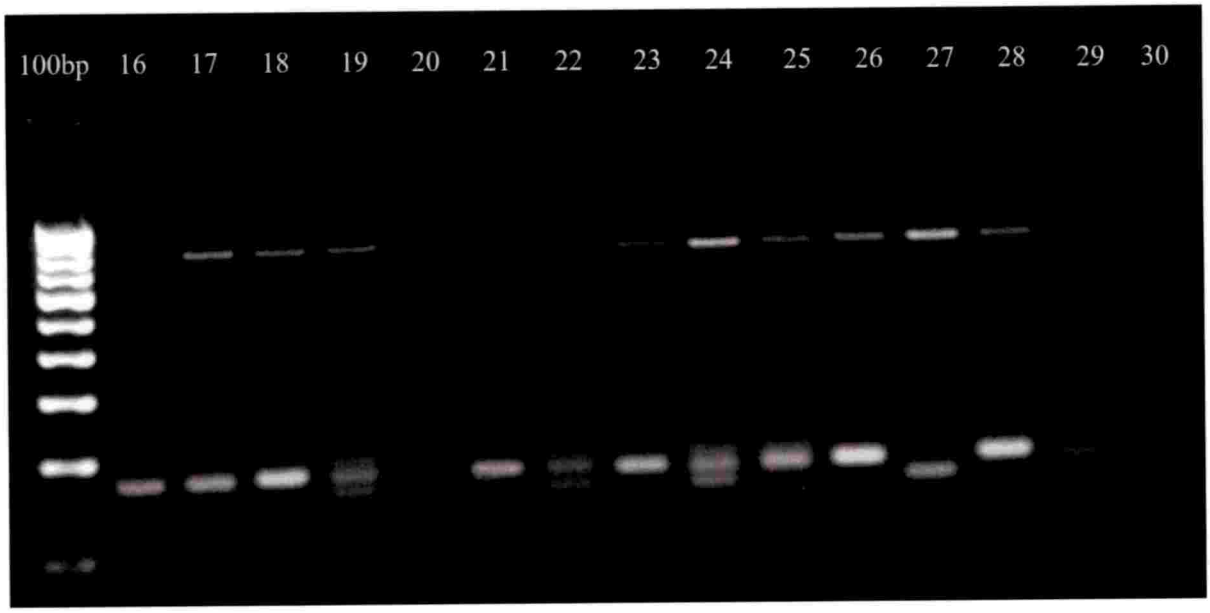


16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
DaH 22-2-3	DaH 58 FG	TCR 308	TCR 319	Sree karthika	Sree keerthi	Sree swathy	Da 377	Da 489	TCR 113	Da 820	DaH 17-5	Da 810	Orissa elite	Sree neelima

Plate 18: Agarose gel profile of the SSR primer Dab2D08 for 30 accessions

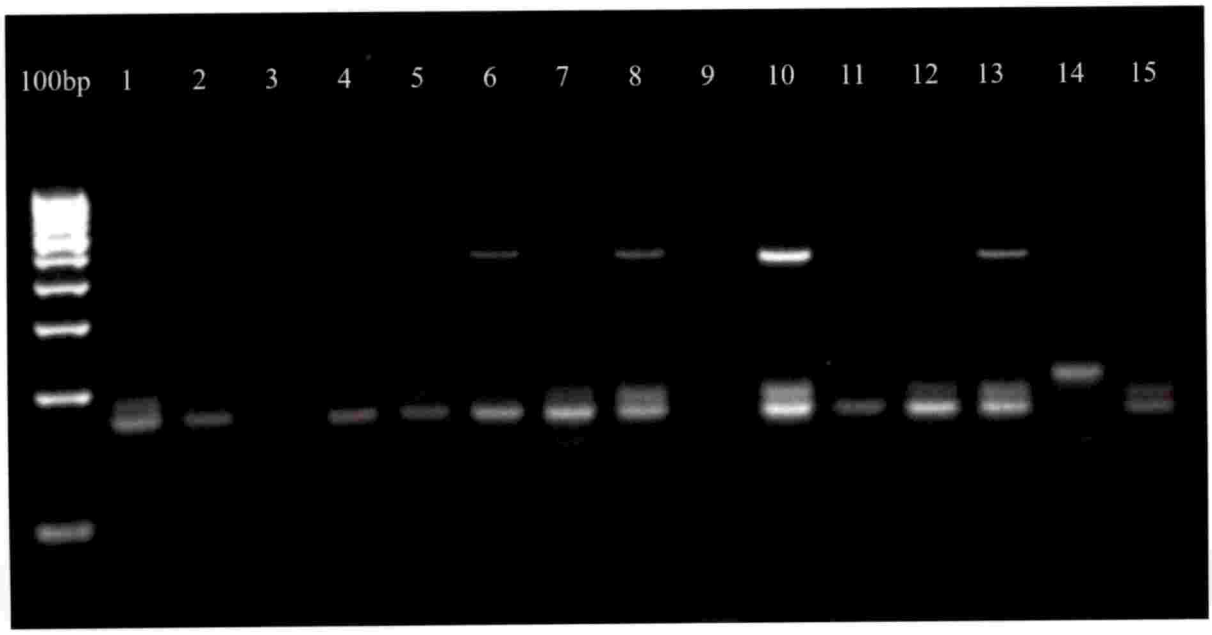


1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Da 110	Da 198	Da 200	Da 209	Da 210	Da 293	Da 374	DaH 9-196	Da 264	Da 340	Da 817 V	JAS 2	TCR 226	TCR 64	Da 12

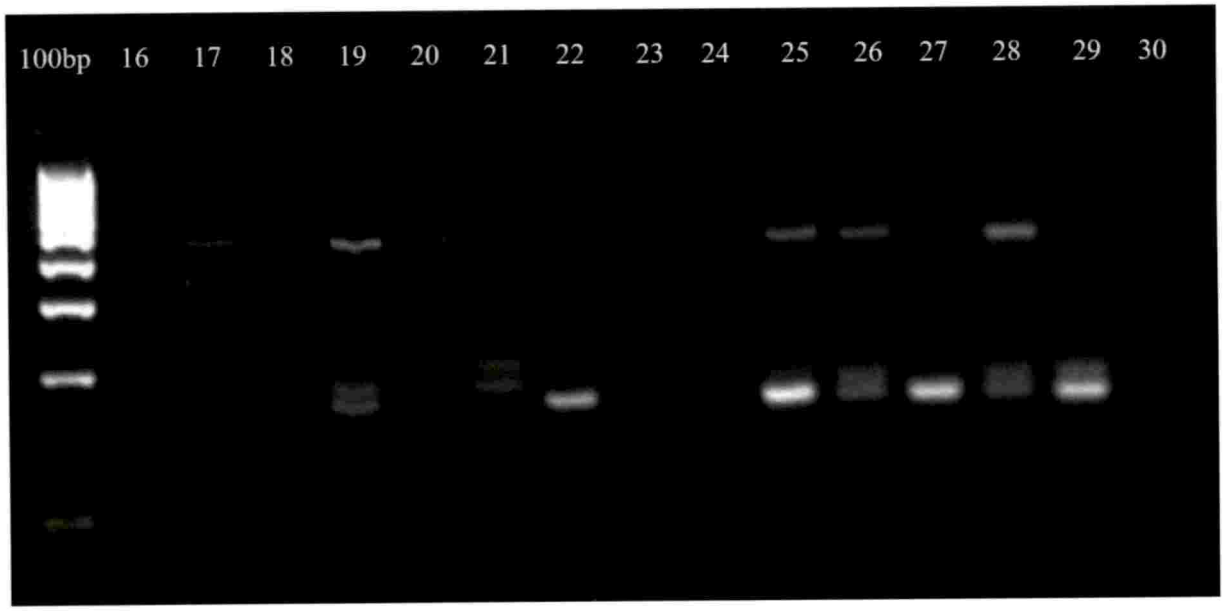


16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
DaH 22-2-3	DaH 58 FG	TCR 308	TCR 319	Sree karthika	Sree keerthi	Sree swathy	Da 377	Da 489	TCR 113	Da 820	DaH 17-5	Da 810	Orissa elite	Sree neelima

Plate 19: Agarose gel profile of the SSR primer Dab2E07 for 30 accessions

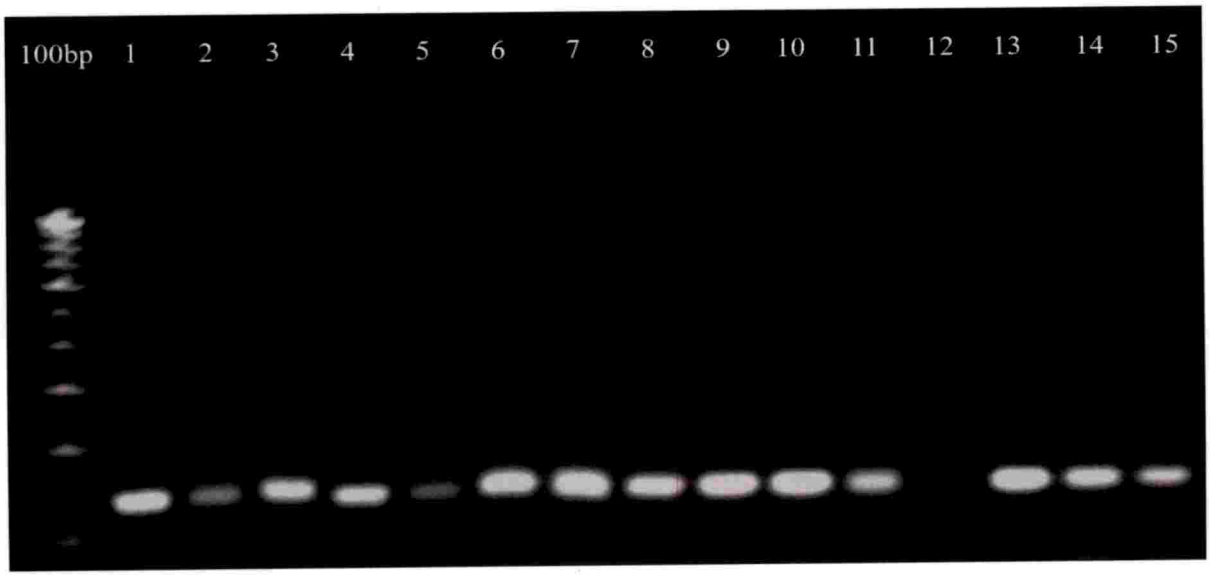


1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Da 110	Da 198	Da 200	Da 209	Da 210	Da 293	Da 374	DaH 9-196	Da 264	Da 340	Da 817 V	JAS 2	TCR 226	TCR 64	Da 12

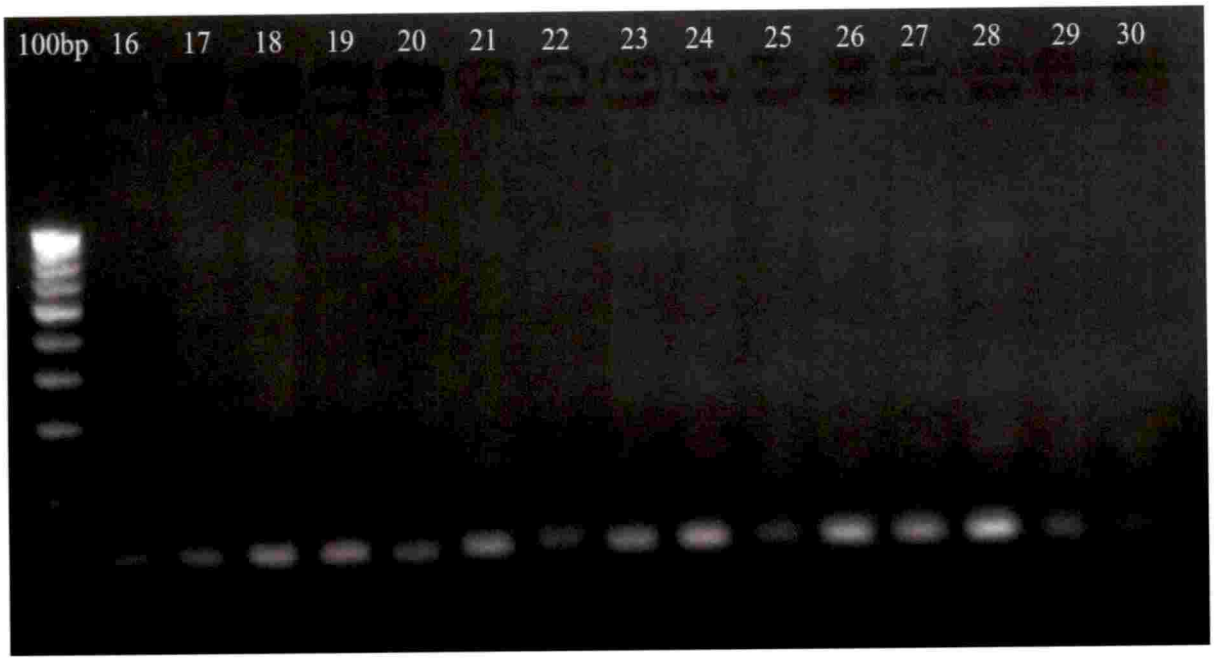


16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
DaH 22-2-3	DaH 58 FG	TCR 308	TCR 319	Sree karthika	Sree keerthi	Sree swathy	Da 377	Da 489	TCR 113	Da 820	DaH 17-5	Da 810	Orissa elite	Sree neelima

Plate 20: Agarose gel profile of the SSR primer Yam SSR 1 for 30 accessions

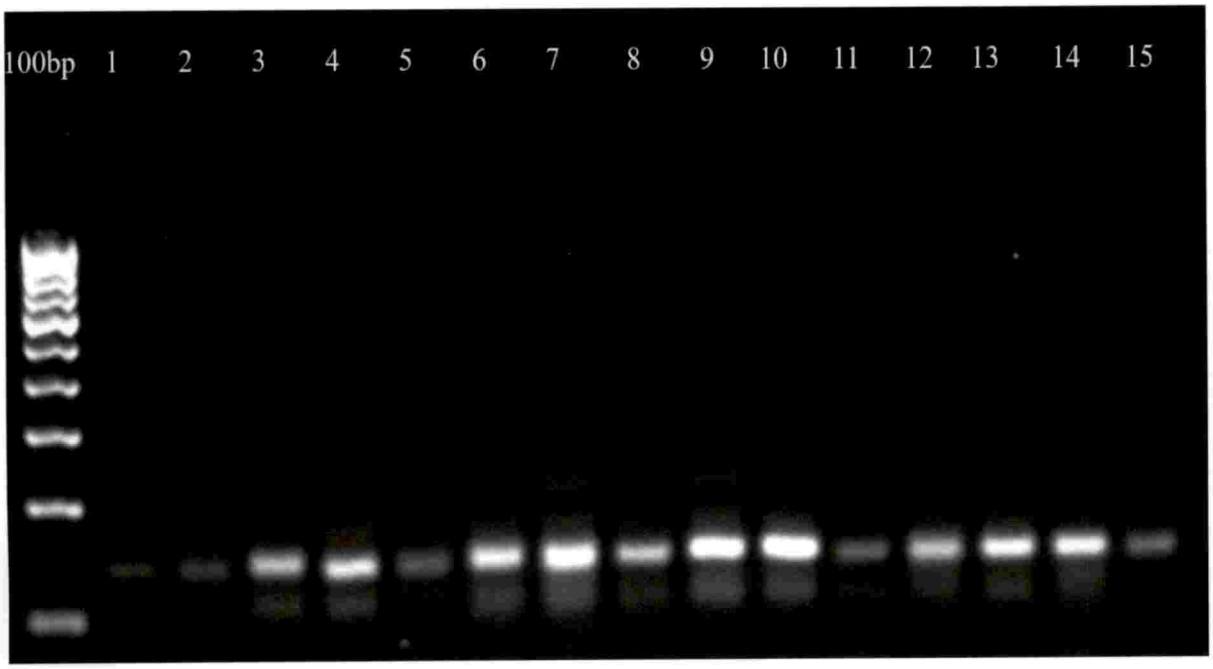


1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Da 110	Da 198	Da 200	Da 209	Da 210	Da 293	Da 374	DaH 9-196	Da 264	Da 340	Da 817 V	JAS 2	TCR 226	TCR 64	Da 12

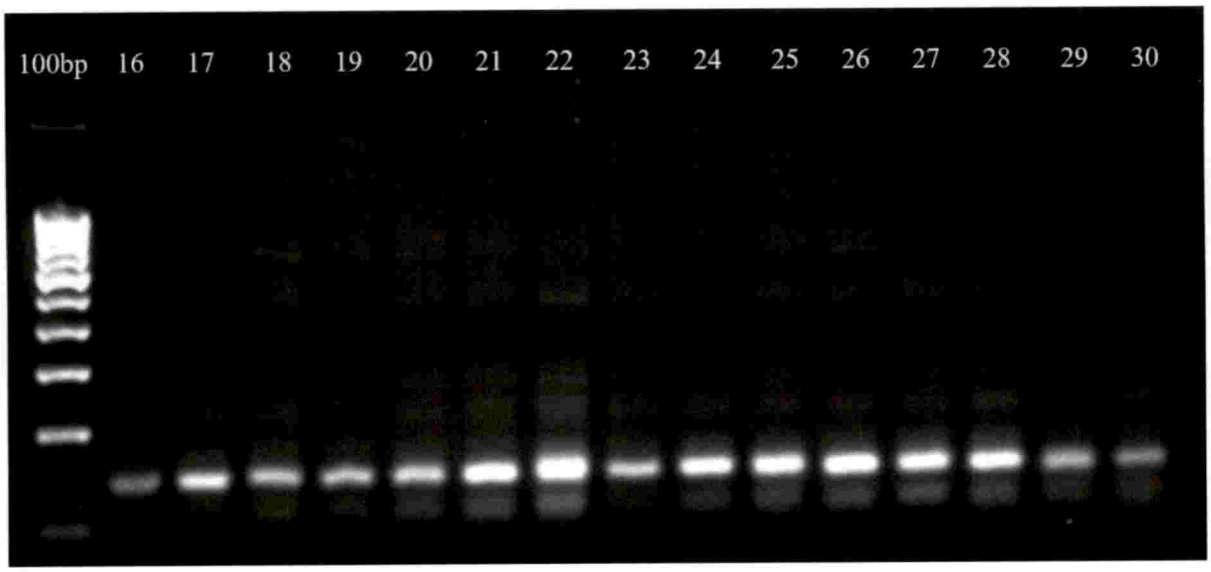


16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
DaH 22-2-3	DaH 58 FG	TCR 308	TCR 319	Sree karthika	Sree keerthi	Sree swathy	Da 377	Da 489	TCR 113	Da 820	DaH 17-5	Da 810	Orissa elite	Sree neelima

Plate 21: Agarose gel profile of the SSR primer Yam SSR 2 for 30 accessions

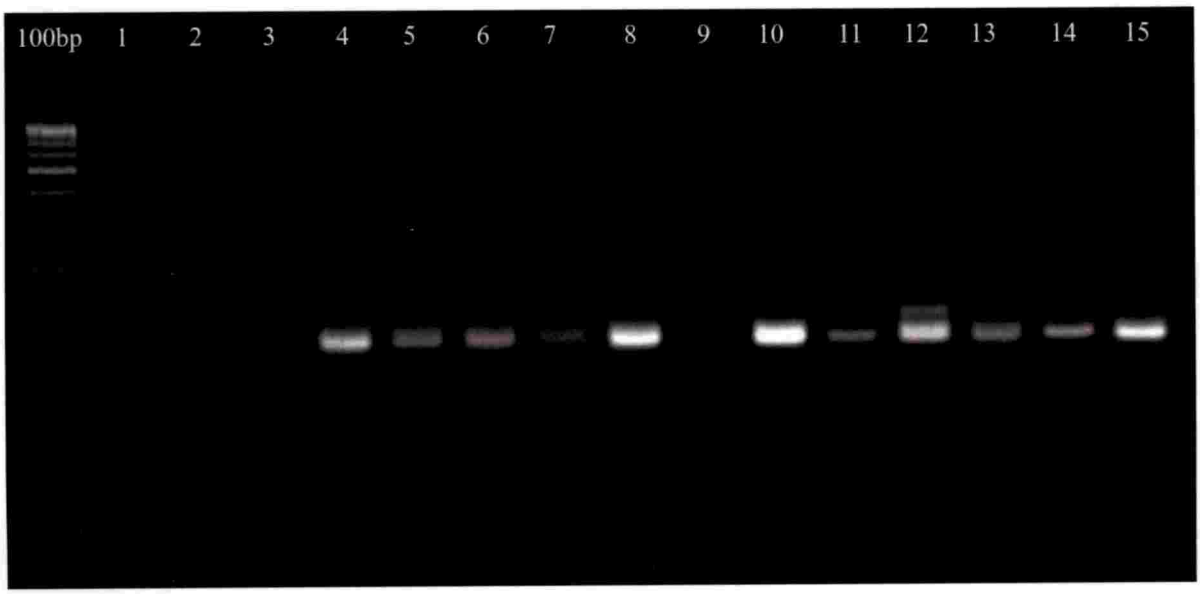


1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Da 110	Da 198	Da 200	Da 209	Da 210	Da 293	Da 374	DaH 9-196	Da 264	Da 340	Da 817 V	JAS 2	TCR 226	TCR 64	Da 12

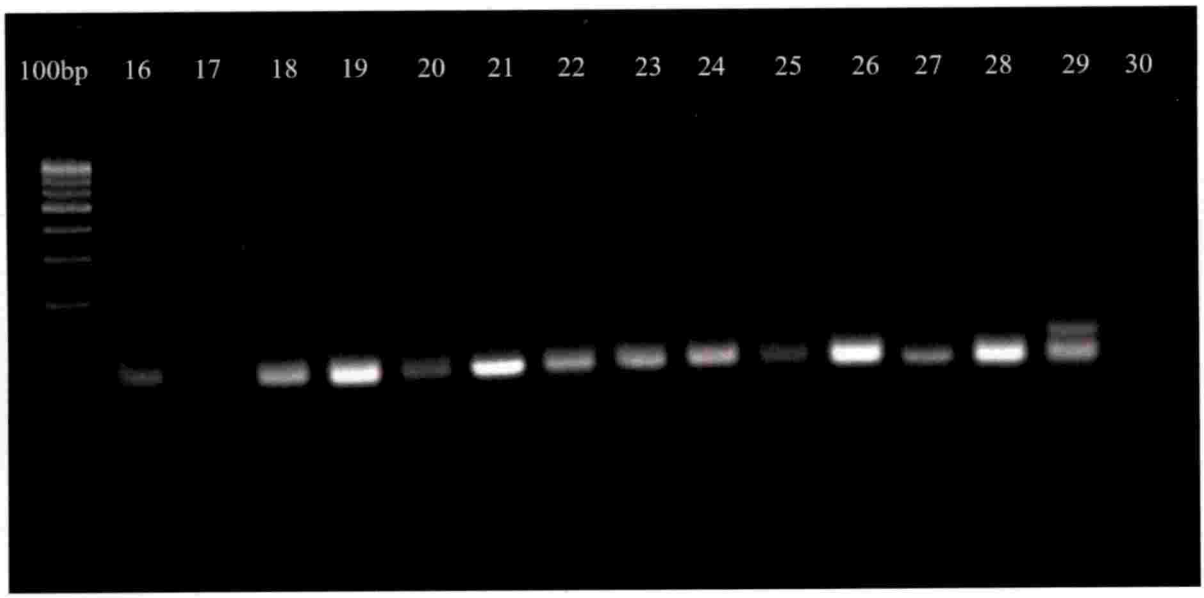


16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
DaH 22-2-3	DaH 58 FG	TCR 308	TCR 319	Sree karthika	Sree keerthi	Sree swathy	Da 377	Da 489	TCR 113	Da 820	DaH 17-5	Da 810	Orissa elite	Sree neelima

Plate 22: Agarose gel profile of the SSR primer Yam SSR 3 for 30 accessions

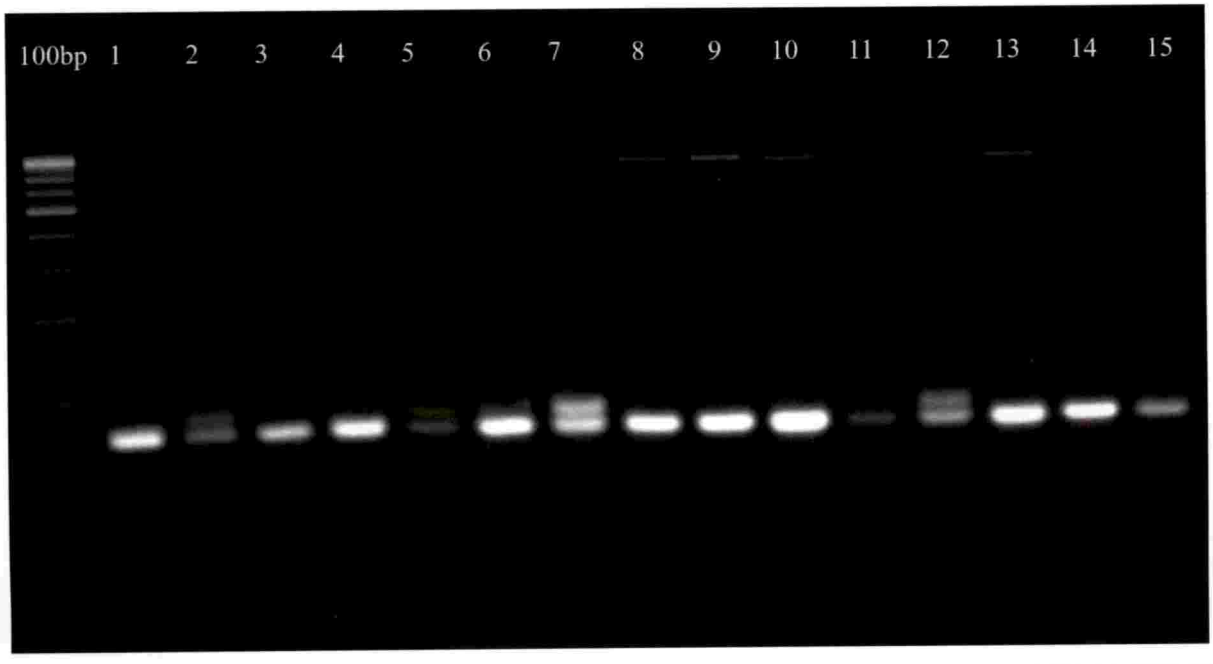


1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Da 110	Da 198	Da 200	Da 209	Da 210	Da 293	Da 374	DaH 9-196	Da 264	Da 340	Da 817 V	JAS 2	TCR 226	TCR 64	Da 12

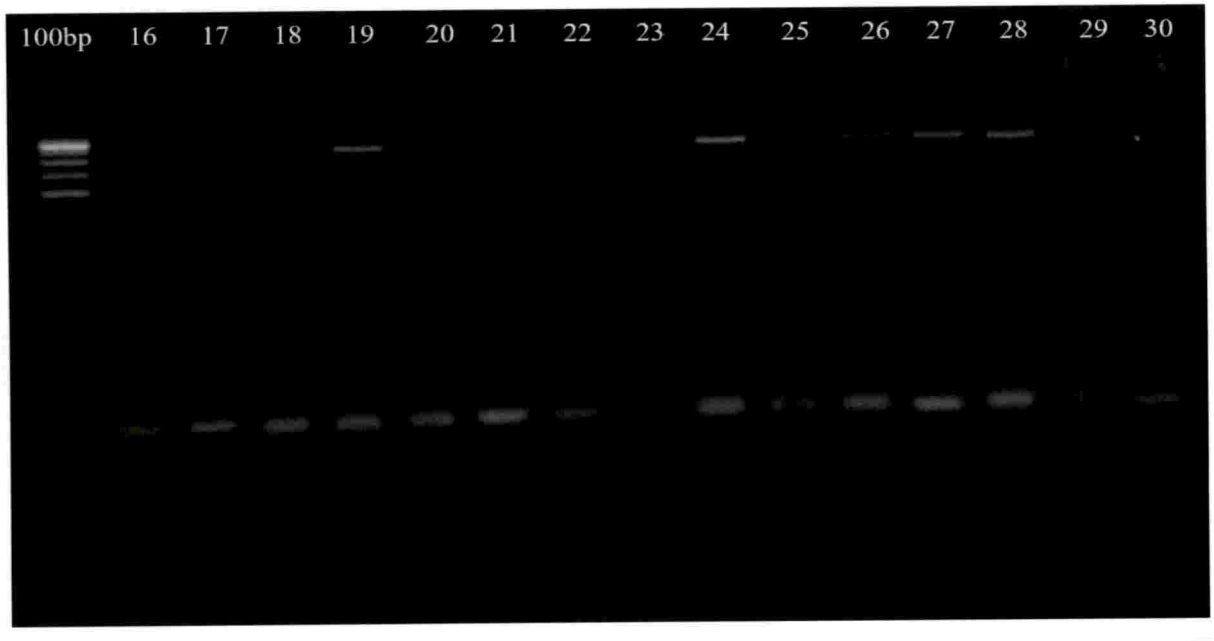


16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
DaH 22-2-3	DaH 58 FG	TCR 308	TCR 319	Sree karthika	Sree keerthi	Sree swathy	Da 377	Da 489	TCR 113	Da 820	DaH 17-5	Da 810	Orissa elite	Sree neelima

Plate 23: Agarose gel profile of the SSR primer Yam SSR 4 for 30 accessions

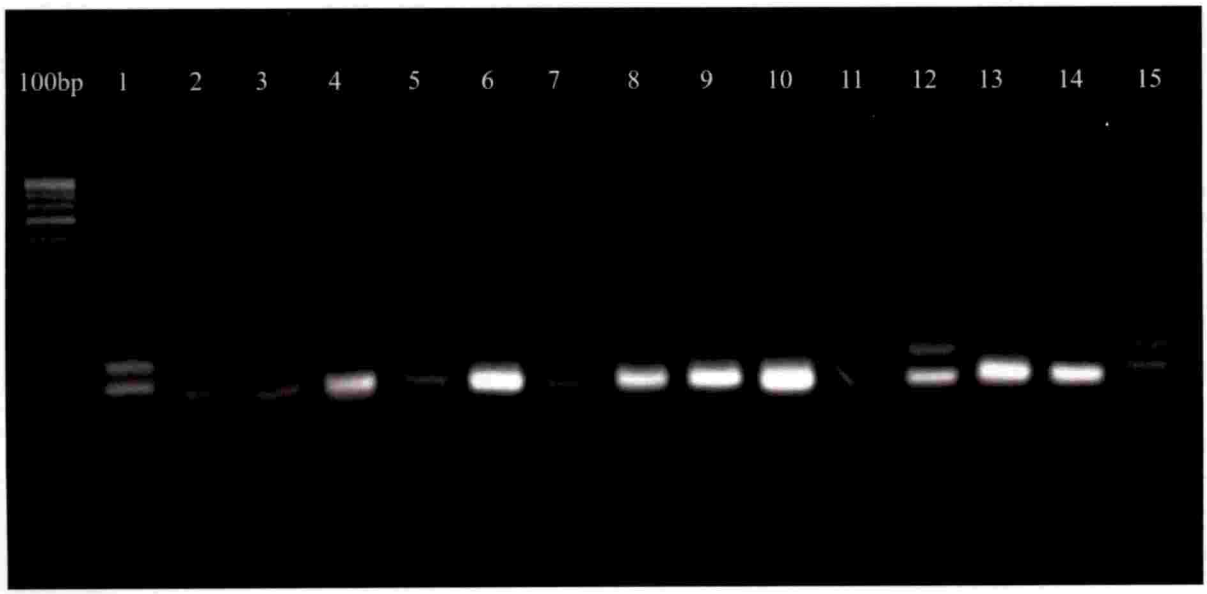


1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Da 110	Da 198	Da 200	Da 209	Da 210	Da 293	Da 374	DaH 9-196	Da 264	Da 340	Da 817 V	JAS 2	TCR 226	TCR 64	Da 12

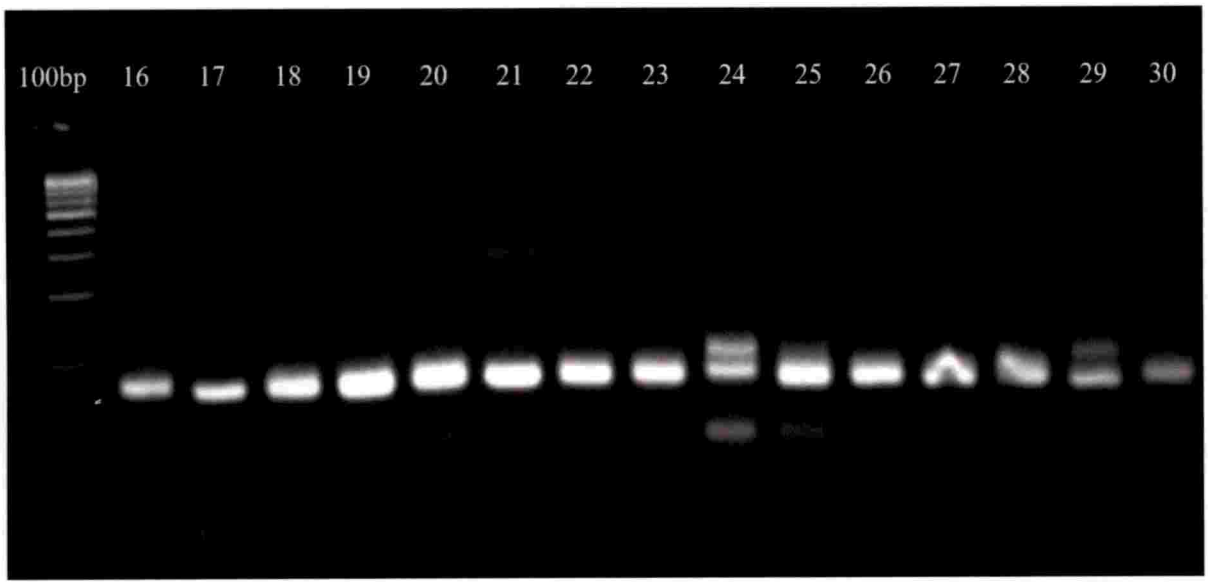


16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
DaH 22-2-3	DaH 58 FG	TCR 308	TCR 319	Sree karthika	Sree keerthi	Sree swathy	Da 377	Da 489	TCR 113	Da 820	DaH 17-5	Da 810	Orissa elite	Sree neelima

Plate 24: Agarose gel profile of the SSR primer Yam SSR 5 for 30 accessions



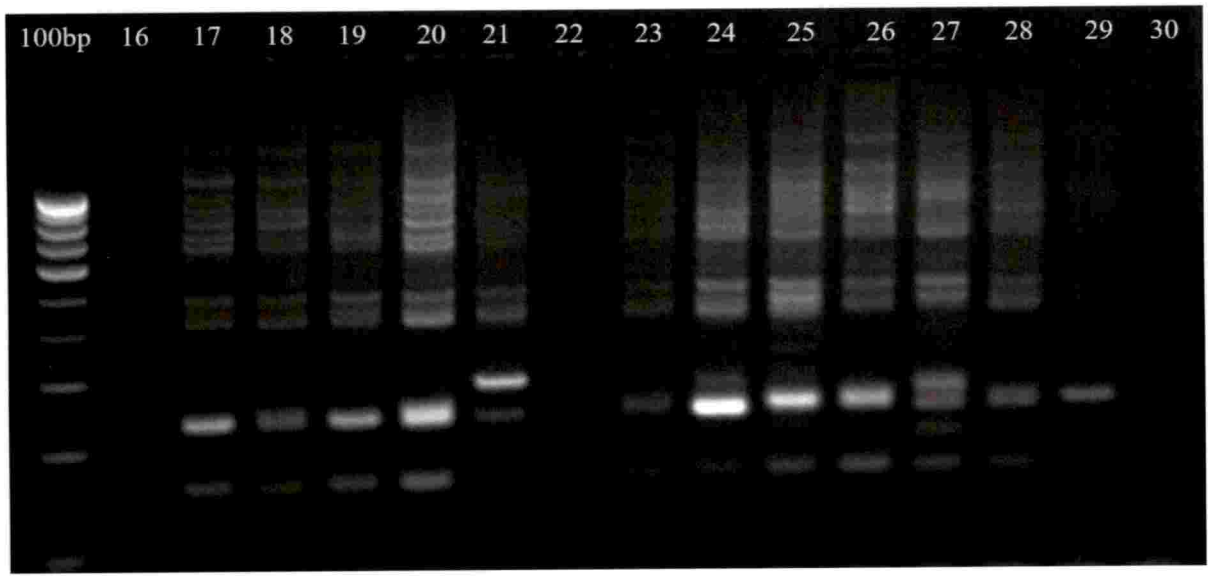
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Da 110	Da 198	Da 200	Da 209	Da 210	Da 293	Da 374	DaH 9-196	Da 264	Da 340	Da 817 V	JAS 2	TCR 226	TCR 64	Da 12



16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
DaH 22-2-3	DaH 58 FG	TCR 308	TCR 319	Sree karthika	Sree keerthi	Sree swathy	Da 377	Da 489	TCR 113	Da 820	DaH 17-5	Da 810	Orissa elite	Sree neelima

Plate 25: Agarose gel profile of the SSR primer Yam SSR 6 for 30 accessions

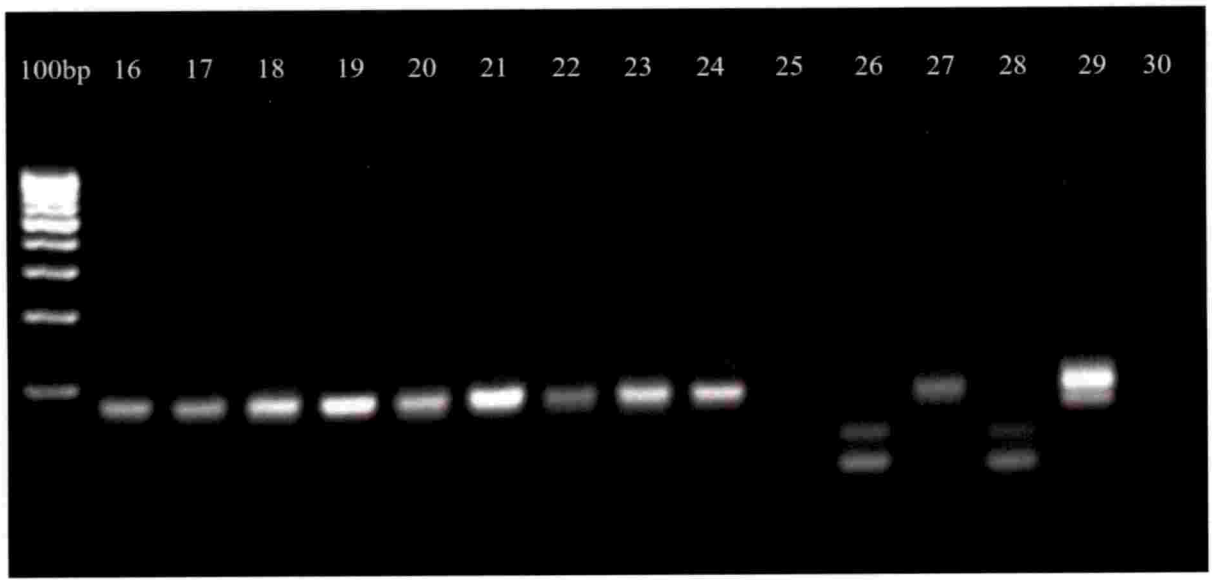
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Da 110	Da 198	Da 200	Da 209	Da 210	Da 293	Da 374	DaH 9-196	Da 264	Da 340	Da 817 V	JAS 2	TCR 226	TCR 64	Da 12



16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
DaH 22-2-3	DaH 58 FG	TCR 308	TCR 319	Sree karthika	Sree keerthi	Sree swathy	Da 377	Da 489	TCR 113	Da 820	DaH 17-5	Da 810	Orissa elite	Sree neelima

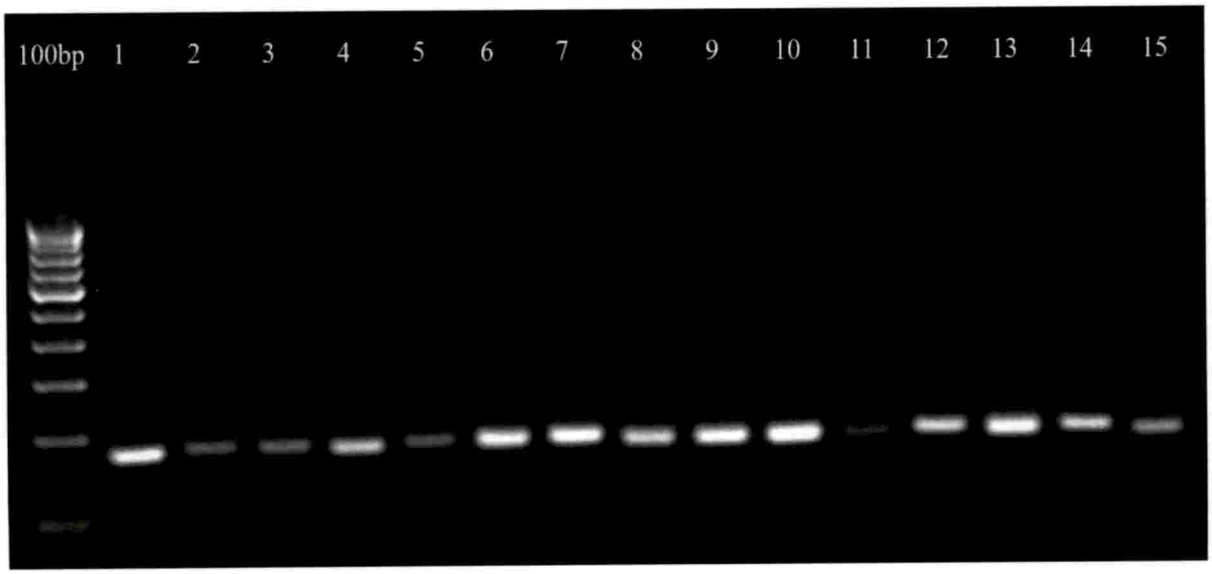
Plate 26: Agarose gel profile of the SSR primer Yam SSR 8 for 30 accessions

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Da 110	Da 198	Da 200	Da 209	Da 210	Da 293	Da 374	DaH 9-196	Da 264	Da 340	Da 817 V	JAS 2	TCR 226	TCR 64	Da 12

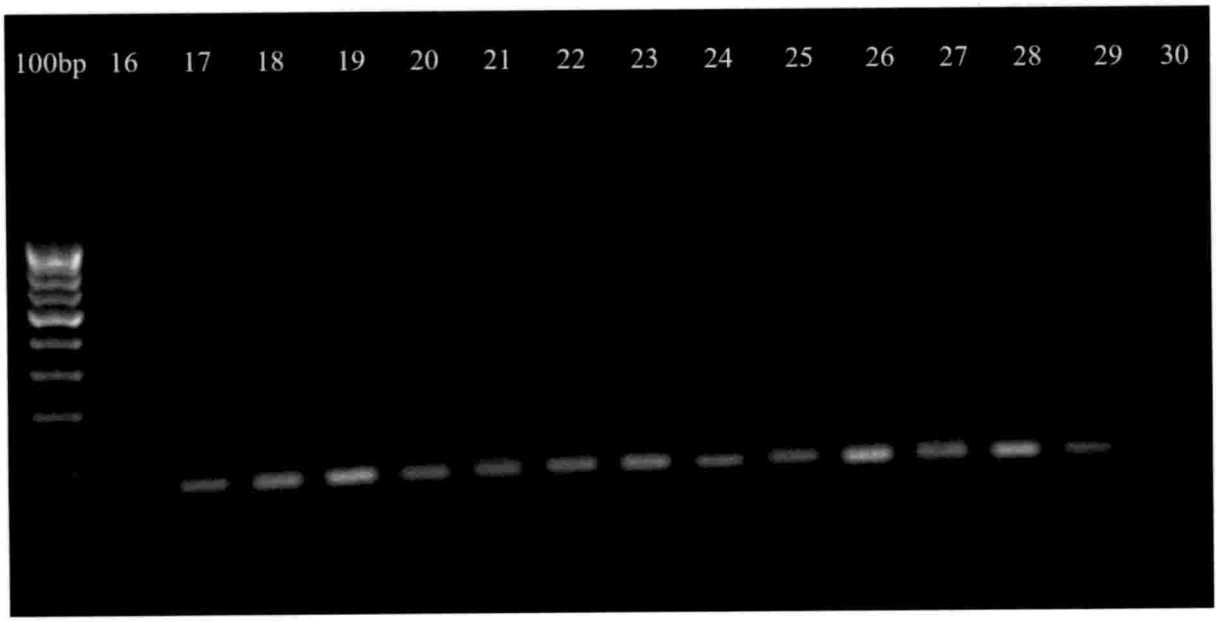


16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
DaH 22-2-3	DaH 58 FG	TCR 308	TCR 319	Sree karthika	Sree keerthi	Sree swathy	Da 377	Da 489	TCR 113	Da 820	DaH 17-5	Da 810	Orissa elite	Sree neelima

Plate 27: Agarose gel profile of the SSR primer Yam SSR 9 for 30 accessions

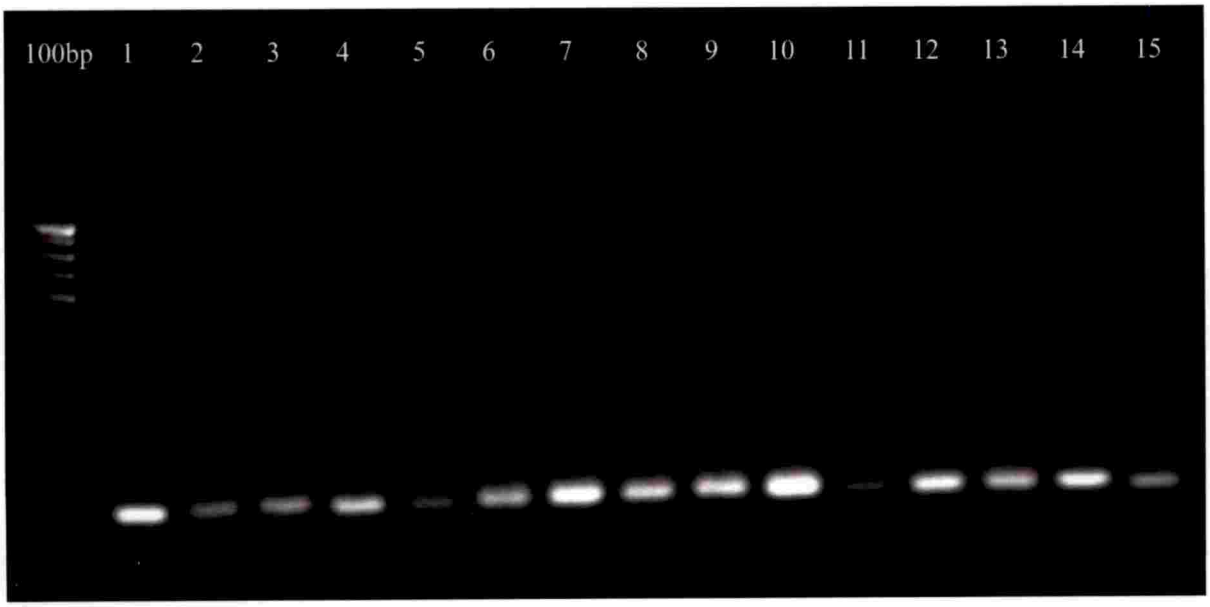


1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Da 110	Da 198	Da 200	Da 209	Da 210	Da 293	Da 374	DaH 9-196	Da 264	Da 340	Da 817 V	JAS 2	TCR 226	TCR 64	Da 12

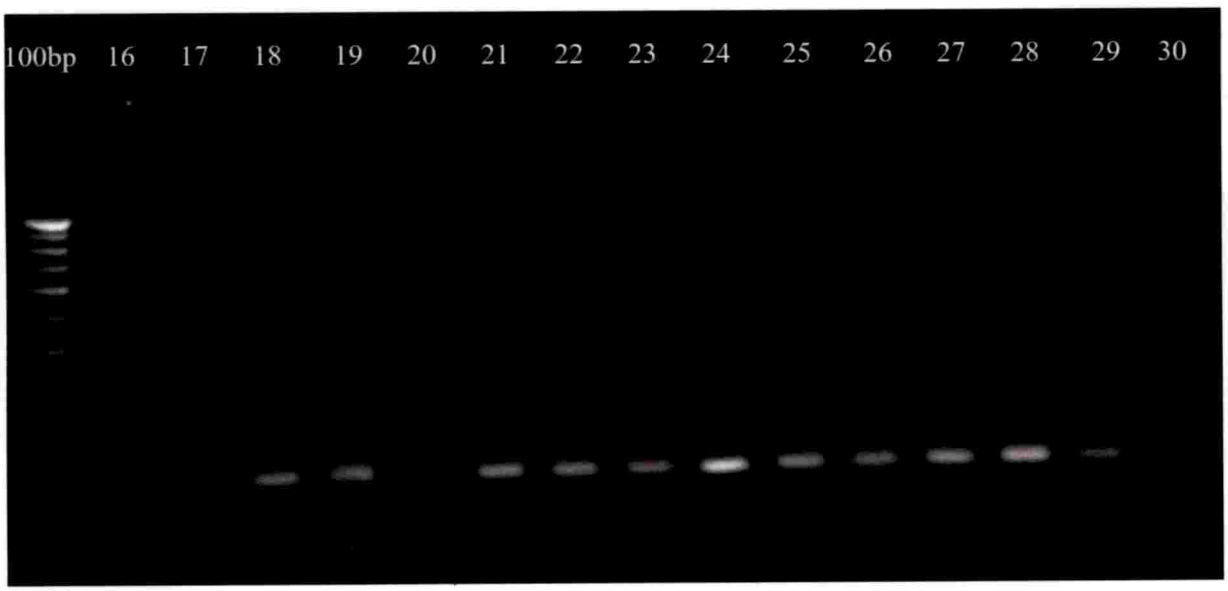


16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
DaH 22-2-3	DaH 58 FG	TCR 308	TCR 319	Sree karthika	Sree keerthi	Sree swathy	Da 377	Da 489	TCR 113	Da 820	DaH 17-5	Da 810	Orissa elite	Sree neelima

Plate 28: Agarose gel profile of the SSR primer Yam SSR 12 for 30 accessions

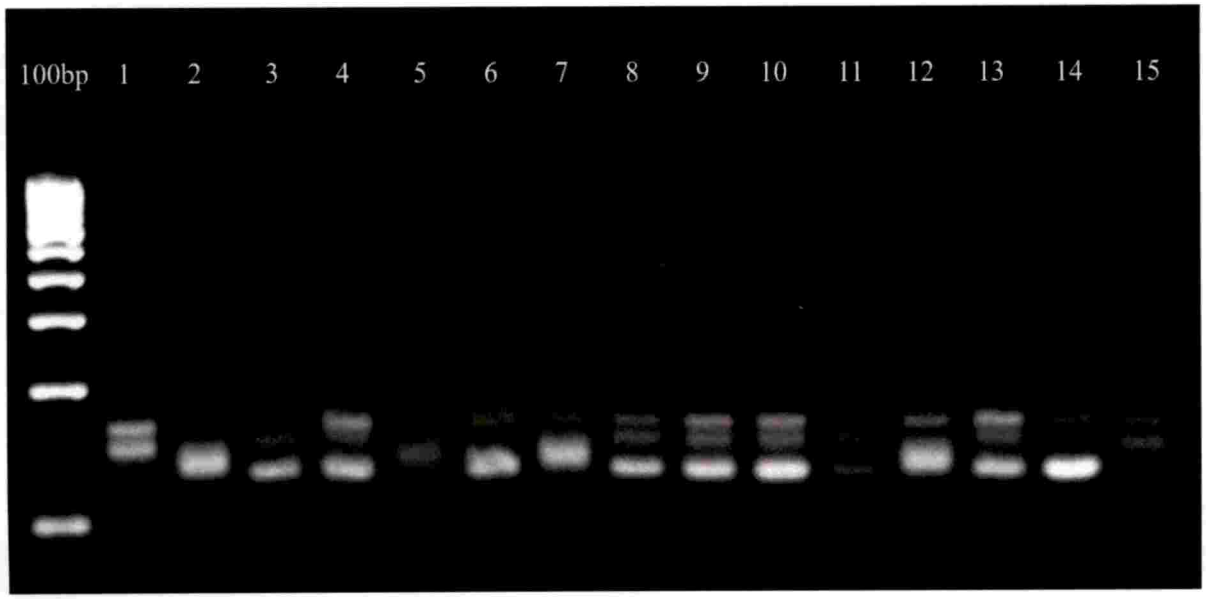


1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Da 110	Da 198	Da 200	Da 209	Da 210	Da 293	Da 374	DaH 9-196	Da 264	Da 340	Da 817 V	JAS 2	TCR 226	TCR 64	Da 12

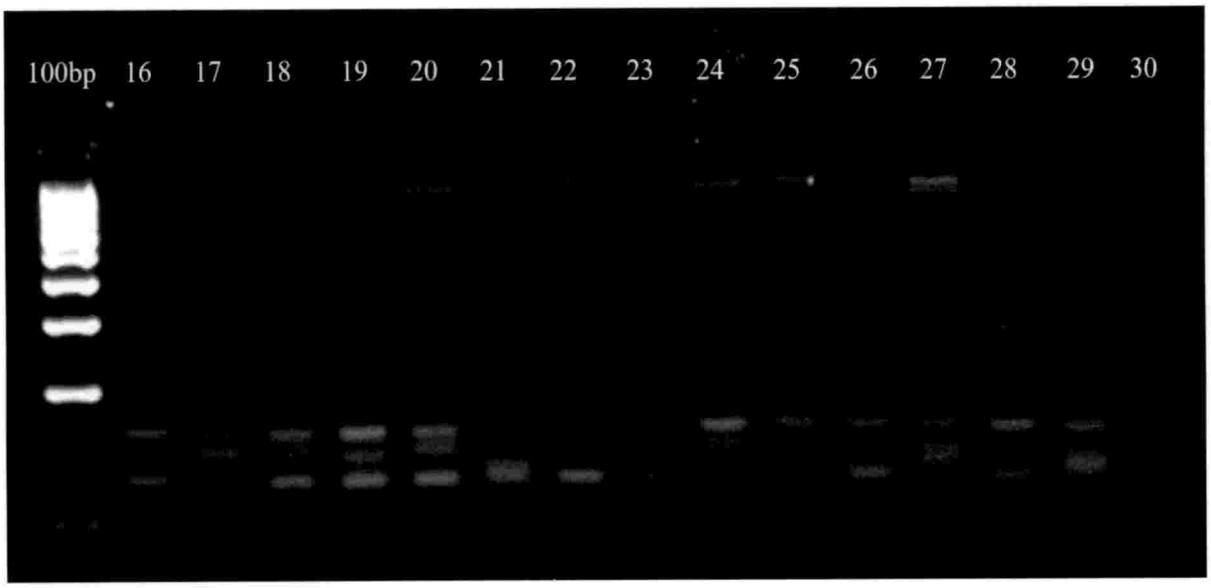


16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
DaH 22-2-3	DaH 58 FG	TCR 308	TCR 319	Sree karthika	Sree keerthi	Sree swathy	Da 377	Da 489	TCR 113	Da 820	DaH 17-5	Da 810	Orissa elite	Sree neelima

Plate 29: Agarose gel profile of the SSR primer Yam SSR 15 for 30 accessions

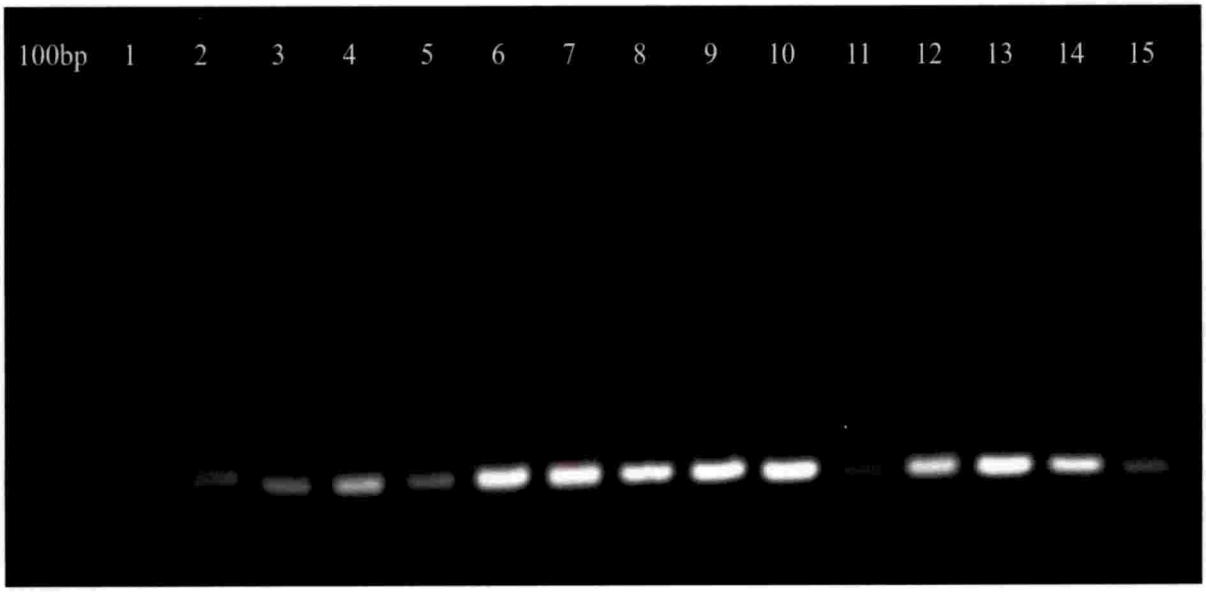


1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Da 110	Da 198	Da 200	Da 209	Da 210	Da 293	Da 374	DaH 9-196	Da 264	Da 340	Da 817 V	JAS 2	TCR 226	TCR 64	Da 12

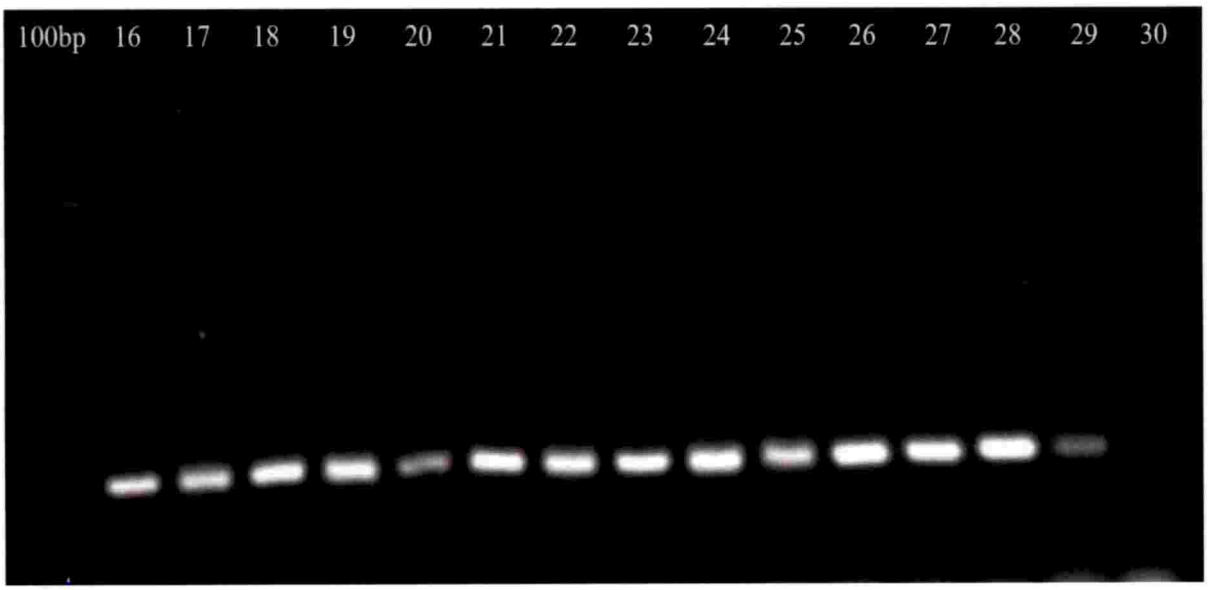


16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
DaH 22-2-3	DaH 58 FG	TCR 308	TCR 319	Sree karthika	Sree keerthi	Sree swathy	Da 377	Da 489	TCR 113	Da 820	DaH 17-5	Da 810	Orissa elite	Sree neelima

Plate 30: Agarose gel profile of the SSR primer Yam SSR 16 for 30 accessions

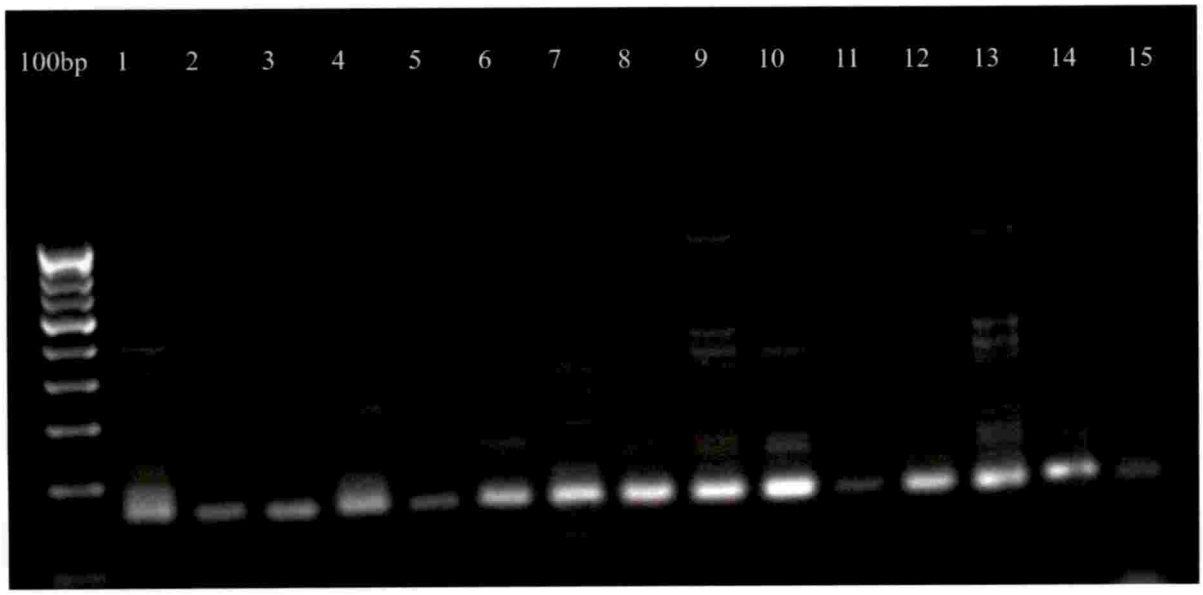


1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Da 110	Da 198	Da 200	Da 209	Da 210	Da 293	Da 374	DaH 9-196	Da 264	Da 340	Da 817 V	JAS 2	TCR 226	TCR 64	Da 12

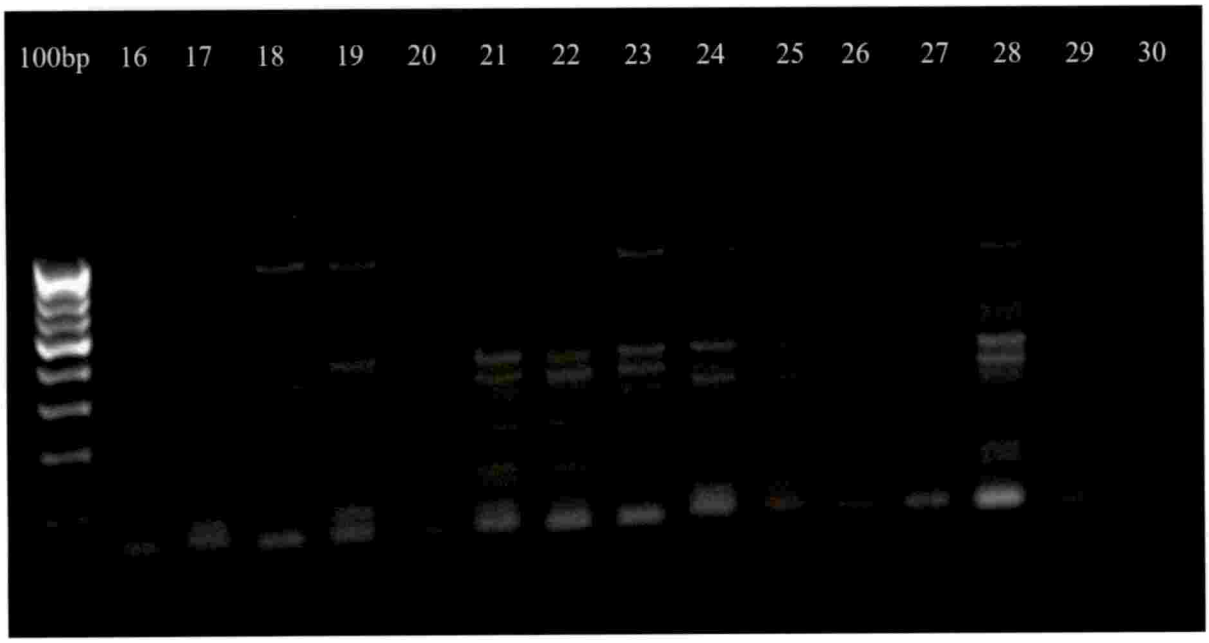


16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
DaH 22-2-3	DaH 58 FG	TCR 308	TCR 319	Sree karthika	Sree keerthi	Sree swathy	Da 377	Da 489	TCR 113	Da 820	DaH 17-5	Da 810	Orissa elite	Sree neelima

Plate 31: Agarose gel profile of the SSR primer Yam SSR 18 for 30 accessions



1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Da 110	Da 198	Da 200	Da 209	Da 210	Da 293	Da 374	DaH 9-196	Da 264	Da 340	Da 817 V	JAS 2	TCR 226	TCR 64	Da 12



16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
DaH 22-2-3	DaH 58 FG	TCR 308	TCR 319	Sree karthika	Sree keerthi	Sree swathy	Da 377	Da 489	TCR 113	Da 820	DaH 17-5	Da 810	Orissa elite	Sree neelima

Plate 32: Agarose gel profile of the SSR primer Yam SSR 19 for 30 accessions

band size of 358.74bp. The primers Dab2E07, Yam SSR 9 and Yam SSR 12 showed 100% polymorphism.

The expected heterozygosity values ranged from 0 to 0.6612. The highest PIC value (0.5872) was expressed by Yam SSR 16 followed by Yam SSR 6 (0.5023). All the primers recorded PIC value <0.5872. The highest PIC value (0.5872) was recorded by Yam SSR16 followed by Yam SSR6 (0.5023). The PIC value of primers Da3G04, Yam SSR 2, Yam SSR 12, Yam SSR 15, Yam SSR 18 and Yam SSR 19 was found to be zero. The gene diversity values ranged from 0 to 0.32. The primers Da3G04, Yam SSR 2, Yam SSR 3, Yam SSR 15, Yam SSR 18 and Yam SSR 19 showed gene diversity value zero. Among the SSR primers tested, Yam SSR12 recorded the highest gene diversity index followed by Dab2E07.

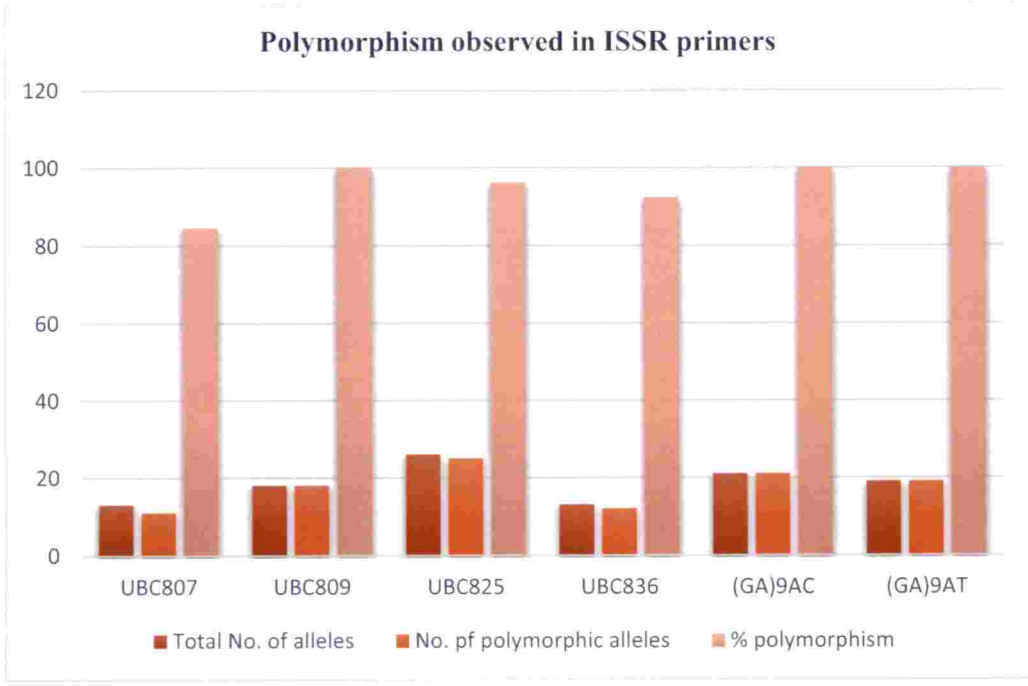
Table 17: Genetic characteristics of the SSR markers used

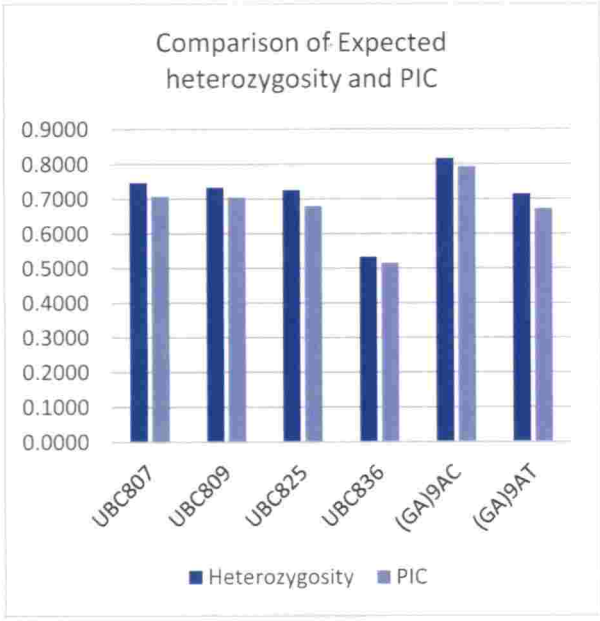
Marker	Total No. of alleles	No. of polymorphic alleles	% polymorphism	He	PIC	Gene Diversity
Da3G04	1	0	0	0.0000	0.0000	0.0000
Dab2D08	2	1	50.00	0.1172	0.1103	0.0622
Dab2E07	2	2	100.00	0.4494	0.3484	0.2822
YamSSR1	2	1	50.00	0.1172	0.1103	0.0622
YamSSR2	1	0	0	0.0000	0.0000	0.0000
YamSSR3	2	0	0	0.5000	0.3750	0.0000
YamSSR4	2	1	50.00	0.2778	0.2392	0.1600
YamSSR5	2	1	50.00	0.2778	0.2392	0.1600
YamSSR6	3	2	66.66	0.5792	0.5023	0.2793
YamSSR8	2	1	50.00	0.4844	0.3671	0.2100
YamSSR9	3	3	100.00	0.3788	0.3473	0.2141
YamSSR12	1	1	100.00	0.0000	0.0000	0.3200
YamSSR15	1	0	0	0.0000	0.0000	0.0000
YamSSR16	3	2	66.66	0.6612	0.5872	0.2230
YamSSR18	1	0	0	0.0000	0.0000	0.0000
YamSSR19	1	0	0	0.0000	0.0000	0.0000

Among the different markers tested, ISSR primer (GA)9AC recorded the highest polymorphism information content (0.7929) while gene diversity index was maximum (0.32) for the SSR primer, Yam SSR 12.

Graphical representations depicting the efficiency of ISSR, RAPD and SSR primers are provided in figure 1, 2 and 3 respectively.

Graphical representation of the efficiency of primers





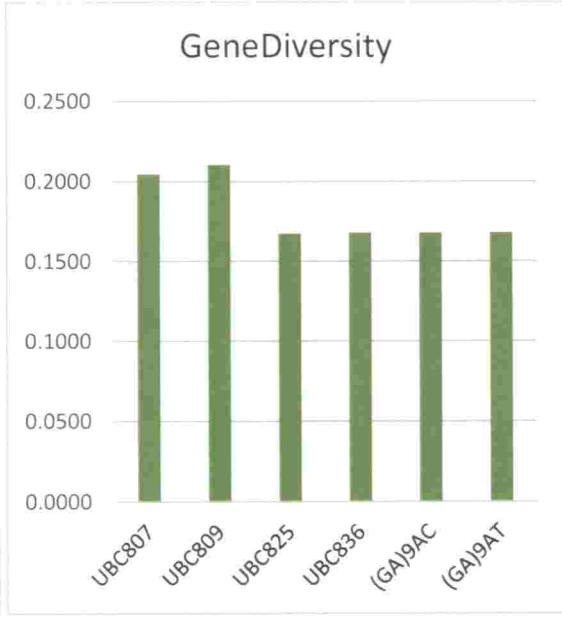
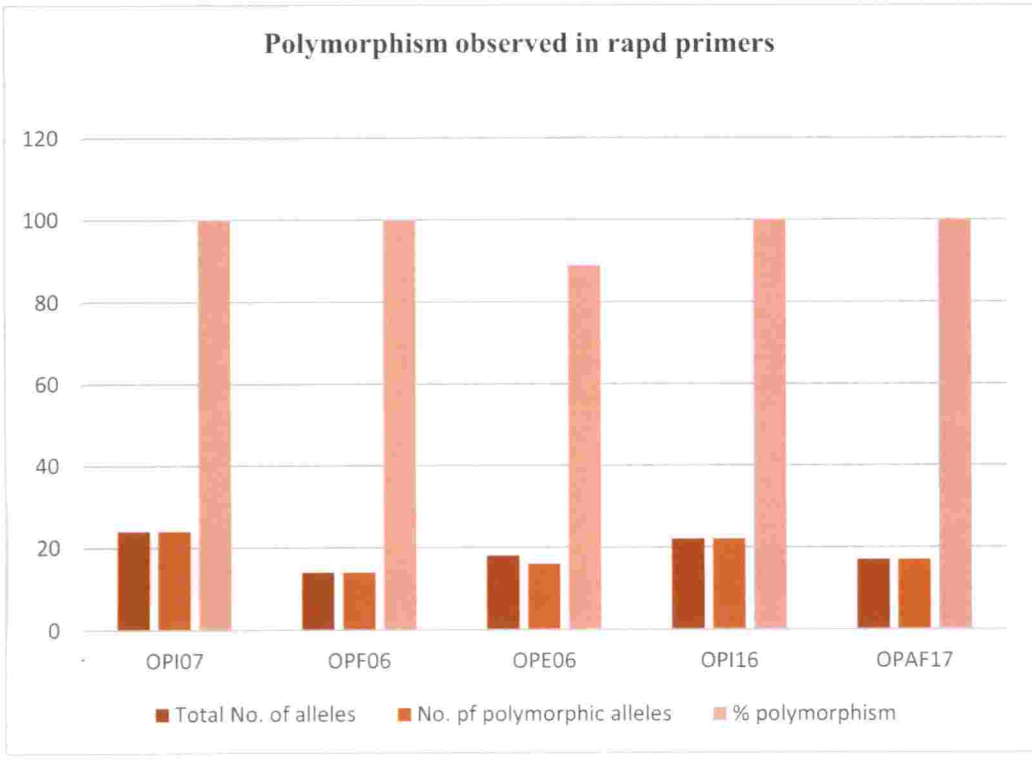


Figure 1: Graphical representation of the efficiency of ISSR primers



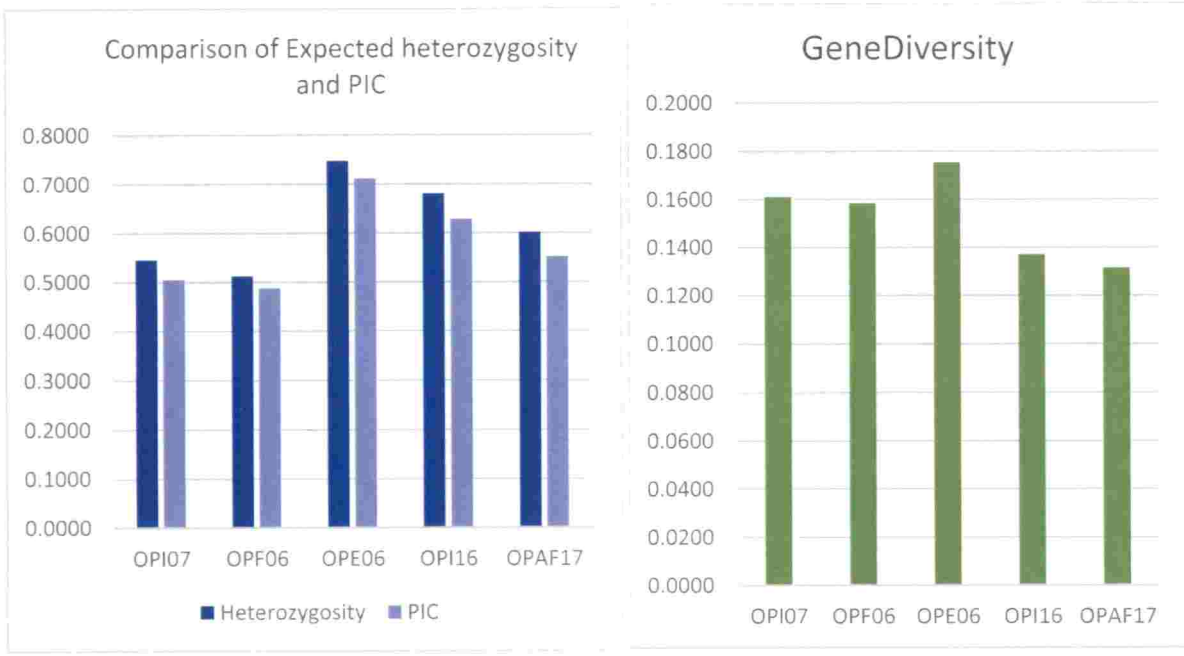
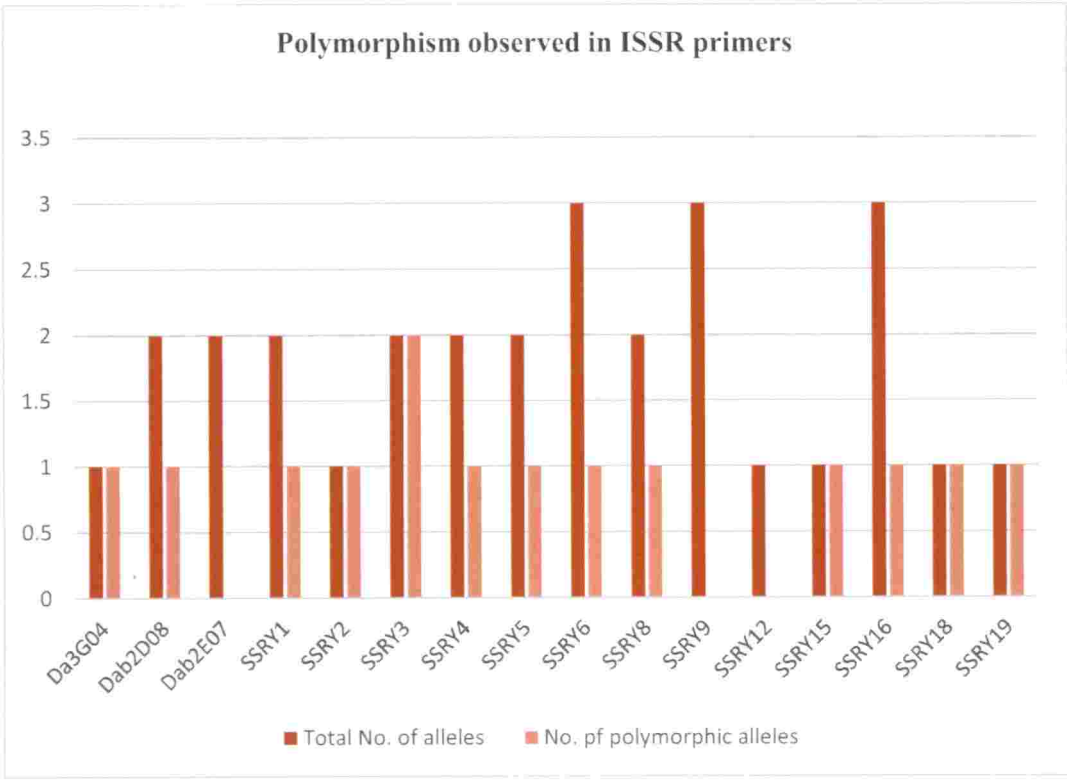


Figure 2: Graphical representation of the efficiency of RAPD primers



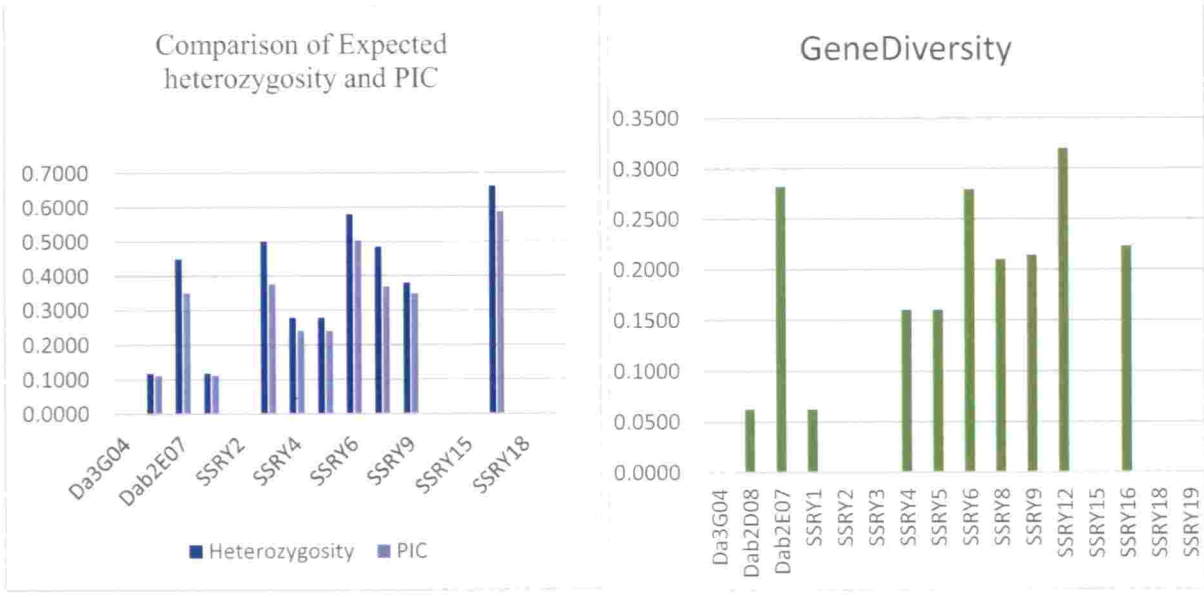


Figure 3: Graphical representation of the efficiency of SSR primers

4.4 Cluster Analysis

Cluster analysis was done and dendrograms were generated using UPGMA cluster analysis with 30 accessions of greater yam using ISSR, RAPD and SSR primers.

4.4.1 Characterization of 30 accessions using ISSR Primers

Dendrogram was constructed with 30 accessions of greater yam using 6 ISSR primers and is provided in the figure 4. Accessions were divided into two major clusters at 36% similarity level. Cluster A was again divided into two subclusters in which Da 198 is found to be an outlier. Cluster B includes the two highly susceptible genotypes Orissa Elite and Sree Neelima and the showed 38% similarity. At 50% similarity level, the dendrogram was grouped into three subclusters and two outliers as presented in the table 18. Subcluster 1 is the largest containing 23 genotypes, which were further grouped into several small subclusters.Two resistant accessions, TCR 308 and Da 340 were found to possess 91% similarity. The second subcluster (1B) grouped three highly resistant varieties Da 200, Sree karthika and Sree keerthi together.

Table 18: Clustering pattern of 30 greater yam accessions based on ISSR primers

Clusters	Sub-Clusters	Accessions
Cluster 1	1A	Da 110,Da 489,Da 12,Da 377, Sree Swathy, DaH 9-196, Da 64, TCR 308, Da 340, Da 820, TCR 226, DaH 17-5, Da 810, TCR 319, Da 209, Da 817V, TCR 113, Da 22-2-3, DaH 58FG,Da 210, Da 374, Da 374, Da 293, JAS 2
	1B	Da 200, Sree Karthika, Sree Keerthi
	Outlier 1	TCR 64
	Outlier 2	Da 198
Cluster 2		Orissa Elite, Sree Neelima

4.4.2 Characterization of 30 accessions using RAPD Primers

Dendrogram was constructed with 30 accessions of greater yam using 5 RAPD primers and is provided in the figure 5. Accessions were divided into two major clusters at 27 % similarity level. The first cluster grouped 27 accessions together which were then subclustered into 4 a t0.37 dissimilarity level. Cluster 2

that grouped three highly susceptible genotypes together was again divided into two in which TCR 64 is an outlier. At 37% similarity level, the dendrogram depicted four subclusters and 2 outliers as presented in the table 19. Orissa elite and Sree Neelima, two highly susceptible genotypes were clustered together and showed 47% similarity.

Table 19: Clustering pattern of 30 greater yam accessions based on RAPD primers

Clusters	Sub-Clusters	Accessions
Cluster 1	1A	Da 110, Da 12, Da 209, Da 817V, Da 293, Da 374, TCR 319, DaH9-196, Da 377, TCR 113, Da 820, DaH 17-5, Sree Swathy, TCR 226, Da 264, Da 340, Da 210, Da 810, Sree Keerthi, Da 200, Da 22-2-3, DaH 58 FG
	1B	JAS 2, Da 489
	1C	TCR 308, Sree Karthika
	Outlier 1	Da 198
Cluster 2	Outlier 2	TCR 64
	2A	Orissa Elite, Sree Neelima

4.4.3 Characterization of 30 accessions using SSR Primer

Dendrogram was constructed with 30 accessions of greater yam using 16 SSR primers and is provided in the figure 6. Accessions were divided into two major clusters at 69 % similarity level. Cluster A was again divided into two major clusters at 77% similarity. Da 820 and Da 810, two susceptible accessions were found to possess 100% similarity. At 80% similarity level, the 30 genotypes were divided into three subclusters and two outliers as given in the table 20. TCR 226 showed 100% similarity with six resistant accessions including Sree Karthika, DaH 9-196, Da 22-2-3, TCR 308, Da 264, and Da 340.

Table 20: Clustering pattern of 30 greater yam accessions based on SSR primers

Clusters	Sub- Clusters	Accessions
Cluster 1	1A	Da 110, Da 817V, Da 12, Da 489, DaH 17-5
	1B	Da 198, Da 210, Da 374, JAS 2, Sree Keerthi, Da 377, Da 209, Da 293, TCR 319, Sree Swathy, Orissa elite, Da H 9-196, Da 22-2-3, TCR 308, Sree Karthika, Da 264, TCR 226, Da 340, Da H 58 FG

	Outlier 1	Sree Neelima
	Outlier 2	TCR 64
Cluster 2	2A	Da 200, Da 820, Da 810, TCR 113

Figure 4: Cluster dendrogram based on ISSR markers

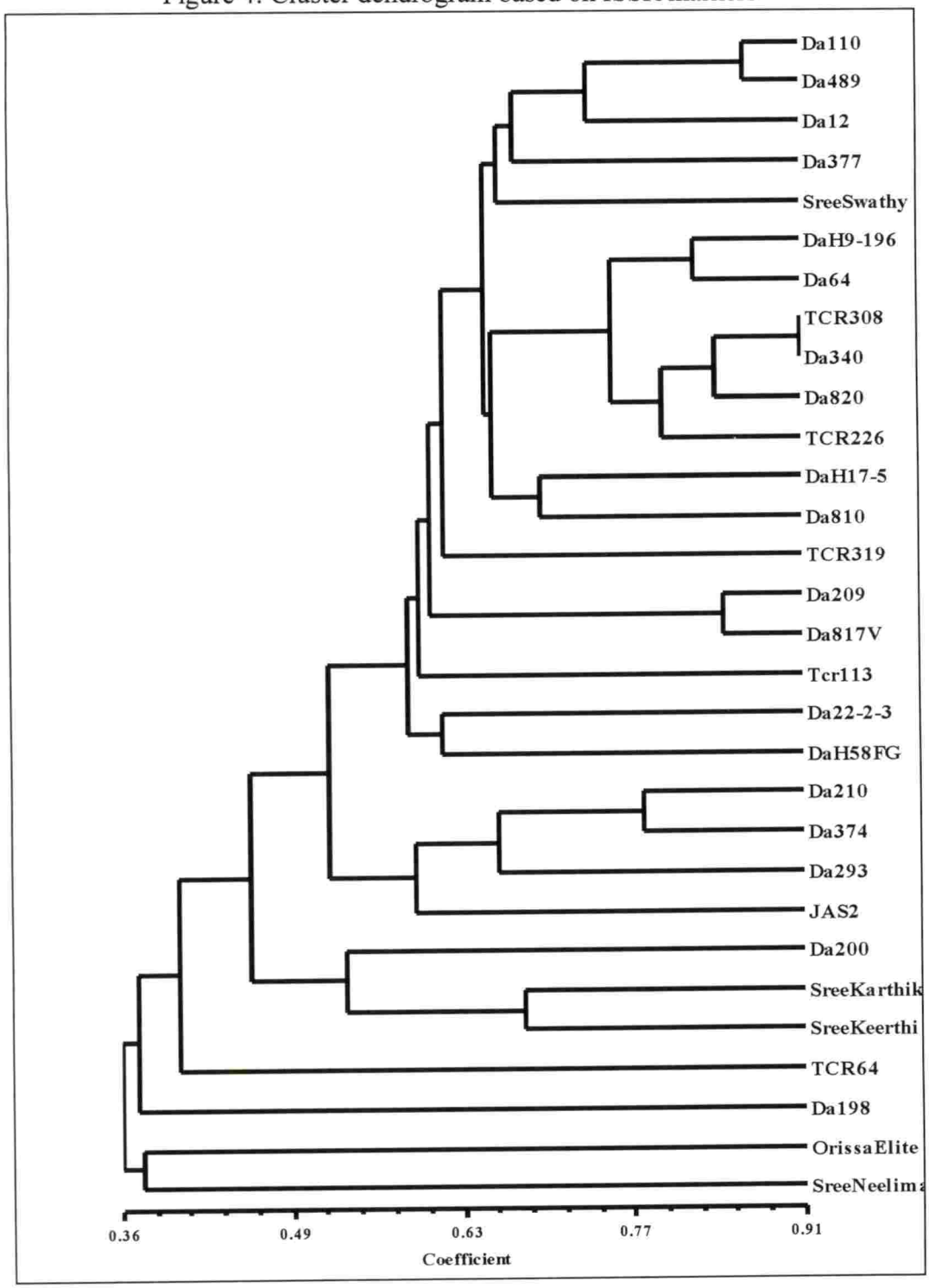


Figure 5: Cluster dendrogram based on RAPD markers

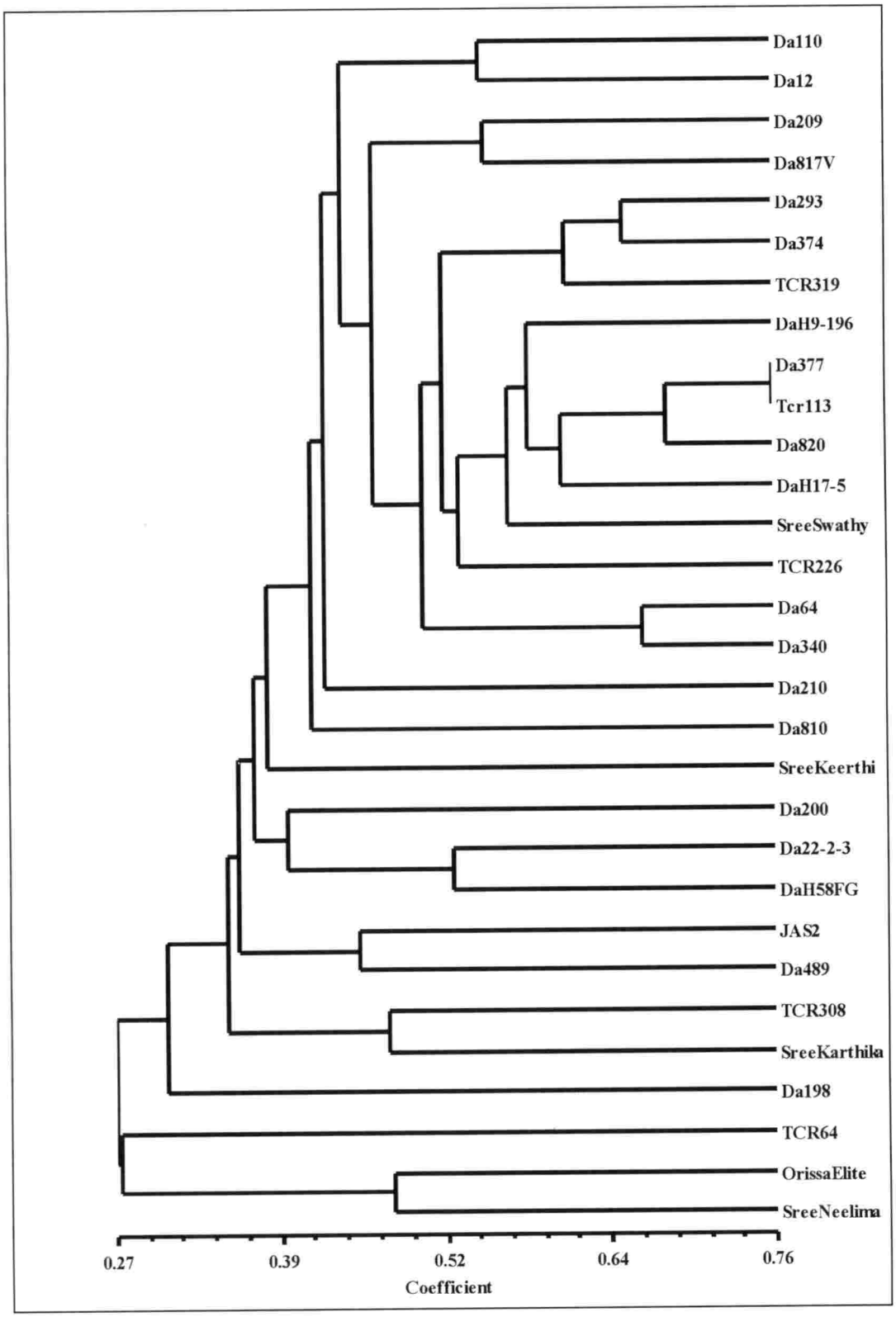


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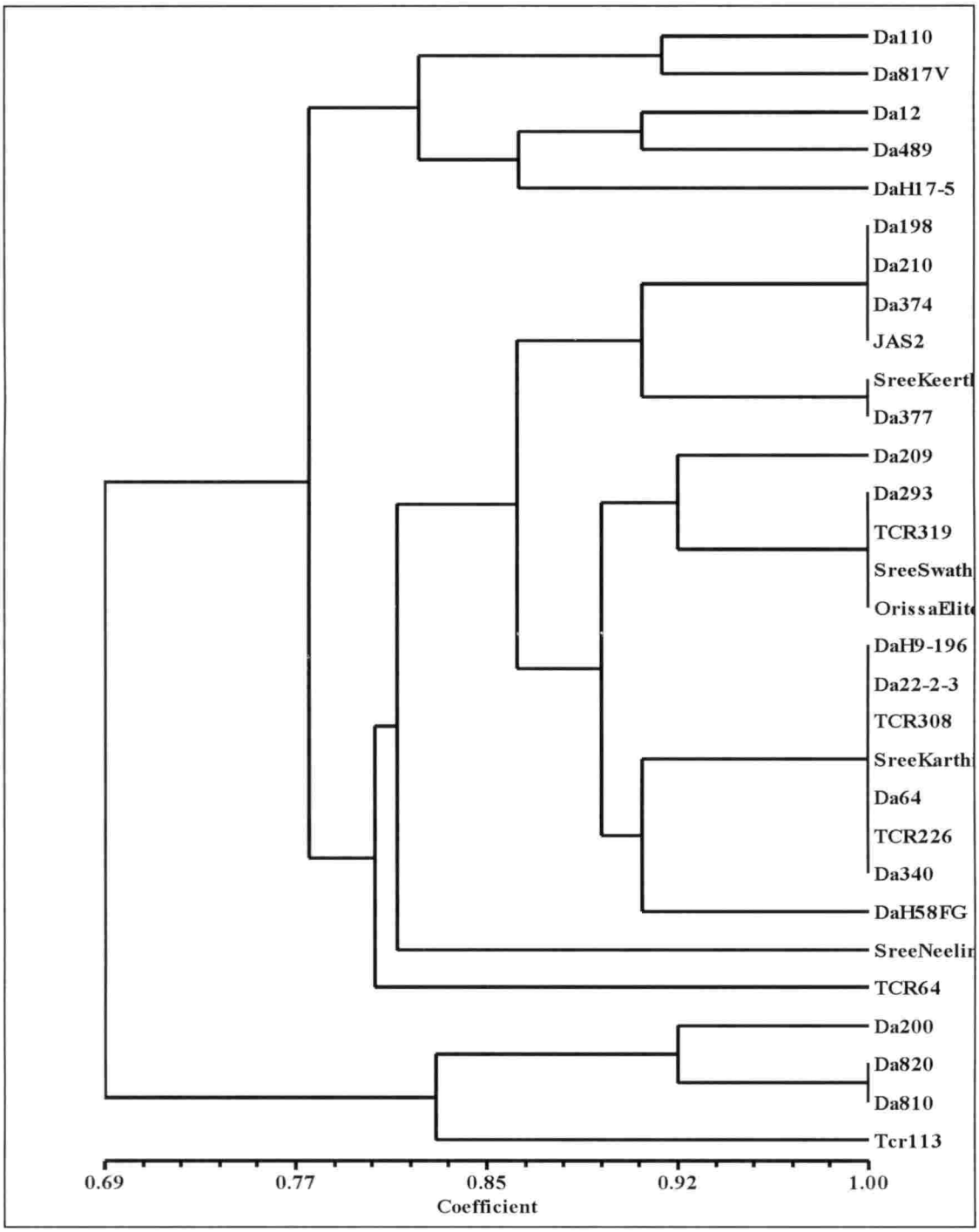
Figure 6: Cluster dendrogram based on SSR markers



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4.4.4 Correlation of Molecular Data

Mantel test was done to assess correlation between the different markers used.

Table 21: r-values and p-values obtained for the three marker combinations

	r-value	p-value
RAPD vs ISSR	0.5139	3.8702
SSR vs ISSR	0.0576	0.4597
SSR vs RAPD	0.0033	0.0271

Mantel test revealed non-significant correlation of the clustering of greater yam genotypes between SSR and other markers. However significantly high correlation of 0.51385 existed between clustering of genotypes based on RAPD and ISSR markers.

4.4.5. Principal component analysis

4.4.5.1 Principal component analysis using ISSR

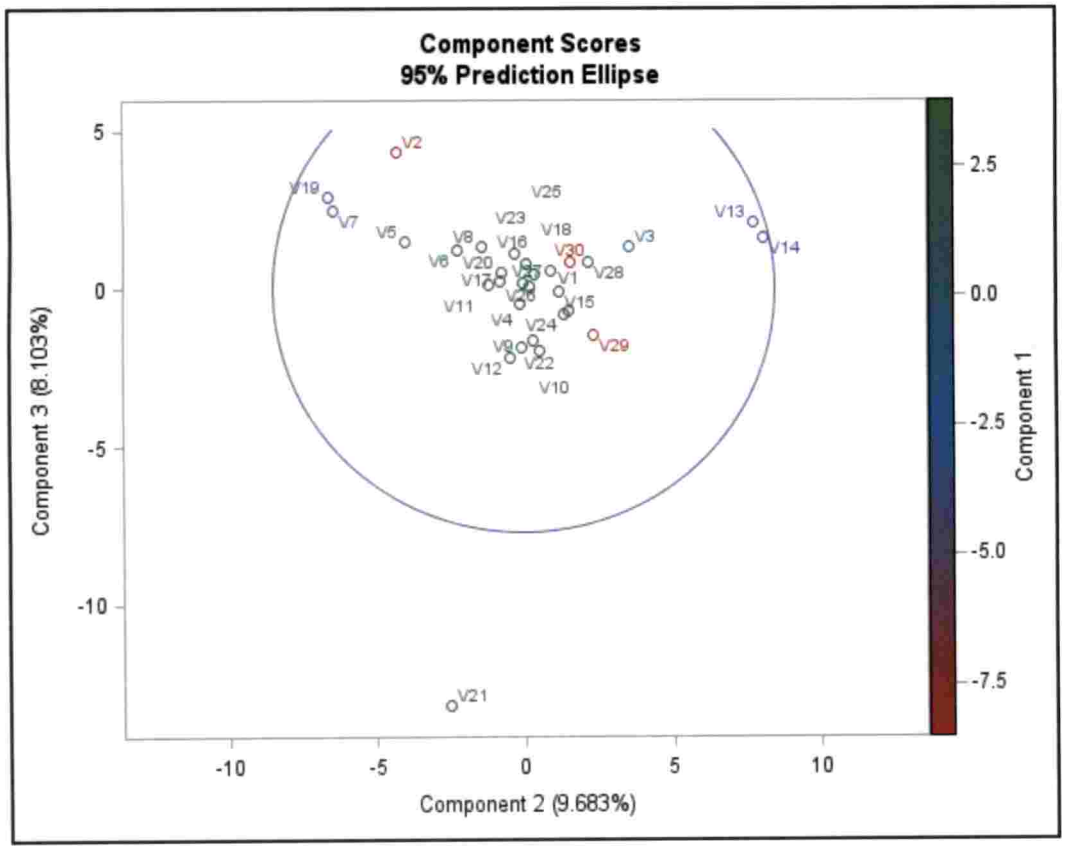
The patterns of variation and the relative importance of each amplicon in explaining the observed variability was assessed through principal component analysis (PCA). The result of PCA grouped the variables into principal components based on ISSR amplicons, among which the first three explained 29.57% of the total variability (Table 22).

Table 22: Eigen values, proportion cumulative variance of the first ten principal components for ISSR markers in 30 genotypes of yams.

Eigenvalues of the Correlation Matrix				
	Eigenvalue	**Difference**	**Proportion**	**Cumulative**
1	12.2510628	2.1810132	0.1178	0.1178
2	10.0700496	1.6434280	0.0968	0.2146
3	8.4266216	1.0672345	0.0810	0.2957
4	7.3593871	1.2521670	0.0708	0.3664
5	6.1072201	0.3323152	0.0587	0.4251
6	5.7749048	0.6845661	0.0555	0.4807
7	5.0903388	0.4173959	0.0489	0.5296
8	4.6729429	0.4007834	0.0449	0.5745
9	4.2721595	0.3276343	0.0411	0.6156
10	3.9445252	0.0615677	0.0379	0.6536

Based on first three principal components, grouping of genotypes was done based on ISSR markers (Fig 7). Genotypes V14 (Sree Keerthy) and V21 (TCR 64) were found to be outliers.

Fig 7: Projection of greater yam genotypes and ISSR markers on PC-I, PC-II and PC-III



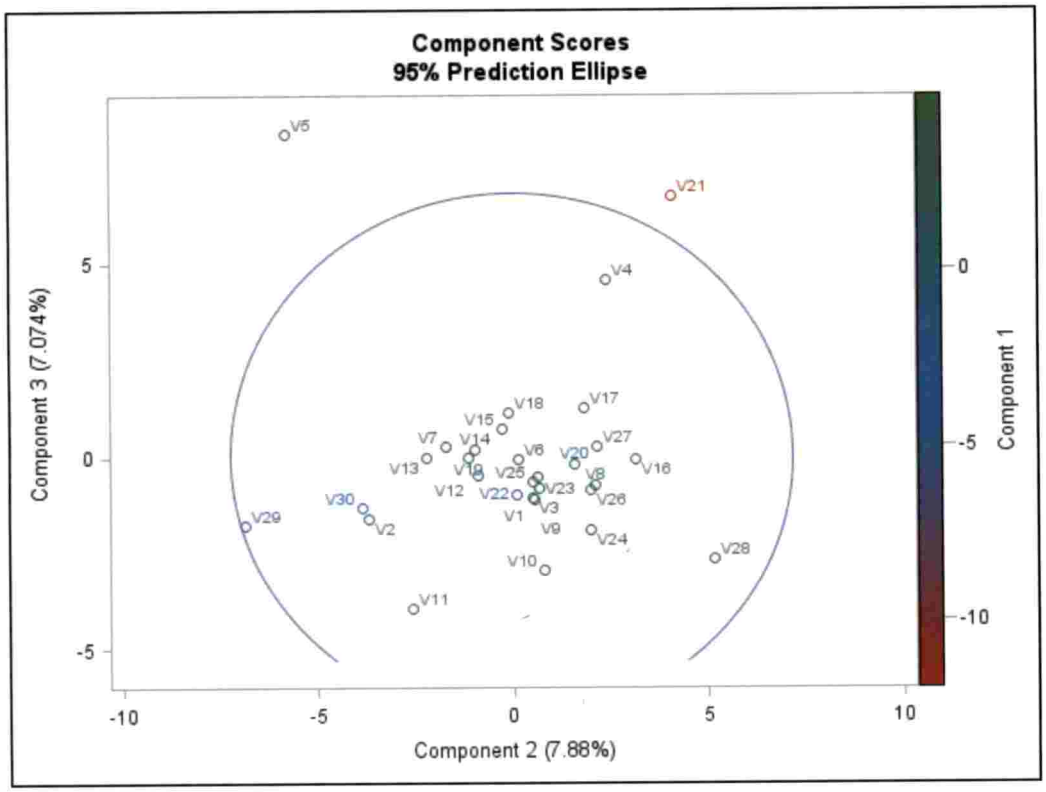
4.4.5.2 Principal component analysis using RAPD

The patterns of variation and the relative importance of each amplicon in explaining the observed variability was assessed through principal component analysis (PCA) based on RAPD marker data. The result of PCA grouped the variables into principal components among which the first three explained 24.19% of the total variability (Table 23).

Table 23: Eigen values, proportion cumulative variance of the first ten principal components for RAPD markers in 30 genotypes of yams.

Eigenvalues of the Correlation Matrix				
	Eigenvalue	**Difference**	**Proportion**	**Cumulative**
1	8.49314795	1.24310422	0.0923	0.0923
2	7.25004373	0.74195453	0.0788	0.1711
3	6.50808920	0.26200039	0.0707	0.2419
4	6.24608881	0.60963231	0.0679	0.3098
5	5.63645650	0.49723413	0.0613	0.3710
6	5.13922237	0.37262543	0.0559	0.4269
7	4.76659694	0.31349315	0.0518	0.4787
8	4.45310378	0.18733554	0.0484	0.5271
9	4.26576824	0.45193037	0.0464	0.5735
10	3.81383787	0.10159839	0.0415	0.6149

Fig 8: Projection of greater yam genotypes and RAPD markers on PC-I, PC-II and PC-III



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Based on first three principal components, grouping of genotypes was done based on RAPD marker data (Fig 8). Genotypes V5 (Da 210) and V21 (TCR 64) were found to be outliers.

4.4.5.3 Principal component analysis using SSR

The patterns of variation and the relative importance of each amplicon in explaining the observed variability was assessed through principal component analysis (PCA) based on SSR marker data. The result of PCA grouped the variables into eight principal components among which the first three explained 68.64% of the total variability (Table 24).

Table 24: Eigen values, proportion and cumulative variance of the first eight principal components for SSR markers in 30 genotypes of yams.

Eigenvalues of the Correlation Matrix				
	Eigenvalue	**Difference**	**Proportion**	**Cumulative**
1	2.99507616	1.13244648	0.3328	0.3328
2	1.86262968	0.54248144	0.2070	0.5397
3	1.32014824	0.38506649	0.1467	0.6864
4	0.93508175	0.31522968	0.1039	0.7903
5	0.61985207	0.05283183	0.0689	0.8592
6	0.56702024	0.05735784	0.0630	0.9222
7	0.50966240	0.31913296	0.0566	0.9788
8	0.19052944	0.19052944	0.0212	1.0000

The grouping of genotypes based on first two principal components of SSR marker data is presented in Fig 9. Genotypes V4 (Da 209) and V18 (Da817V) and V27 (DaH17-5) were found to be highly divergent from other genotypes. Among the three different types of molecular markers *viz.* ISSR, RAPD and SSR used in the present study, SSR markers were found to be better in elucidating variation among greater yam genotypes based on principal component analysis.

Fig 9: Projection of greater yam genotypes and SSR markers on PC-I ad PC-II

4.5. Identification of molecular markers linked to anthracnose disease

Based on field and laboratory screening, 17 genotypes were identified as resistant and 13 genotypes as susceptible. The frequency distribution of the different alleles in resistant and susceptible genotypes with regard to ISSR, RAPD and SSR markers were recorded. The difference between the percentage of the resistance genotypes and susceptible genotypes with specific alleles and their deviation were calculated and shortlisted alleles with respect to ISSR, RAPD and SSR markers were given in Tables 25, 26 and 28 respectively.

4.5.1 Identification of ISSR marker

Out of 15 ISSR primers tested on greater yam accessions 110 amplicons were obtained on 30 genotypes of greater yam. Twenty three amplicons were shortlisted as shown in table 25. The revalidation of markers on three reference highly resistant genotypes *viz.* Da110, DaH9-196 and JAS-2 and three highly susceptible genotypes *viz.* Da810, Orissa elite and Sree Neelima were carried out and the results are given in Table 27.

Table 25: List of ISSR markers shortlisted based on association with anthracnose resistance

Name of the marker	Size of the amplicon	No. of resistant genotypes with the amplicon	No. of susceptible genotypes with the amplicon	% of resistant genotypes with the amplicon(A)	% of susceptible genotypes with the amplicon(B)	Deviation (A-B)
UBC 836	332	15	9	88.24	69.23	19.00
	1754	13	8	76.47	61.54	14.93
UBC 825	435	14	8	82.35	61.54	20.81
	615	15	8	88.24	61.54	26.70
	1176	17	11	100.00	84.62	15.38
	1703	12	7	70.59	53.85	16.74
UBC 809	788	17	11	100.00	84.62	15.38
UBC 807	312	4	0	23.53	0.00	23.53
	416	12	7	70.59	53.85	16.74
	591	17	10	100.00	76.92	23.08
	1328	16	9	94.12	69.23	24.89
(GA)9AT	375	2	0	11.76	0.00	11.76
	388	9	4	52.94	30.77	22.17
	774	2	0	11.76	0.00	11.76
	800	2	0	11.76	0.00	11.76
	1180	2	0	11.76	0.00	11.76
	1291	13	8	76.47	61.54	14.93
	1837	7	3	41.18	23.08	18.10
(GA)9AC	579	13	8	76.47	61.54	14.93
	636	2	0	11.76	0.00	11.76
	956	13	7	76.47	53.85	22.62
	1034	16	8	94.12	61.54	32.58
	1180	17	10	100.00	76.92	23.08

Table 26: List of RAPD markers with associated with anthracnose resistance

Name of the marker	Size of the amplicon	No. of resistant genotypes with the amplicon	No. of susceptible genotypes with the amplicon	% of resistant genotypes with the amplicon (A)	% of susceptible genotypes with the amplicon (B)	Deviation (A-B)
OPI-07	1544	13	5	76.47	38.46	38.01
OPF-06	1181	8	2	47.06	15.38	31.67
	1323	2	0	11.76	0.00	11.76
	2154	14	9	82.35	69.23	13.12
	2217	2	0	11.76	0.00	11.76
OPE-06	1333	3	0	17.65	0.00	17.65
OPI-16	849	2	0	11.76	0.00	11.76
	1009	4	0	23.53	0.00	23.53
	1451	2	0	11.76	0.00	11.76
	1461	2	0	11.76	0.00	11.76
	1488	9	5	52.94	38.46	14.48
	1603	10	6	58.82	46.15	12.67
OPAF-17	808	15	10	88.24	76.92	11.31
	963	17	11	100.00	84.62	15.38

4.5.1.2 Re-validation of selected markers on reference varieties

For an effective marker to be useful in marker assisted selection for anthracnose resistance, it should produce specific alleles in resistant genotypes and should not amplify the same allele in susceptible genotypes. The results revealed the association of UBC 836 (1754bp), UBC 807 (312 bp), (GA)9AT (388, 1837bp) with anthracnose resistance. UBC 836 (1754bp) was amplified in all the highly resistant varieties *viz.* Da110, DaH 9/196 and JAS2 while it was absent in all the susceptible genotypes. The ISSR primer UBC 807 amplified the amplicon of size 312bp in highly resistant genotypes *viz.* Da110, while it is absent in all the susceptible genotypes.

Table 27: Validation of selected markers on reference varieties

Genotype		Da 110	DaH 9-196	JAS 2	Da 810	Orissa Elite	Sree Neelima
		R	R	R	S	S	S
UBC836	1754	1	1	1	0	0	0
UBC807	312	1	1	0	0	0	0
(GA)9AT	388	0	1	1	0	0	0
(GA)9AT	1837	1	1	0	0	0	0

4.5.2 Identification of RAPD marker

Out of 5 RAPD primers tested on greater yam accessions 95 amplicons were obtained on 30 genotypes of greater yam, 110 amplicons were obtained. Fourteen amplicons were shortlisted (Table 27). The revalidation of markers on three reference highly resistant genotypes *viz.* Da110, DaH9-196 and JAS-2 and three highly susceptible genotypes *viz.* Da810, Orissa elite and Sree Neelima were carried out and could not reveal any association of the amplicons with anthracnose resistance.

4.5.3 Identification of SSR marker

Out of 16 SSR markers tested on greater yam accessions 95 amplicons were obtained on 30 genotypes of greater yam, 29 alleles were amplified. Five alleles were shortlisted (Table 28). The revalidation of markers on three reference highly resistant genotypes viz. Da110, DaH9-196 and JAS-2 and three highly susceptible

genotypes viz. Da810, Orissa elite and Sree Neelima were carried out and could not reveal any association of the alleles with anthracnose resistance.

Table 28: List of SSR markers associated with anthracnose resistance

Name of the marker	Size of the amplicon	No. of resistant genotypes with the amplicon	No. of susceptible genotypes with the amplicon	% of resistant genotypes with the amplicon (A)	% of susceptible genotypes with the amplicon (B)	Deviation (A-B)
SSR 06	135	12	6	70.59	46.15	24.43
SSR 08	159	14	7	82.35	53.85	28.51
SSR 12	175	15	9	88.24	69.23	19.00
SSR 16	133	16	10	94.12	76.92	17.19
Dab2E07	170	10	5	58.82	38.46	20.36

Among the markers evaluated for association with anthracnose disease resistance in greater yam, the SSR and RAPD markers could not reveal any association. In the present investigation, the ISSR primer UBC 836 (1754 bp) was found to be the best marker associated with anthracnose disease followed by UBC 807 (312 bp) and (GA)9AT (388bp, 1837 bp). However these amplicons needs to be further validated through sequencing and also in mapping population so as to identify the best molecular marker to be used in marker assisted breeding of greater yam for developing anthracnose disease resistant genotypes.

5. DISCUSSION

Yam is one of the most important staple starchy food crops of the tropics and subtropics, and is particularly important in West Africa, where approximately 93% of the world's annual yam production of about 51.4 mt (FAO, 2007) is produced. Yam belongs to the order Liliflorae, family *Dioscoreaceae*, and genus *Dioscorea*. About 600 species of yam have been identified and among these only 6 are edible yams (Coursey, 1967a; Burkill, 1960). *D. alata* has many superior characteristics when compared to *D. rotundata* for sustainable production, *viz.* high yield potential, ease of propagation, early vigour for weed suppression, and high shelf life and hence is the most widely cultivated yam species. Greater yam tubers possess a high nutritional content with an average crude protein content of 7.4%, starch content of 75±84%, and vitamin C content ranging from 13.0 to 24.7 mg/100g (Muzac-Tucker *et al.,* 1993). A study based on the heredity of microsatellite markers has shown that the basic chromosome number of this species is x = 20 and not x = 10 as previously assumed (Arnau *et al.,* 2009). This species was considered to be highly polyploid with six levels of ploidy (2n = 30, 40, 50, 60, 70 and 80) (Sharma and De., 1956). However, it is now accepted that it has only three cytotypes (2n = 40, 60 and 80) and that the most common forms are diploids, followed by triploids and tetraploids are rare (Arnau *et al.,* 2009). Its major limitation in the field is the susceptibility of most cultivars to anthracnose disease caused by *Colletotrichum gloeosporioides* that exerts a devastating impact on productivity. The deployment of durable host plant resistance in *D. alata* against yam anthracnose disease will contribute significantly to a high level and stability of field performance.

5.1 FIELD SCREENING

In the present investigation to identify anthracnose disease resistant genotypes, a total of 45 greater yam genotypes were used for field screening. Whole plant area scoring method was done in which the lesions were rated visually on a 0-5 scale based on the percentage of lesion appeared on leaves and vines, where 0= no infection, 1= 1-10%, 2= 10-25%, 3= 25-50%, 4= 50-75% and 5= >75%. Die back of yam vines were observed in some of the genotypes *viz.* Orissa elite, Sree Neelima and DaH 24-6-3 and hence were identified as highly susceptible

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genotypes. Da 340 and TCR 142 were found to be highly resistant to anthracnose disease along with the released varieties viz. Sree Keerthy and Sree Swathy.

5.2 LAB SCREENING

Excised leaf assay was carried out using 40 yam genotypes with varying resistance levels to anthracnose. Leaves of similar age and size (leaves at nodes 3 to 4, counted from the top) were used and were inoculated with pure cultures of *Colletotrichum gloeosporioides.* Lesions formed were rated visually at 3 days after inoculation on a 0-5 scale based on the percentage of lesion per inoculated droplet, where 0= no infection, 1= 1-10%, 2= 10-25%, 3= 25-50%, 4= 50-75% and 5= >75%. Many of the accessions including Sree Neelima, Orissa Elite, TCR 64, TCR 226 etc. developed large brown spots with yellow halo and were classified as susceptible whereas accessions such as Sree Karthika, Sree Keerthi, and Sree Swathy didn't show any infection and hence were categorized as resistant genotypes. Similar study was done by Poolsawat *et al.* (2012) for laboratory and field evaluations of anthracnose resistance in grapevine. The reaction of different accessions to the disease was classified into resistant and susceptible categories as presented in Table 13.

Field and lab screening gave comparable results in most of the genotypes. However lab screening was found to be more effective. Amusa (2001) also reported that *in vitro* screening of' cassava and yam clones using toxic metabolites compared favourably with field screening based on natural epidemics. He emphasized that using toxic metabolites appears to be a more effective technique for screening for disease resistance than conventional inoculation with plant pathogens in conformity with the present results.

5.3 MOLECULAR MARKER ANALYSIS

Isolated DNA from 30 greater yam accessions including 17 resistant and 13 susceptible genotypes. DNA samples were diluted to a concentration of 10ng/µl using nuclease free water, irrespective of the varying concentrations for molecular marker analysis. Standardization of PCR conditions was undertaken for different types of molecular markers so as to obtain clear and consistent amplicons under reduced cycle duration. Changing annealing temperature for certain SSR primers

gave better results.

In the present study, molecular markers *viz.* ISSR and RAPD primers were selected based on previous reports in Yams (Mignouna *et al.*, 2002; Narina *et al.*, 2011; Sartie *et al.*, 2012). Among the SSR primers used, four were already reported and the rest twenty four were newly designed using bioinformatics tools. For preliminary screening of ISSR, 15 ISSR primers were used of which, only 7 were selected based on their polymorphism and repeatability. Eleven RAPD primers were screened of which, only 5 were selected based on their polymorphism and repeatability. Six RAPD primers were eliminated after preliminary screening due to poor amplification profile. For preliminary screening of SSR, 26 SSR were used of which, only 16 were selected based on their polymorphism and repeatability.

The ISSR markers are useful in studying variation in microsatellite regions that are scattered particularly in nuclear genome. Total number of bands per ISSR primer ranged from 13 (UBC 807 & UBC 836) to 26 (UBC 809). All the bands of UBC 825, (GA)9AT and (GA)9AC were found to be polymorphic. UBC 809 that recorded the highest number of bands (26) also recorded the highest number of polymorphic bands (25) followed by UBC 827 with nine bands. UBC 836 showed the lowest number of polymorphic bands (11) and the highest number of monomorphic bands (2). The polymorphism of the ISSR primers studied ranged from 84.61% to 100%. UBC 807 recorded the lowest polymorphism (84.61%) among the primers followed by UBC 836 (92.31%).

The expected heterozygosity value (He) of the ISSR primers ranged between 0.53 (UBC 836) to 0.81 ((GA)9AC). The polymorphism Information content (PIC) of the primers ranged from 0.5164 (UBC 836) to 0.7929 ((GA)9AC). All the primers recorded high PIC value of >0.6. The gene diversity values ranged from 0.1680 to 0.2104. The primers UBC 836, (GA)9AC and (GA)9AT recorded the same gene diversity value of 0.1680.

As reported by Mignouna *et al.* (2002), although RAPD markers were adequate for genetic diversity studies, the level of polymorphism detected in mapping populations was low. Total number of bands per RAPD primer ranged from 14 to 24. All the primers except OPE 06 showed 100% polymorphism. The

primer OPI 07 recorded the highest number of bands (24) whereas the primer OPF 06 produced the lowest number of bands (14). The primer OPE 06 exhibited 88.89% polymorphism and 100% polymorphism was observed in the rest four primers.

The expected heterozygosity values ranged from 0.5120 (OPF 06) to 0.7476 (OPE 06). OPF 06 exhibited the lowest PIC value 0.4870. The highest PIC value of 0.7117 was recorded by OPE 06 followed by OPI 16 (0.6287), OPAF 17 (0.5515) and OPI 07 (0.5048). All the primers showed gene diversity values <0.2. The highest gene diversity value was expressed by the primer OPE 06 (0.1754) and the lowest value is 0.1314 expressed by OPAF 17.

The results obtained were contradictory to that reported by Mignouna *et al.* (2002). They found that the markers $OPI7_{1700}$ and $OPE6_{950}$ are closely linked in coupling phase with *Dcg-1,* a single locus that contributes to anthracnose resistance in the tetraploid greater yam breeding line, TDa 95/00328.

Among the sixteen SSR markers studied, both the number of alleles per marker and the number of polymorphic alleles ranged from 1 to 3. Yam SSR 06, Yam SSR 09, and Yam SSR 16 showed highest number of bands (3) in which, all the three bands of Yam SSR 09 were found to be polymorphic. But the bands produced by Yam SSR 02, Yam SSR 03, Yam SSR 15,Yam SSR 18 and Yam SSR 19 were highly monomorphic. The primer DabD08 produced two unique bands with the accessions Da 209 and Da 817V at a band size of 358.74bp. The primers Dab2E07, Yam SSR 9 and Yam SSR 12 showed 100% polymorphism.

The expected heterozygosity values ranged from 0 to 0.6612. The highest PIC value (0.6612) was expressed by Yam SSR 16 followed by Yam SSR 6 (0.5792). The expected heterozygosity measure is helpful in establishing the informativeness of a locus. Loci with expected heterozygosity of 0.5 or less are not very useful for large-scale parentage analysis (Otoo et al., 2009). However in the present finding all the primers recorded PIC value <0.5872. The highest PIC value (0.5872) was recorded by Yam SSR16 followed by Yam SSR6 (0.5023). The high heterozygosity values suggest that spontaneous hybridization must have contributed to the ancestry of some of the accessions and improvement by farmers in agreement with the findings of Obidiegwu *et al.*(2009). The PIC value of primers

Da3G04, Yam SSR 2, Yam SSR 12, Yam SSR 15, Yam SSR 18 and Yam SSR 19 was found to be zero. The gene diversity values ranged from 0 to 0.32. The primers Da3G04, Yam SSR 2, Yam SSR 3, Yam SSR 15, Yam SSR 18 and Yam SSR 19 showed gene diversity value zero. Among the SSR primers tested, Yam SSR12 recorded the highest gene diversity index followed by Dab2E07. However, Otoo *et al.*(2015), reported high gene diversity (0.94) and the average observed heterozygosity of 0.77 for the SSR markers studied on greater yam germplasm in Ghana in contrary to low values recorded in the present study. In the present investigation, most of the SSR primers were designed using bioinformatics tools for identifying the best marker linked to anthracnose resistance

5.4. GENETIC DIVERSITY STUDIES

Cluster analysis was done and dendrograms were generated using UPGMA cluster analysis with 30 accessions of greater yam using ISSR, RAPD and SSR primers.

Based on ISSR primers, accessions were divided into two major clusters at 36% similarity level. Cluster A was again divided into two subclusters in which Da 198 is found to be an outlier. Cluster B includes the two highly susceptible genotypes Orissa Elite and Sree Neelima that recorded38% similarity. Two resistant accessions, TCR 308 and Da 340 were found to possess 91% similarity. Three highly resistant varieties Da 200, Sree Karthika and Sree Keerthi were grouped together.

In the clustering pattern based on RAPD primers, accessions were divided into two clusters at 27 % similarity level. The first cluster grouped 27 accessions together which were then subclustered into 4 at 0.37 dissimilarity level. Cluster 2 that grouped three susceptible genotypes together was again divided into two in which TCR 64 is an outlier. At 37% similarity level, the dendrogram was grouped into four subclusters and 2 outliers. Orissa Elite and Sree Neelima, two highly susceptible genotypes were again clustered together and recorded 47% similarity.

Based on SSR primers, accessions were divided into two major clusters at 69 % similarity level. Cluster A was again divided into two major clusters at 77% similarity. Da 820 and Da 810, two susceptible accessions were found to possess

100% similarity. At 80% similarity level, the 30 genotypes were divided into three subclusters and two outliers. TCR 226 showed 100% similarity with six resistant accessions including Sree Karthika, DaH 9-196, Da 22-2-3, TCR 308, Da 264 and Da 340 and hence resistant genotypes seemed to be grouped together because most of the SSR primers were designed specifically for linkage with anthracnose resistance.

The patterns of variation and the relative importance of each amplicon in explaining the observed variability was assessed through principal component analysis (PCA) based on ISSR, RAPD and SSR marker data. Among the three different types of molecular markers *viz.* ISSR, RAPD and SSR used in the present study, SSR markers were found to be better in elucidating variation among greater yam genotypes based on principal component analysis. Tostain *et al.* (2007) also found SSR markers as discriminatory enough in diversity studies of yam in conformity with the present results.

Mantel test was done to assess correlation between the markers used. Mantel test revealed non-significant correlation of the clustering of greater yam genotypes between SSR and other markers. However significantly high correlation of 0.51385 existed between clustering of genotypes based on RAPD and ISSR markers.

5.5 IDENTIFICATION OF RESISTANCE LINKED MOLECULAR MARKERS

Out of 6 ISSR primers tested on greater yam accessions, 110 amplicons were obtained on 30 genotypes of greater yam. Twenty three amplicons were shortlisted. From the shortlisted amplicons, 4 were selected for re-validation on three highly resistant genotypes viz. Da110, DaH9-196 and JAS-2 and three highly susceptible genotypes *viz.* Da810, Orissa Elite and Sree Neelima. The results indicated the association of three ISSR markers (UBC 807, UBC 836 and (GA)9AT) with anthracnose resistance in greater yam.

Mignouna *et al.*,2002 reported two RAPD markers *viz.* OPW 18850 and OPX15850 linked to Yam Mosaic Virus resistance. However in the present investigation, none of the RAPD markers showed high association with anthracnose resistance in greater yam.

To improve the efficiency of MAS, it is important that the recombination

frequency between the target gene and the marker be as low as possible, and this could be achieved by identifying markers as close as possible to the gene (Hittalmani *et al.*, 2000). The presence of UBC 836 (1754bp) in the three resistant genotypes and absence in three susceptible genotypes used for final validation suggests it as a good marker system for further studies on fine mapping of the resistance gene region in greater yam. The primers UBC 807 (312 bp), (GA)9AT (388, 1837bp) were also found to be present in the two resistant genotypes, but absent in all the three highly susceptible genotypes used for validation and hence can be selected as specific markers linked to anthracnose resistance. Such studies have been conducted on anthracnose resistance genes in common bean cultivar 'G2333' (Young *et al.*, 1998) and on blast resistance genes in rice (Hittalmani *et al.*, 2000).

6. SUMMARY

Yams (*Dioscorea* sp.) are staple food crops for millions of people in tropical and subtropical regions. *Dioscorea alata*, also known as greater yam, is one of the major cultivated species and most widely distributed throughout the tropics. The most serious foliage disease of the Anthracnose, cause by an epiphytic fungus *Colletotrichum gleosporioides.* The disease appears as very small brown or blackish spots on the leaves and stem. The spots enlarge, the uninfected leaf tissue tends to yellow, and eventually the leaves die, but may hang on the vine for long periods. Premature dieback of leaves and stems drastically reduces yield. It can cause yield reduction of upto 80% and so is a major constraint faced by farmers. Hence the present study aims to identify greater yam accessions with high resistance to anthracnose disease and to identify molecular markers (RAPD, ISSR and SSR) linked to anthracnose resistance in greater yam that can be used for marker assisted breeding in future.

Field screening and laboratory screening was done by whole plant area scoring method and Excised leaf assay technique respectively. In both the methods, the lesions observed were rated visually on a 0-5 scale based on the percentage of lesion on leaves and vines, where 0= no infection, 1= 1-10%, 2= 10-25%, 3= 25-50%, 4= 50-75% and 5= >75%. This helped to classify the 40 genotypes into resistant and susceptible groups, from which 17 resistant and 13 susceptible genotypes were selected for molecular marker analysis.

DNA was isolated from the selected 30 accessions for molecular studies. Standardization of PCR conditions was undertaken for different types of molecular markers so as to obtain clear and consistent amplicons under reduced cycle duration. Changing annealing temperature for certain SSR primers gave better results. Molecular markers *viz*. ISSR, SSR and RAPD primers were selected based on previous reports in yams. A set of 24 newly synthesized SSR primers were also used. For preliminary screening of ISSR, 15 ISSR primers were used of which, only 6 were selected based on their polymorphism and repeatability. For preliminary screening of RAPD, 11 RAPD were used of which, only 5 were selected based on their polymorphism and repeatability. Twenty six SSR primers were screened of

which, 16 were selected based on their polymorphism and repeatability.

Total number of bands per ISSR primer ranged from 13 (UBC 807 & UBC 836) to 26 (UBC 809). All the bands of UBC 825, (GA)9AT and (GA)9AC were found to be polymorphic. UBC 809 that recorded the highest number of bands (26) also recorded the highest number of polymorphic bands (25) followed by UBC 827 with nine bands. UBC 836 showed the lowest number of polymorphic bands (11) and the highest number of monomorphic bands (2). The polymorphism of the ISSR primers studied ranged from 84.61% to 100%. The expected heterozygosity value (He) of the ISSR primers ranged between 0.53 (UBC 836) to 0.81 ((GA)9AC). The polymorphism Information content (PIC) of the primers ranged from 0.5164 (UBC 836) to 0.7929 ((GA)9AC). All the primers recorded high PIC value of >0.6. The gene diversity values ranged from 0.1680 to 0.2104.

Total number of bands per RAPD primer ranged from 14 to 24. All the primers except OPE 06 showed 100% polymorphism. The primer OPI 07 recorded the highest number of bands (24) whereas the primer OPF 06 produced the lowest number of bands (14). The primer OPE 06 exhibited 88.89% polymorphism and 100% polymorphism was observed in the rest four primers. The expected heterozygosity values ranged from 0.5120 (OPF 06) to 0.7476 (OPE 06). OPF 06 exhibited the lowest PIC value 0.4870. The highest PIC value of 0.7117 was recorded by OPE 06 followed by OPI 16 (0.6287), OPAF 17 (0.5515) and OPI 07 (0.5048). The highest gene diversity value was expressed by the primer OPE 06 (0.1754) and the lowest value is 0.1314 expressed by OPAF 17.

Among the sixteen SSR markers studied, both the number of alleles per marker and the number of polymorphic alleles ranged from 1 to 3. Yam SSR 06, Yam SSR 09, and Yam SSR 16 showed highest number of bands (3) in which, all the three bands of Yam SSR 09 were found to be polymorphic. The primer DabD08 produced two unique bands with the accessions Da 209 and Da 817V at a band size of 358.74bp. The primers Dab2E07, Yam SSR 9 and Yam SSR 12 showed 100% polymorphism. The expected heterozygosity values ranged from 0 to 0.6612. The highest PIC value (0.6612) was expressed by Yam SSR 16 followed by Yam SSR 6 (0.5792). All the primers recorded PIC value <0.5872. The highest PIC value

(0.5872) was recorded by Yam SSR16 followed by Yam SSR6 (0.5023). The gene diversity values ranged from 0 to 0.32. Among the SSR primers tested , Yam SSR12 recorded the highest gene diversity index followed by Dab2E07.

Separate dendrograms were generated for the three classes of primers using UPGMA cluster analysis with 30 accessions of greater yam. Dendrograms showed the partition of many of the resistant and susceptible genotypes in different clusters. Based on ISSR primers, accessions were divided into two major clusters at 36% similarity level. Cluster A was again divided into two subclusters in which Da 198 is found to be an outlier. Cluster B includes the two highly susceptible genotypes Orissa elite and Sree Neelima and the showed 38% similarity. Two resistant accessions, TCR 308 and Da 340 were found to possess 91% similarity. Three highly resistant varieties Da 200, Sree Karthika and Sree Keerthi were grouped together. In the clustering pattern based on RAPD primers, accessions were divided into two clusters at 27 % similarity level. The first cluster grouped 27 accessions together which were then subclustered into 4 at 0.37 dissimilarity level. Cluster 2 that grouped three susceptible genotypes together was again divided into two in which TCR 64 is an outlier. Orissa elite and Sree Neelima, two highly susceptible genotypes were clustered together and showed 47% similarity. The dendrogram produced by SSR primers was unique and the accessions were divided into two major clusters at 69 % similarity level. Cluster A was again divided into two major clusters at 77% similarity. Da 820 and Da 810, two susceptible accessions were found to possess 100% similarity. At 80% similarity level, the 30 genotypes were divided into three subclusters and two outliers. TCR 226 showed 100% similarity with six resistant accessions including Sreekarthika, DaH 9-196, Da 22-2-3, TCR 308, Da 264, and Da 340 and so it can be called as a highly tolerant genotype.

Among the three different types of molecular markers *viz.* ISSR, RAPD and SSR used in the present study, SSR markers were found to be better in elucidating variation among greater yam genotypes based on principal component analysis. Mantel test revealed non-significant correlation of the clustering of greater yam genotypes between SSR and other markers. However significantly high correlation of 0.51385 existed between clustering of genotypes based on RAPD and ISSR

markers.

The results indicated the association of three ISSR markers (**UBC 807, UBC 836 and (GA)9AT)** with anthracnose resistance in greater yam. The presence of UBC 836 (1754bp) in the three resistant genotypes and absence in three susceptible genotypes, used as reference for final validation, suggests it as the best marker studied. The primers UBC 807 (312 bp), (GA)9AT (388, 1837bp) were also found to be present in the two resistant genotypes, but absent in the three susceptible genotypes used for validation and hence can be selected as specific markers linked to anthracnose resistance.

The results suggests UBC 807, UBC 836 and (GA)9AT as good markers associated with anthracnose resistance. However these markers needs further validation on mapping population preferably recombinant breeding lines that can be developed using the highly resistant genotypes identified in the present investigation. Also highly resistant genotypes *viz.* Da110, JAS2 and DaH9/196 identified can be further evaluated to develop a variety with high level of resistance to anthracnose disease for cultivation in Kerala.

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APPENDIX I

CTAB Extraction Buffer

Tris- HCl (pH 8.0)	100 mM	
EDTA	25 mM	
NaCl	1.5 M	
CTAB	2.5%	
β-mercaptoethanol	0.2% (v/v)	freshly added prior to DNA extraction
PVP	1 % (w/v)	

APPENDIX II

Wash solution

Ammonium acetate	15mM
Ethanol	75%

APPENDIX III

TE buffer (10X)

Tris- HCl (pH 8.0)	10 mM
EDTA	1 mM

APPENDIX IV

NaCl	2M
PEG	4%

APPENDIX V

TBE Buffer (10X)

Tris base	107g
Boric acid	55g
0.5 M EDTA (pH 8.0)	40 ml

Final volume made up to 1000 ml with distilled water and autoclaved before use.

Identification of molecular markers linked to anthracnose resistance in greater yam (*Dioscorea alata* L.)

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Abstract of the thesis
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ABSTRACT

Yam (*Dioscorea* spp) is a multi-species, polyploid and vegetatively propagated tuber crop that is cultivated widely in the tropics and subtropics. Yam belongs to the order Liliflorae, family Dioscoreaceae, and genus *Dioscorea* and are mainly depended for tubers, which are enlarged storage organs containing food reserves in the form of starch and carbohydrate. *Dioscorea alata* L., commonly known as water yam is one amongst the top ten most important yam species that contribute to food security of millions of people of the world. The major limitation to the sustainable production of *D. alata* is its susceptibility to anthracnose disease, caused by the fungus *Colletotrichum gleosporioides* and it can cause yield reduction upto 80%. Hence the present study was carried out with an objective to identify resistant genotypes and molecular markers linked to genes conferring resistance to anthracnose in greater yam using ISSR, RAPD and SSR primers.

Field and laboratory screening was done by whole plant area scoring method and excised leaf assay technique respectively. In both the methods, the lesions observed were rated visually on a 0-5 scale based on the percentage of lesion on leaves and vines, where 0= no infection, 1= 1-10%, 2= 10-25%, 3= 25-50%, 4= 50-75% and 5= >75%. This helped to classify the 40 genotypes used into resistant and susceptible groups, from which 17 resistant and 13 susceptible genotypes were selected for molecular marker analysis.

DNA was isolated from 30 greater yam (*Dioscorea alata* L.) genotypes. Based on preliminary screening, 6 ISSR, 5 RAPD and 24 SSR primers with high polymorphism were selected for the molecular characterization of greater yam genotypes.

Total number of bands per ISSR primer ranged from 13 (UBC 807 & UBC 836) to 26 (UBC 809). All the bands of UBC 825, (GA)9AT and (GA)9AC were found to be polymorphic. The polymorphism of the ISSR primers studied ranged from 84.61% to 100%. The expected heterozygosity value (He) of the ISSR primers ranged between 0.53 (UBC 836) to 0.81 ((GA)9AC). The polymorphism Information content (PIC) of the primers ranged from 0.5164 (UBC 836) to 0.7929 ((GA)9AC). The gene diversity values ranged from 0.1680 to 0.2104. Total number

of bands per RAPD primer ranged from 14 to 24. All the primers except OPE 06 showed 100% polymorphism. The expected heterozygosity values ranged from 0.5120 (OPF 06) to 0.7476 (OPE 06). All the primers showed gene diversity values <0.2. Among the sixteen SSR markers studied, the number of alleles per marker ranged from 1 to 3 while the number of polymorphic alleles ranged from 0 to 3. The expected heterozygosity values ranged from 0 to 0.6612. All the primers recorded PIC value <0.5872. The gene diversity values ranged from 0 to 0.32.

Mantel test revealed non-significant correlation of the clustering of greater yam genotypes between SSR and other markers. However significantly high correlation of 0.51385 existed between clustering of genotypes based on RAPD and ISSR markers. Among the three different types of molecular markers *viz.* ISSR, RAPD and SSR used in the present study, SSR markers were found to be better in elucidating variation among greater yam genotypes based on principal component analysis.

The frequency of the different alleles in resistant and susceptible genotypes with regard to ISSR, RAPD and SSR markers were recorded. The difference between the percentage of the resistance genotypes and susceptible genotypes with specific alleles and their deviation were calculated and shortlisted with respect to ISSR, RAPD and SSR markers. The results indicated the association of three ISSR markers (**UBC 807, UBC 836** and **(GA)9AT)** with anthracnose resistance in greater yam. The presence of UBC 836 (1754bp) in the three resistant genotypes and absence in three susceptible genotypes, used as reference for final validation, suggests it as the best marker studied.

The results suggests UBC 807, UBC 836 and (GA)9AT as good markers associated with anthracnose resistance. However these markers needs further validation on mapping population preferably recombinant breeding lines that can be developed using the highly resistant genotypes identified in the present investigation. Also highly resistant genotypes *viz.* Da110, JAS2 and DaH9/196 identified can be further evaluated to develop a variety with high level of resistance to anthracnose disease for cultivation in Kerala.