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In Vitro propagation and identification of molecular markers linked to dwarfness in white yam (*Dioscorea rotundata* Poir.)

by

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(2010-09-103)

THESIS

Submitted in partial fulfillment of the

requirement for the degree of

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M.Sc. Integrated Biotechnology

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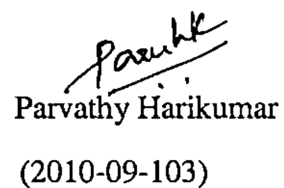
KERALA, INDIA

2015

DECLARATION

I hereby declare that the thesis entitled "***In vitro* propagation and identification of molecular markers linked to dwarfness in white yam (*Dioscorea rotundata* poir.)**" is a bonafide record of research done by me and that the thesis has not previously formed the basis for the award of any degree, diploma, fellowship or other similar title, of any other University or Society.



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Produce tuber reduce hunger

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Certified that this thesis, entitled "***In vitro* propagation and identification of molecular markers linked to dwarfness in white yam (*Dioscorea rotundata* Poir.)**" is a record of research work done independently by **Parvathy Harikumar (2010-09-103)** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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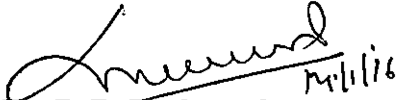

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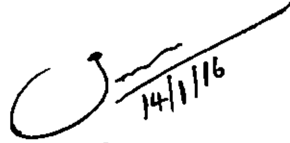

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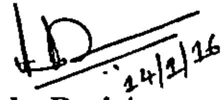

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LIST OF ABBREVIATIONS

^{0}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
$AgNO_3$	Silver nitrate
APS	Ammonium persulfate
Bind Silane	3- Methacryloxypropyltrimethoxysilane
bp	Base pair
cm	Centimetre
cM	centimorgan
CAPS	Cleaved Amplified Polymorphic Sequence
CTAB	Cetyl trimethyl ammonium bromide
CTCRI	Central Tuber Crops Research Institute
DNA	Deoxyribo nucleic acid
dNTPs	Deoxy nucleotide tri phosphates
EDTA	Ethylene diamine tetra acetic acid

FAO	Food and Agriculture Organization
FAOSTAT	Food and Agriculture Organization Statistical Database
F_1	Filial 1
G	Gram
GLM	Generalized Linear Model
h	Hour
ha	Hectare
IPC	Integral plate chamber
IPGRI	International Plant Genetic Resources Institute
ISSR	Inter simple sequence repeat
KAU	Kerala Agricultural University
kbp	Kilo base pair
kg	Kilogram
kcal	Kilocalories
M	Molar
mg	Milligram
$MgCl_2$	Magnesium Chloride
min	Minute
ml	Millilitre
mm	Millimetre
m*M*	Millimolar

NaCl	Sodium chloride
NaOH	Sodium hydroxide
NBPGR	National Bureau of Plant Genetic Resources
ng	Nanogram
nm	Nanometre
OD	Optical Density
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PVP	Polyvinyl pyrrolidone
QTL	Quantity Trait Loci
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
RNase	Ribonuclease
rpm	Revolution per minute
s	Second
SSR	Simple sequence repeat
SCAR	Sequence Characterized Amplified Regions
STS	Sequence Tagged Site
Taq	*Thermus aquaticus*
TBE	Tris-borate EDTA buffer
TE	Tris- EDTA buffer

TEMED	N,N ,N',N'-tetramethylethylenediamine
T_m	Melting temperature
Tris HCl	Tris (Hydroxy Methyl) aminomethane hydrochloride
U	Enzyme unit
UV	Ultraviolet
V	Volt
v/v	volume/volume
W	Watt
w/v	Weight/volume

INTRODUCTION

1. INTRODUCTION

The English term 'Yam' is believed to be originated from tribal African word "niam" meaning "to sample" or "to taste"(Coursey *et al.*, 1967).Yam (Dioscorea genus of family Dioscoreaceae) is an important food and nutritional security crop grown in tropical countries for its underground tubers. It is a source of income and also a part of socio-cultural events. Currently yams are cultivated in about 50 tropical countries on 4.6 million ha worldwide with an annual production of about 52 million tons (FAO, 2007).

It is a leading source of calories for over 300 million people in Africa, Asia, parts of South America, as well as the Caribbean and the South Pacific islands (Degras, 1983).It contains higher value of protein (2.4%) and substantial amount of vitamins (thiamine, riboflavin and ascorbic acid) and minerals like calcium, phosphorous and iron than any other common tuber crops (Oyenuga, 1968). It also has medicinal properties such as the steroidal sapogenins, used in the production of cortisone and synthetic steroid hormones (Narula *et al.,* 2007).Yams rank as the fourth most important tuber crop in economic terms (Mignouna *et al.*, 2005), after potatoes (*Solanum tuberosum* L.), cassava (*Manihote sculenta* Crantz.) and sweet potatoes (*Ipomoea batatas* (L.) Poir.). It has a relatively small genome size estimated at 550 Mbp/l C for *D. alata* and 800 Mbp/l C for *D. rotundata* (Mignouna *et al.*, 2002).

It is a polyploid and clonally propagated plant. In most species they are replanted and produced annually, while in others they are perennial. As crops, yams are harvested on every season and replanted using tuber pieces to regenerate the plant. Unlike other tropical root and tuber crop species, once harvested yam can be stored for 4-6 months in ambient tropical conditions without significant deterioration of the nutritional properties. Tubers often dried and later milled into flour for reconstituting as a stiff paste (*fufu*), mainly consumed in West Africa (Lebot, 2009).

Greater yam, white yam, lesser yam and aerial yam are the major edible yams grown in India. White yam (*Dioscorea rotundata* Poir) is grown as a major food crop in African countries. It is emerging as an important tuber crop in India as well as in Kerala and it is well known for its yield potential. Central Tuber Crops Research Institute holds a field gene bank of 1100 *Dioscorea* accessions including 158 white yam genotypes. Use of stakes for trailing white yam led to high cost of cultivation limiting the spread of the crop. Recent studies revealed that the most critical problems facing the farmers cultivating yams includes, non availability of adequate staking materials and its high cost that accounts for 30 percent of the cost of cultivation. High labour cost for harvesting also necessitates the development of varieties with dwarf plant stature. A dwarf mutant, Sreedhanya was released in 1993 as the first dwarf variety suitable for planting with closer spacing without any staking.

Conventional breeding of yam is time consuming due to various factors including the long growth cycle. Despite their economic and socio cultural importance, there is limited knowledge about the origin, phylogeny, diversity, and genetics of these wild yams due to research neglect and several biological constraints (Mignouna *et al.*, 2007).Therefore process of developing new varieties through conventional plant breeding can take almost 25 years. Indeed, such a procedure is laborious and time consuming, involving several crosses, several generations, and careful phenotypic selection, and the linkage drag (tight linkage of the undesired loci with the desired loci) may make it further difficult to achieve the desired objective. Now, however, biotechnology has considerably shortened the time to 7-10 years for new crop varieties through marker assisted selection (MAS).

The identification of DNA markers linked to key traits will make it possible to accelerate the gene transfer process. Advent of DNA marker technology, development of several types of molecular markers and molecular breeding strategies offered possibilities to plant breeders and geneticists to overcome many of the problems faced during conventional breeding. A number of molecular marker systems are now available based on the need. Use of linked molecular marker also would allow indirect selection for desirable traits in early segregating generations at the seedling stage independent of environmental influences. DNA markers shows variations at sequence level, not influenced by environment and it will be expressed in all tissues. Some of the commonly used molecular markers are RFLP, RAPD, SSR, ISSR, SCAR, CAPS, STS etc.

Micropropagation is the practice of rapidly multiplying stock plant material to produce a large number of progeny plants, using modern plant tissue culture methods. It is used to multiply novel plants that have been genetically modified or bred through conventional plant breeding methods. It can be used for plants that does not produce seeds or not respond well to vegetative propagation.

In vitro propagation helps to avoid problems related to *ex vitro*. Yam propagation by seeds using conventional methods is slow and not adequate for rapid multiplication. Tuber yield is drastically reduced by viral and nematode infections. Through infected tubers it is transmitted to the next generation and it also deteriorate the quality of the tuber. In yam cultivation, the most important problem faced by farmers is the non availability of quality planting material, because part of the tuber itself is saved as planting material for next season. Quality of planting material should also be assured to ensure good sprouting and less contamination. So the necessity of standardization of protocol for *in vitro* propagation is inevitable. It helps in mass propagation of bushy yam and enhances the availability of planting materials to farmers.

Hence the present study aims at identification of molecular markers linked to dwarfness in white yam and also elucidation of genetic diversity among white yam accessions based on molecular markers. The study also aims at developing method for rapid multiplication of quality planting material of bushy yam through standardization of micropropagation protocol.

Review of Literature

2. REVIEW OF LITERATURE

2.1 ROOT AND TUBER CROPS

Root and tuber crops are plants that are grown for their modified, thickened roots or stems, which generally develop underground (Bradshaw, 2010).They are rich in carbohydrates and are commonly used as a dietary staple, livestock feed, raw material for the production of industrial products such as starch and alcohol, or processed into various food products. The tropical root and tuber crops are the oldest on the earth. In wet tropics, they are the only staple and fed extensive populations before the introduction of cereals. They represent the second most important set of food crops in developing countries, following the cereals.

The important tuber crops around the world are cassava, potato, sweet potato, yams and cocoyams. Some of these are root tubers like cassava and sweet potato and others are stem tubers like potato and cocoyams while yam is an intermediate between stem and root tuber. With low inputs it can be raised and it will be a source of income and employment in marginal areas especially for women. Mostly consumed by poor all over the world, root and tuber crops promises food security and they also plays high esteem culturally. They are cash crops and are used for animal feed or as raw material for industrial processing. With increasing world population the demand for carbohydrates also will increase and being rich source of carbohydrates, root and tuber crops can play an essential role in ensuring food security especially to low income population.

2.2 Yams

Yams are considered as crops of ancient origin which were domesticated before 5000B.C. They are members of genus *Dioscorea* which produces tubers, bulbils or rhizomes having economic importance (Lawton and Lawton, 1969). They are consumed as staple food and are rich in starch and energy. The most important part of yams is tuber. It can be eaten as boiled yam, fufu or fried in oil. Vitamin C has been found in unpeeled yam slices. It consists of pharmacologically active substances including dioscorine, saponin and sapogenin. Dioscorine is an alkaloid, which is a heart stimulant. Yams are considered to be monocots, despite occasional evidence of the existence of a second cotyledon. They belong to the family dioscoreacea within the order dioscoreales (Ayensu and Coursey, 1972). The genus dioscorea is the largest genus of the family. The family includes ten genera and 650 species and are mainly tropical and subtropical and semi temperate in distribution. Cytologically yams have a basic chromosome number n=10. But various degree of polyploidy exists even within the same species. Thus different races with 2n= 40, 60, 80 or 100 chromosomes for *D.bulbifera* and 2n=40 , 90 and 100 for *D.esculenta* have been reported (Raghavan, 1958).

Highest chromosome number count for *D.cayenensis* is 2n =140. Mostly highest chromosome number and smallest chromosome size occur in more tropical *Dioscorea* species, while the smallest numbers and largest sizes occurs mostly in temperate species. The tubers vary in size and shape, averaging 3-8 pounds but sometimes reaching 60 pounds or more. Aerial tubers may develop in the axils of the leaves, especially when vines run on the ground (Coursey, 1967). They grow best in the rain fed areas where the annual rainfall ranges from 1000 to 3000mm, having temperature range between 20°C to 31°C. It can also be raised under irrigation. There are hundreds of cultivars among the cultivated species. Most important species in

economic point of view of the genus *Dioscorea* are *D.rotundata, D.alata, D.cayenensis, D.esculenta, D.dumetorum, D.bulbifera, D.trifida, D.opposita* and *D.japonica.*

Taxonomically the genus is divided in to section, in which species fall. The section enantiophylum contain most of the economically important yam species (*D.rotundata, D.alata, D.cayenensis, D.opposita* and *D.japonica*) characterised by the fact that vines twine to the right that is clockwise direction when viewed from the ground upwards. The most important yam on worldwide basis is *Dioscorea rotundata* Poir (White yam or white guinea yam) grown on larger area compared to other yam species. The *Dioscorea rotundata-cayenensis* complex accounts for the ninety five percent of yam production worldwide. It is native to West Africa and two species *viz.* *D.abyssinica* Hochst ex Kunth and *D.praehensilis* Benth are believed to be the wild relatives of *D.rotundata* (Waitt, 1961).

D. rotundata is the most important African yam, especially in the forest zone, and is probably a hybrid of other African yam, *D. cayenensis*, which is a savannah species. *D. rotundata* plants have $2n = 40$ chromosomes. Cultivars of *D. rotundata* were classified into early and late maturing cultivars. The early maturing cultivars are harvested twice in a year. The first harvest occurs 3-5 months after sprouting and tubers are used for culinary preparations. The second harvest occurs after end of the vegetative cycle. Early maturing cultivar requires more fertile soil than late maturing cultivars. Late maturing cultivars are harvested once in a year at the end of the vegetative cycle and each plant produces many tubers of different weights that vary depending on the cultivars. The late maturing cultivars are best adapted for chips production (Dumont *et al.*, 2006).The most important pests affecting these species are nematode and potyvirus. Most of the varieties cultivated are accessions selected from farmer's field from among existing land races. Breeding in yams as compared to

other tuber crops such as potato or cassava had literally not been attempted recently. It was only in 1960's that the first breeding programme was undertaken.

The lack of knowledge about the origin, diversity and genetics of these species has extremely limited the effectiveness of genetic improvement programmes (Arnau *et al.*, 2010).Major constraint on breeding programme is that large number of varieties does not flower or produce only reduced number of flowers. This is due to exclusive vegetative propagation over a long time and it is one of the problematic genera for cytogenetic studies because of the small size of the chromosomes, sticky nature and their satellites get confused as chromosomes due to same length. Recently some cytogenetic studies and marker studies have been used to characterise the diversity of germplasam collections of *D. rotundata/ D. cyanensis*.

The acquisition of knowledge about the genetic diversity of the species at both agronomic and cytogenetic levels is essential for the effective genetic improvement programme. Hence in yams, being an essential tuber crop more research studies based on markers and cytogenetics needs to be conducted.

2.3 **Origin and Distribution**

Dioscorea spp. is found throughout the tropics and they are the only root crops in which the Asian and African species developed independently of each other. *Dioscorea rotundata* mainly originated in West Africa. It is especially found in forest zone, and is probably a hybrid of the other African yam, *D. cayenensis*, which is a savannah species. *D.alata* is the most widely distributed cultivated yam species in the world and is also one of the oldest cultivated species. They originated from South-east Asia/Melanesia. Exchange and distribution of species was done by the influence of Portuguese explorers.

Table 1. Geographical origin of the yam species

Dioscorea spp.	Common names	Geographical origin
D. alata	Greater,water,winged yam	South-east Asia,Melanesia
D. bulbifera	Aerial,bulbil-bearing yam	SouthAmerica,Africa, Asia, Melanesia
D. cayenensis	Yellow guinea yam	West Africa
D.esculenta	Lesser yam,Asiatic yam	South-eastAsia, Melanesia
D.opposita-japonica	Chinese,Japanese yam	Japan,China
D. nummularia	Spiny yam,wild yam	Melanesia
D. pentaphylla	Five-leaved yam	South-eastAsia, Melanesia
D. rotundata	**White Guinea yam**	**West Africa**
D.transversa	Marou,Wael	Australia, Melanesia
D. trifida	Aja,aje,cush-cush,yampi	South America

2.4 Taxonomy

The genus *Dioscorea* is the type genus of the family Dioscoreacea and is the largest genus within this family of about 644 species (Govaerts *et al.*, 2007). The genus name *Dioscorea* was chosen by Linne in honour of the greek medico and herbalist Dioscorides, who lived in the first century AC. Plants of the genus *Dioscorea* are angiosperms and are taxed below monocotyledon plant class Liliopsida, the subclass Liliidae that comprises the orders Asparagales, Orchidales,

Pandanales, Lilliales and Dioscoreales. Dioscoreales comprises three plant families, namely the Dioscoreacea, called the yam plants, the Trilliaceae and the Smilacaceae. Among the other tropical plants in the family of Dioscoreaceae, *Tamus communis* is the only representative in the temperate regions. All *Dioscorea* species are dioecious twining climbers producing dry capsules, although occasionally both male and female flowers can be found on the same plant.

All species of economic importance are tuberous. The genus *Dioscorea* is divided into sections which have taxanomic status. The main food yam species belong to five different sections: Enantiophyllum (*D. alata, D. cayenensis, D. nummularia, D.opposita, D.rotundata* and *D.transversa*), Combilium (*D. esculenta*), Opsophyton (*D. bulbifera*), Macrogynodium (D. *trifida*) and Lasiophyton (*D. pentaphylla*).The order Dioscoreales is characterized by some dicotyledonous features, i.e. reticulate-veining, stalked net nerving leaves, circular arranged vascular bundles in the stem cross-section and lateral position of the pistil. *Dioscoreacea* plant family show a second delayed cotyledon, which renders the family interesting for the discussion of possible phylogenetic relations between mono and dicotyledonous plants, even if the traditional division of the angiosperms in mono- and dicotyledonous plants was formally given up with the introduction of the Magnoliopsida as distal class of the angiosperms.

2.5 **Morphology**

The stem of *Dioscorea* is unable to support the weight of the leaves and have to climb by twining, but there are no specialized organs such as tendrils. The direction of twining, anticlockwise or clockwise is a characteristic of each taxonomic section. Species of the Enantiophyllum section twine to the right (clockwise) and those of the Combilium, Opsophyton, Macrogynodium and Lasiophyton sections twine to left

(anticlockwise).The stems may be winged, spiny or spineless, hairy or glabrous and circular, rectangular or polygonal in section (Coursey, 1967). After sprouting the stem can remain erect to a height of almost a metre before it needs some support. Certain species have spiny stems like *D. cayenensis, D. rotundata, D. esculenta* and D. *nummularia,* mostly at base portion for self protection and to assist the supporting stem.

The length of stem varies greatly between species, from a few metres for D. *esculenta* to more than 15m for D. *nummularia.* The leaves are carried on long petioles and are usually simple and cordate, but can also be lobed, consisting of three leaflets (*D. trifida*). Each leaf or leaflet has three primary nerves joining at the tip of the lamina. The leaves vary in size between species, between cultivars within species and between different parts of a single plant. Average area is in the range 50-200cm^2. Many species have glands on the leaves producing extrafloral nectarines attractive to ants, due to the aggressive behaviour of ants that protect the young shoot apex of the yam stem against predators (MvKey *et al.*, 1998).The phyllotaxy is spiral, can be opposite or alternate depending on the species, and quite often it can be alternate on the lower part of the stem and opposite on the upper part.

The flowers are usually unisexual and many cultivars rarely flower and set fertile seeds. In general, there are more male than female plants and male flowers are usually more numerous than female ones. The individual flowers are small 2-4 mm in diameter. The male and female flowers were borne in axillary spikes. The flower composed of a calyx of three sepals and a corolla of three petals. There are usually two whorls of three stamens each. The stamens are usually erect and inserted towards the centre of the receptacle. The female flower has a trilocular ovary located below the corolla and there are three stigmas. The flowers are supposedly entomophilous (Coursey, 1967). They are insignificant in colour but sweetly scented and usually

pollinated by night flying insects. Each loculus of ovary contains two ovules (Govaerts *et al.*, 2007).

The fruits are dry dehiscent trilocular capsules (1-3cm long) and can produce six seeds literally. They are not more than three times long as they are wide, with two ovules in each of the three locules. The seeds are usually lenticular, not ridged, with wings all round the margins or restricted to their base or apex. They are flat and light and their wings are an efficient aid to their wind dispersion. When seed germinates, the plantlet establishes itself with the emergence of a radicle outside the thin seed coat, which is followed immediately by the emergence of the first chlorophyllous leaf. The hypocotyledon zone develops and the leaf spreads out as the petiole extends. The first radicle then produces two or three roots and during the following weeks, a complete root system develops rapidly. Thick and long roots develop rapidly after sprouting and reach considerable distances, 3-4m in radius around the plant.

Coming to the yam tuber, it lacks the anatomical characteristics of a modified stem structure. It has no buds or eyes, no scale leaves and no terminal bud at the distal end of the tuber. Some species form perennial tubers, which become larger and more lignified as the plant ages. There is tremendous variation in size, form and number of tubers per plant within and between species. The Enantiophyllum species usually produce one to three large tubers, while *D. esculenta* (Combilium) and *D. trifida* (Macrogynodium) produce a greater number of smaller tubers. The shape of the tuber in species producing small tubers is generally regular and their skin usually thinner than species producing larger tubers. Annual and perennial-producing tubers spend dry part of the year in dormancy from one to six months.

In *D. rotundata,* the stems are glabrous, fistulose and streaked. When a new stem develops, it is thick with abundant prickles and pairs of cataphylls. Stout branches develop at right angles from the axils of these cataphylls. Paired leaves arise from

bracts and are opposite, with a long petiole and are oval or almost round shape. The petioles are as long as the leaves, wide, glabrous and poorly indented at their base. Laminas have seven veins and their lower surface is paler than upper surface (Lebot, 2009).

The male flowers are white, borne on axillary spikes always shorter than the length of the lamina, filiform and glabrous. Flowers are sessile, scattered and solitary. The male inflorescences are approximately 5-8 cm long and can carry 20-30 flowers with three yellow sepals and petals. The female flowers are borne on spikes measuring up to 15-20 cm long and resulting in capsules with a cordate apex. Growth cycle is 6-8 months and the tubers may be produced in pairs or in small groups of four or five and have a long dormancy period of up to 5 months. Tuber shape varies but flesh colour does not have much change. Tuber skin is dark and smooth and nearly free of rootlets (Dumont *et al.*, 2006).

2.6 Nutritional Value

In the diet of tropical regions yam essentially is a staple food, rich in starch and the daily consumption per head on a day accounts 0.5-1.0kg in the yam belt of West Africa. In Yams, protein, mineral and vitamin content are higher. Raw yam consist of 73 percentage water, 2.1g of protein, 101kcal of energy, 0.2g fats, 1.0 mg ash, 20mg calcium, 69mg Phosphorous,0.6 mg iron, 600mg potassium,0.10 mg thiamine,0.5mg niacin,0.04 riboflavin and 9 mg ascorbic acid per 100mg fresh weight. It consists of 15.9% - 30.2% of dry base starch. *D. dumetorum* is the yam species with the highest nutritional qualities, containing high protein and mineral values.

2.7 Molecular Marker Studies Linked to Dwarf gene in Crops

For developing new varieties in plants, by conventional method it takes lot of time, almost 25 years. It is time consuming due to lot of factors including long growth cycle. Due to the advancement of biotechnology, we can go for less, time consuming procedures like Marker assisted selection (MAS), that is identification of DNA markers linked to key traits, helpful for easy gene transfer. This method can overcome the shortcomings in traditional breeding, thus increasing the accuracy and efficiency of selection. It is especially valuable for traits with low to moderate heritability, which are difficult to be improved by traditional selection. Marker Assisted Selection (MAS) has proven to be useful in speeding up genetic improvement in agronomic plant species. Molecular markers, linkage maps, and QTL mapped on the whole genome are essential for MAS. In many important agronomic plant species, a large number of DNA markers and linkage maps have been developed. Many QTLs for important traits have been mapped on the whole genomes, setting up the basis for rapid genetic improvement through MAS (Lee *et al.*, 2015). Several DNA- based marker systems are available for genetic finger printing of plants like RAPD, ISSR, SSR etc. The development of genetic maps allows the use of marker-assisted selection (MAS).

The present study aims at identifying markers linked to dwarf plant type in yams.Molecular markers linked to dwarfness has been identified in *Brassica.* SCAR (Sequence Characterize Amplified Region) has been developed for optimal tagging of the dwarf *Bzh* gene in *Brassica napus*. The usefulness of this marker in breeding dwarf rapeseed lines was reported (Barret *et al.,* 1998). Restriction fragment length polymorphism (RFLP) analysis was done in oats (*Avena sativa* L.). The RFLP markers closely linked to the three dwarfing genes in distinct regions of the oat

genome contributed to plant height and were used for characterizing new genetic sources for dwarfness in oats (Milach *et al.*, 1997).

PCR-based markers were developed to detect the point mutations responsible for the two major semi dwarfing genes *Rht-B1b* (*Rht1*) and *Rht-D1b* (*Rht2*) in wheat (Ellis *et al.*, 2002). These markers had utility in marker-assisted selection of the *Rht-B1b* and *Rht-D1b* genes in wheat breeding programs (Sourdille *et al.*, 1998). *Sterol methyltransferase 1 (STE1) catalyzes D7* sterol C-5 desaturation in the conversion of 24-methylenecholesterol to campestral in brassinosteroid (BR) biosynthesis, and *STE1* has been proven to influence plant architecture by controlling the BR levels in *Arabidopsis*. *TaSTE* (wheat), the ortholog of *AtSTE1* (*Arabidopsis*), was cloned and mapped to wheat chromosome homologous group 3 in the interval between simple sequence repeats (SSRs) markers CWM48.1 and WMC532, with genetic distances of 17.7 and 7.6 cM, respectively. Both linkage and association analyses revealed that *TaSTE-A1* was significantly associated with plant height in bread wheat (Zhang *et al.*, 2013) Fine mapping of a semi-dwarf gene in a centromeric region in rice (*Oryza sativa* L.) had been done and identified a recessive gene, named *sd-c*, that controls plant height and tiller number that helped in breeding semi dwarf indica rice cultivars (Chen *et al.*, 2013).

Association analysis identified six marker loci linked to plant height in Sorghum (Hari *et al.*, 2013). Identification and fine mapping of *qPH6*, a novel major quantitative trait locus for plant height in rice (Yuan *et al.*, 2015). Molecular markers linked to genes affecting plant height in wheat were identified using a doubled-haploid population. Plant height in wheat (*Triticum aestivum* L.) is known to be under polygenic control. Crosses involving genes *Rht-B1* and *Rht-D1*, located on chromosomes 4BS and 4DS, respectively, have shown that these genes have major effects. Two RFLP loci were found to be linked to these two genes (*Xfba1-4B* with *Rht-B1* and *Xfba211-4D* with *Rht-D1*) by genotyping a population of F_1-

derived doubled-haploid lines ['Courtot' (*Rht-B1b+Rht-D1b*)×'Chinese Spring'] (Cadalen *et al*., 1998).

Screening of molecular markers linked to dwarf trait in crape myrtle by bulked segregant analysis was undertaken .Markers used were 41 SSR primers and 384 AFLP primer combinations , in which an amplified fragment length polymorphism marker, M53E39-92, which was 23.33 cM from the loci controlling the dwarf trait, was identified. These results provide basic information for marker-assisted selection in Lagerstromia and cloning of dwarf genes (Ye *et al.,* 2015).Genetic enhancement of Dubraj, specialty rice through pedigree approach employing marker based selection for plant stature, aroma and grain was carried out. This study reported the successful recovery of the premium grain quality traits of Dubraj in IET 21044 (L9), a derivative from Pusa 44/Dubraj cross combination using pedigree approach employing markers associated with plant stature, aroma and grain quality and stringent phenotypic selection. The validation of the superior yield potential and premium grain quality of IET 21044 in the multi location evaluation trials over a 4 year period resulted in its release thus making it the first semi dwarf, high yielding, short grain aromatic rice released in India at the national level. The study was a successful demonstration of the limitless potential of the marker technology in breeding specialty rice (Patnaik *et al*., 2015). Genetic control of plant height in European winter wheat cultivars includes the study that two *Rht-1* semi-dwarfing genes are the major sources of variation and other small- or medium-effect QTL located on chromosomes 6A and 5B explaining 11.0 and 5.7 % of the genotypic variance and potentially epistatic QTL enable fine adjustments in plant height (Würschum *et al*., 2015).

A consensus linkage map analysis and identification of major QTL for stem height was identified in oil palm (*Elaeis guinensis* Jacquin) using dominant and co-dominant markers to facilitate mapping of QTL.Constructed a consensus linkage map for oil

palm using co-dominant markers (*i.e.* microsatellite and SNPs) and two F_1 breeding populations generated by crossing *Dura* and *Pisifera* individuals. Four hundreds and forty-four microsatellites and 36 SNPs were mapped onto 16 linkage groups. The map length was 1565.6 cM, with an average marker space of 3.72 cM. A genome-wide scan of QTL identified a major QTL for stem height on the linkage group 5, which explained 51% of the phenotypic variation. Genes in the QTL were predicted using the palm genome sequence and bioinformatic tools. The linkage map supplies a base for mapping QTL for accelerating the genetic improvement, and will also be useful in the improvement of the assembly of the genome sequences. Markers linked to the QTL may be used in selecting dwarf trees. Genes within the QTL will be characterized to understand the mechanisms underlying dwarfing (Lee *et al.*, 2015).

Konan *et al.* (2011) studied microsatellite gene diversity within Philippines dwarf coconut palm (*Cocos nucifera* L.) resources at Port-Bouët, Côte d'Ivoire in which twelve SSR markers were analyzed for genetic diversity and genetic relationship among 25 palms representing five philippines coconut green dwarf accessions and found out low genetic diversity within Philippines dwarf coconut accessions. Rajesh *et al.* (2013) developed RAPD-derived SCAR marker associated with tall-type palm trait in coconut. Plant material for tall and dwarf accessions were selected and pooled. RAPD analysis was performed using 200 different decamer oligonucleotide primers and 1 primer (OPA 09) showed well reproducible polymorphic bands between the tall and dwarf plant type pool. The marker OPA09260 was present exclusively in tall accessions and absent in the dwarf cultivar, so the marker was excised, purified, cloned , sequenced and converted to SCAR.

Divashuk *et al.* (2013) studied reduced height genes and their importance in winter wheat cultivars grown in southern Russia. The results on this study showed that coupling *Rht-B1e* with *Rht8* or *Rht-B1b* with *Rht8* was more successful compared with the effects of other genes or their combinations. They reported that *Rht-B1e* can

be recommended for use in breeding programs and the presence of a molecular marker for this allele simplifies its transfer to elite wheat germplasm. Fine genetic mapping of a locus controlling short internode length in melon (*Cucumis melo* L.) was studied using 76 SSR markers positioned on 15 linkage groups spanning 462.84 cM, the location of *mdw1* was localized to Chromosome 7and developed gene-based CAPS markers from the CKX, GPCR, ERECTA, and *SqE* gene in melon and analyzed the association between these markers and the *mdw1* locus conferring the shortened internodes in Korean melon line PNUD1(Hwang *et al.*, 2014).

Genetic effects of dwarfing gene *Rht-5* on agronomic traits in common wheat (*Triticum aestivum* L.) and QTL analysis on its linked traits are studied using 21 pairs of SSR primers and it is used for screening polymorphism. Only three primer pairs including *Xbarc*102, *Xwmc*623 and Xgwm533b identified polymorphisms (Daoura *et al.*, 2014).

2.8 **Molecular Marker Studies in Yams**

Genetic linkage maps based on AFLP markers have been constructed for *Dioscorea tokoro*, a wild yam (Terauchi and Kahl, 1999) and for the cultivated species, *D. rotundata* (Mignouna *et al.*, 2002a) and *D. alata* (Mignouna *et al.*, 2002b). The *D. rotundata* map is based on 341 markers segregating in an intraspecific F_1 cross. Separate maternal and paternal linkage maps were constructed, comprising 12 and 13 linkage groups, respectively. To date, only about 60 SSR markers developed from seven different yam species, *D. tokoro* (Terauchi and Konuma, 1994), *D. rotundata* (Mignouna and Dansi, 2003), *D. japonica* (Misuki *et al.*, 2005), *D. alata, D. abyssinica, D. praehensilis* (Tostain *et al.*, 2006) *and D. trifida* (Hochu *et al.*, 2006) are available in yams.

The bulked segregant analysis approach was successfully used for the identification of RAPD markers linked to YMV and anthracnose resistance genes. Two RAPD markers, OPW18850 and OPX15850, closely linked in coupling phase with the dominant YMV- resistance locus *Ymv-1*, were identified (Mignouna *et al.*, 2002c). Similarly, two RAPD markers, OPI171700 and OPE6950, closely linked in coupling phase with the anthracnose resistance locus, *Dcg-1*, were identified (Mignouna *et al.*, 2002d). These RAPD markers will be easier to use for indirect selection once converted into co- dominant PCR-based sequence characterized amplified regions (SCARs).

Mignouna *et al.* (2005) studied the efficiency of different molecular markers in yams. The efficiency of RAPD, AFLP and SSR markers for the assessment of genetic relationships, and for cultivar identification and discrimination among 45 West and Central African white yam cultivars belonging to 22 morphotypes/cultivar groups was investigated. The order of merit based on this criterion in this study was AFLPs (GI = 2.56), SSRs (GI = 0.39) and RAPDs (GI = 0.35). In yams microsatellite markers linked to anthracnose disease tolerance were available (Narina *et al.*, 2012).

Mapping populations were developed for genetic analysis in yams (*Dioscorea rotundata* Poir. and *Dioscorea alata* L.) F_1 full-sibmapping populations were developed from crossing male and female parents of *Dioscorea rotundata* Poir. And *Dioscorea alata* L. that differ in specific traits of interest towards identification of molecular markers linked to those traits. Success in hybridization was validated based on DNA analysis with 145 SSR markers on agarose gel. Traits for which the populations were developed included multiple tuber production, cooking quality and virus disease resistance in *D. rotundata* and anthracnose disease resistance, cooking quality and tuber oxidation in *D. alata* (Alieu *et al.*, 2011).

Clonal diversity and estimation of relative clone age in yam (*Dioscorea rotundata*) had done as an application in agro biodiversity studies. Evolutionary history of yam and intra-clonal diversity is studied using 12 microsatellite markers (Scarcelli *et al*., 2013). Genetic diversity of *Dioscorea dumetorum* (Kunth) Pax using Amplified Fragment Length Polymorphisms (AFLP) and cpDNA was studied (Sonibare *et al*., 2010).A total of 1052 AFLP fragments of which 94.1% were polymorphic produced from twelve primer combinations indicated a relatively high level of polymorphism among the 53 accessions of *Dioscorea dumetorum* from six countries in West and Central Africa efficiency of RAPD, ISSR, AFLP and ISTR markers were checked for the detection of polymorphisms and genetic relationships in camote de cerro (*Dioscorea* spp.).Six RAPD, 6 ISSR, 3 ISTR and 4 AFLP primers were used for the analysis. Among the marker systems evaluated, RAPD and AFLP were found to be more efficient in the estimation of molecular diversity of different accessions of *Dioscorea* spp.; it was evident from large values of polymorphism percent, PIC, multiplex ratio and average number of polymorphic bands per primer (Ramírez *et al*., 2014).

Siqueira *et al.* (2014) studied water yam (*Dioscorea alata* L.) diversity pattern with SSR and morphological markers. Genetic diversity and structure analysis among commercial and local varieties of water yam (*Dioscorea alata* L.) in Brazil using microsatellite and morphological markers.12 SSR primers were used to generate DNA profiles of 72 local varieties and 17 commercial accessions of water yam collected from four-different regions in Brazil. Also, four morphological traits were evaluated on individual plants under field conditions. The morphological characterization showed considerable diversity. High polymorphism was found with 100 % polymorphism observed for 11 primers and a discriminating power value of 0.92, on an average. Nemorin *et al.* (2012) studied inheritance pattern of tetraploid *Dioscorea alata* and showed evidence of double reduction using microsatellite marker segregation analysis. A progeny of 188 individuals obtained by crosses between two natural tetraploid parents (Noulelcae, Tepuna), genetically distant, were

analyzed. Segregation patterns of tetraploid *D. alata* varieties (2n = 4x = 80) were determined by studying 20 SSR inheritance markers and by calculating double reduction coefficients. Analyses of these two parameters provide strong evidence that tetraploid *D. alata* are autotetraploid and not allotetraploid.

Genetic diversity and species delimitation in the cultivated and wild Guinea yams (*Dioscorea* spp.) from Southwest Ethiopia as determined by AFLP (amplified fragment length polymorphism) markers. In this study, amplified fragment length polymorphism (AFLP) genetic fingerprinting was used to evaluate and characterize 43 individual plants, belonging to different populations of wild and cultivated Guinea yams. The three primer combinations used in the AFLP analyses generated 158 scorable bands, with an overall polymorphism of 78 % (Mengesha *et al.*, 2013).

Narina *et al.* (2011) had done work on generation and analysis of expressed sequence tags (ESTs) for marker development in yam (*Dioscorea alata* L.).In this study total of 44,757 EST sequences were generated from the cDNA libraries of the resistant and susceptible genotypes. Greater than 56% of ESTs had annotated using MapMan Mercator tool and Blast2GO search tools. Gene annotations had used to characterize the transcriptome in yam and also performed a differential gene expression analysis between the resistant and susceptible EST datasets. From ESTs revealed 1702 unique sequences containing SSRs and 1705 SSR markers were designed using those sequences.

Sartie *et al.* (2012) studied genetic and phenotypic diversity in a germplasm working collection of cultivated tropical yams (*Dioscorea* spp.). Working collection of yam (*Dioscorea* spp.) comprising 53 landraces and seven improved cultivars of four species (*D. alata* L., *D. cayenensis* Lam., D. dumetorum (Kunth) and *D. rotundata* Poir.) was evaluated for phenotypic and genetic diversity. The evaluation involved field assessment of 24 morphological traits and DNA analysis with 32

Simple sequence repeat (SSR) polymorphic markers. The study provides improved understanding about the genetic and phenotypic relatedness among *D. rotundata, D. cayenensis, D. alata* and *D. dumetorum*. Interspecific polymorphic SSR markers were identified that may be used for genetic analysis across yam species. Spatially structured genetic diversity of the Amerindian yam (*Dioscorea trifida* L.) assessed by SSR and ISSR markers in Southern Brazil.Fifty three accessions of *D. trifida* from 11 municipalities in the states of Sa˜o Paulo, Santa Catarina, Mato Grosso and Amazonas were characterized on the basis of eight Simple Sequence Repeats (SSR) and 16 Inter Simple Sequence Repeats (ISSR) markers. The level of polymorphism among the accessions was high, 95 % for SSR and 75.8 % for ISSR. The SSR marker showed higher discrimination power among accessions compared to ISSR, with D parameter values of 0.79 and 0.44, respectively in this study (Nascimento *et al.*, 2013).

Genetic diversity analysis of yams (*Dioscorea* spp.)cultivated in China using ISSR and SRAP markers. In this study, 11 inter simple sequence repeat (ISSR) and 60 sequence related amplified polymorphism (SRAP) techniques were used to assess genetic diversity within 21 yam landraces from seven cultivated populations and observed high level of polymorphism among these landraces, specifically, 95.3 % for ISSR and 93.5 % for SRAP(Wu *et al.*, 2014). The genetic diversity of 52 genotypes of yam germplasm from Sierra Leone was accessed using 14 SSR primers. High diversity of 0.650 was noted among genotypes studied (Norman *et al.*, 2012).

2.9 **Studies on *in vitro* Propagation of Yam**

In vitro propagation is used to multiply novel plants for the production of planting material. *In vitro* plants can be used for the storage of breeding material with degenerated growth, to struggle against genetic erosion and it also provides phytosanitary qualities that is microbe free planting materials. Many workers reported

in vitro propagation in several crops including yams. Mahesh *et al.* (2010) worked with *Dioscorea wightii* and propagated the plant using nodal segment as explants. BA and kinetin was used for the multiplication of nodal segment. Callus initiation was observed in MS medium supplemented with 0.15-1.75μM BA, 0.75-5.0μM kinetin, 0.15-0.30 μM 2iP and shoot formation was observed in all growth regulators tested in BA, Kinetin and 2iP. Chen *et al.* (2003) developed a protocol for rapid *in vitro* propagation of *D. zingiberensis* using stem as explants. Medium supplemented with 4.4μM BAP+1.1 μM NAA produced shoots on nodal segments within 20 days. Callus formed on MS +8.9 μM BA+ 5.4 μM NAA in 30 days, 22.2 μM BAP and 1.1 μM NAA regenerated shoot from callus and for rooting 4.9 μM IBA was used.

Ovono *et al.* (2010) had studied the tuber formation and development of *Dioscorea cayenensis–Dioscorea rotundata* complex under *in vitro* conditions. This study showed the promotive effects of exogenous polyamines on yam tuber formation and development *in vitro*. Application of exogenous polyamines, polyamine metabolism inhibitors, and polyamine precursors in various concentrations positively affected microtuber formation and development in yam nodal cuttings and showed a better development of tuber in the presence of PUT.

Effects of synthetic hormone substitutes and genotypes on rooting and mini tuber production of vines cuttings obtained from white yam (*Dioscorea rotundata*, Poir) was studied and the result showed that rice straw ash and neem leaf powder could serve as substitute to IBA hormone as root promoting substance in yam vine cuttings using carbonized rice husk as planting medium. For soaking method, 5% rice straw ash enhanced rooting of vine cuttings of the genotypes tested. Coconut water at 5% dilution in water was also found useful as a root - promoting substance for vine cuttings (Agele *et al.*, 2010).

Effects of storage conditions on sprouting of microtubers of yam (Dioscorea cayenensis–*D. rotundata* complex) was analyzed and found that conditions like the storage duration, the conditions of humidity, temperature and luminosity during storage and of the size of microtubers affects the sprouting rate. Dormancy phase was observed after 4 weeks of storage, its duration was between 20 and 28 weeks and storage temperature of 25°C permitted a quicker sprouting than 18 °C (Ovono *et al.*, 2010).

Ovono*et al.* (2010) studied the importance of *in vitro* preservation of yam (*Dioscorea cayenensis – D. rotundata* complex) for a better use of genetic resources in concern with the importance of storage duration. According to the storage condition experiments the microtubers were harvested after 9 months of culture and directly transferred on a new medium without hormones, the tubers rapidly sprouted under *in vitro* conditions. Harvested microtubers were also stored dry in jars in sterile conditions during 2 to 18 weeks before *in vitro* sprouting. In this case, microtubers stored during 18 weeks sprouted more rapidly than those stored 8 weeks. The size of the tubers used for the storage had great influence on further sprouting. The upper microtubers in 25 mm could be kept to the darkness, under 50% of relative humidity, in 25°C during at least 18 weeks.

Axillary shoot proliferation and tuberization of *Dioscorea fordii* Prain et Burk is analyzed and found that activated charcoal have played a positive role in axillary shoot proliferation and tuberization of *in vitro* plantlets. Shoot length, frequency of proliferation, fresh weight and dry weight of shoots, frequency of tuberization and mean number of tubers per plantlet (NTPs) were significantly (P B 0.05) greater in liquid medium compared to semi-solid media. Sucrose concentration had the record effect on frequency of tuberization and proliferation. The preferred medium for axillary shoot proliferation and tuberization of *D. fordii* was found to be MS basal

medium supplemented with 1.0 mg·l^{-1} BA, 0.1 mg l^{-1} NAA, 30 g l^{-1} sucrose and 1.5 g l^{-1} AC in liquid culture (Yan *et al.*, 2011).

Olivier *et al.* (2012) studied *in vitro* induction of mini tubers in yam (*Dioscorea cayenensis- D. rotundata* complex) and reported the importance of optimum sucrose level of 4 percent and higher sucrose concentration (16%) inhibited tuberization. Nitrogen in the culture medium was recognized to play an important role in the tuberization of certain plant species and jasmonic acid promoted early tuber formation. Medium used in this study is T-medium, it is a modified MS medium with 6% less nitrogen. Culture media were supplemented with MS vitamins (for MS medium) or thiamine (1 mg/ml for T-medium), myoinositol(100 mg/l) and sucrose (2, 4, 8 and 16%, w/v). The pH of the media was adjusted to 5.8 before autoclaving for 30 min at 1.04 kg/cm^2 and 121_C. JA was filter sterilized and added at concentrations of 0.5, 1.5, 2.5, 3.5 and4.5 µM. Cultures were incubated in a growth chamber at 16 h/8 h (light/dark) photoperiod, 25 ± 3_□C, and 60–70% relative humidity. For each treatment, 30 single nodal explants were used, and one explant was cultured per test tube.

Effect of liquid media culture systems on yam plant growth was studied by (Jova *et al.*, 2011). They found that Temporary Immersion System (TIS) and Constant Immersion System (CIS) with aeration through continuous bubbling in culture medium gave higher results in morphological and physiological plant indicators in comparison with plants obtained in culture systems with passive renewal of internal atmosphere in culture flasks or Static Liquid System (SLS). In Temporary Immersion System, the best results were obtained after six weeks of culture in relation to total length (20.8 cm), axillary bud number (8.6), fresh weight (2.1 g) and dry weight (0.18 g) per plant, as well as photosynthetic pigment content (chlorophyll a, b, and total), net photosynthesis (15.3 µmol C02.m-2.s^{-1}), total transpiration (5.97 mmol H2O.m-2.s^{-1}), stomatal conductance (457 µmol H2O.m^{-2}.s-1) and leaf starch content (45.77

mg.gMF^{-1}). Reducing sugar in culture medium with Temporary Immersion System was completely depleted, and mineral nutrients of lower contents (phosphorus, nitrogen, magnesium, calcium, iron, and manganese) in culture medium from this culture system could be related with plant growth.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The Study entitled "*In vitro* propagation and identification of molecular markers linked to dwarfness in white yam (*Dioscorea rotundata* Poir.)" was carried out at the Division of crop improvement, ICAR-Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram during 2014-2015. In this chapter, details pertaining to the experimental material and procedures used in the study are elaborated.

3.1. Molecular Marker Analysis

In order to study the genetic variability among white yam genotypes and to identify genes linked to dwarfness, the molecular characterization using ISSR, RAPD and SSR markers were carried out among the following white yam genotypes.

3.1.1 Source of plant material

The plant materials used for molecular marker analysis of dwarf gene and genetic diversity studies comprises of thirty accessions of *D.rotundata* collected from germplasam maintained in the field gene bank at CTCRI. For the standardization of protocol for *in vitro* propagation of dwarf yam, nodal explants and shoot tips were obtained from released dwarf variety of *D. rotundata,* sreedhanya from conserved germplasam collection of CTCRI, Trivandrum.

3.1.2 Maintenance of accessions

The collected accessions were initially grown in pots under the shade net house in CTCRI, Trivandrum, with the vines trailed on coir ropes for tall ones. FYM was mixed with potting mixture as manure and vermiculate was spread on top of the potting mixture to hold moisture. The field planting, was done at the end of April

after the pre-monsoon showers. Tuber cuttings were planted on mounds prepared at a spacing of 1m×1m.During mound preparation FYM at the rate of 12 t $ha^{-1}$was applied to the soil. Fertilizers were applied at the rate of 80:60:80kg ha^{-1} of N, P and K in two split does as per the package of practices for yams. Weeding and earthing up were carried out along with each fertilizer application. The vines were supported on stake of about 2m height for tall plants and not necessary for dwarf ones. Harvest was done during January-February by manually digging out the tubers and the tubers were cleaned free of soil and kept in ventilated yam storage house till the next planting season.

Table 2. Accessions of White yam used for the study

SL. No	Accession Code	Sample Name
1	V1	SREEDHANYA
2	V2	DRD 1110
3	V3	DRD 495
4	V4	DRD 1118
5	V5	DRD 1835
6	V6	DRD 1060
7	V7	DRD 1033
8	V8	DRD 920
9	V9	DRD 1142
10	V10	DRD 949
11	V11	DRD 1157
12	V12	DRD 1068
13	V13	DRD 835
14	V14	SD 15
15	V15	DR 2

16	V16	DR 29
17	V17	DRS 47
18	V18	DRS 45
19	V19	DR 130
20	V20	DR 17
21	V21	DR 73
22	V22	DRS 652
23	V23	DR VIOLET
24	V24	DRS 36
25	V25	DRS 1155
26	V26	DRH 1047
27	V27	DRH 657
28	V28	DRH 657 A
29	V29	SREEPRIYA
30	V30	SREESUBHRA

3.1.3 **Source of primers**

The primers were selected from collected literatures showing high polymorphic values in *Dioscorea* species, ordered and shipped from Integrated DNA Technologies, Inc., as lyophilized form.

Table 3. ISSR primers Used

Sl. No	Primer name	Sequence (5'↔3')
1	UBC 808	AGAGAGAGAGAGAGAGC
2	UBC 809	AGAGAGAGAGAGAGACG
3	UBC 810	GAGAGAGAGAGAGAGAT
4	UBC 811	GAGAGAGAGAGAGAGAC
5	UBC 817	CACACACACACACACAA
6	UBC 825	ACACACACACACACACT
7	UBC 827	ACACACACACACACACG
8	UBC 848	CACACACACACACACARG
9	UBC 864	ATGATGATGATGATGATG
10	UBC 818	CACACACACACACACAG
11	UBC 836	AGAGAGAGAGAGAGAGYA
12	$(GA)_9AC$	GAGAGAGAGAGAGAGAGAAC
13	$(GA)_9AT$	GAGAGAGAGAGAGAGAGAAT
14	UBC 14	CGCGCGCGCGCGCGCGTG
15	$(ACC)_6Y$	ACCACCACCACCACCACCY

Table 4. RAPD Primers Used

Sl. No	Primer name	Sequence (5'↔3')
1.	OPW-1	CTCAGTGTCC
2.	OPG-02	GGCACTGAGG
3.	OPG-03	GAGCCCTCCA
4.	OPG-05	CTGAGAGGGA
5.	OPG-08	TCACGTCCAC
6.	OPG-13	CTCTCCGCCA

7.	OPW-16	CAGCCTACCA
8.	OPW-18	TTCAGGGCAC

Table 5. SSR Primers Used

Sl.No	Primer name	Sequence (5'↔3') Forward	Sequence (3'↔5') Reverse
1.	Dab2C05	CCCATGCTTGTAGTTGT	TGCTCACCTCTTTACTTG
2.	Dab2D06	TGTAAGATGCCCACATT	TCTCAGGCTTCAGGG
3.	Dab2E07	TTGAACCTTGACTTTGGT	GAGTTCCTGTCCTTGGT
4.	Da1A01	TATAATCGGCCAGAGG	TGTTGGAAGCATAGAGAA
5.	Dpr3F04	AGACTCTTGCTCATGT	GCCTTGTTACTTTATTC
6.	YM5	AATGAAGAAACGGGTGAGGAAGT	CAGCCCAGTAGTTAGCCCATCT
7.	YM15	TACGGCCTCACTCCAAACACTA	AAAATGGCCACGTCTAATCCTA
8.	YM26	AATTCGTGACATCGGTTTCTCC	ACTCCCTGCCCACTCTGCT
9.	MT 13	TAACAAACAAAAAATGAAAC	TAACAGTGATTGAGCTAGGA
10.	MT 10	TCGTGTCCATCTTGCTGCGT	GAAAAGCGGAGATGAAGAGCA

3.1.4 Glasswares and Instruments in Molecular Biology Lab

Eppendorf tubes, Pestle and mortar, micropipette tips for 1ml, 200ul and 2ul, PCR tubes were autoclaved and used. Eppendorf tube stand, PCR tube holders micropipettes, water bath, icebags, collection bags, scalpels ,scissors, tags, labels, wipes, bottles, conical flasks, spatula, weigh boats, funnel, and syringe are other materials needed for molecular work.

3.1.5 **Instruments**

Ice machine, weighing balance, Water bath, Vortexer , Centifuge, Spectrophotometer, Electrophoresis apparatus, Hot air oven, Autoclave, PCR machine, Alpha imager, UV transilluminator and UV torch,-20 and -80,4°C refrigerator, Genetic analyzer.

3.1.6 **Sample collection for DNA extraction**

Fresh tender young leaves of *Dioscorea rotundata* Poir accessions were collected from germplasm of CTCRI, Trivandrum including 14 dwarf , 1 semi dwarf and 15 tall varieties. Included released dwarf and tall varieties of white yam called Sree Dhanya (dwarf) and SreeSubhra, SreePriya (tall). Leaves were collected in pocket size plastic kits and transferred to lab in an ice box.

3.1.7 **DNA Extraction**

3.1.7.1 **Manual Method**

DNA was extracted from fresh and tender young leaves using modified protocol of Doyle & Doyle, 1987.

Young leaves collected were weighed of about 100-200mg.Grind it in pestle and mortar using liquid nitrogen along with addition of little amount of Polyvinyl pyrrolidine. Added 1ml of extraction buffer (Appendix I) and transferred to autoclaved 2ml eppendorf tubes and added 10ul Proteinase K. Vortexed and incubated it at 37°C for 30 minutes with intermittent shaking. Centrifuged at10,000 rpm for 15 minutes at room temperature and supernatant was collected. Collected supernatant was mixed with equal volume of chloroform:isoamylalcohol. Centrifuged at 10,000 rpm for 10 minutes at room temperature. Supernatant collected and chloroform:isoamylalcohol extraction was repeated. Supernatant in fresh eppendorf

tubes were mixed with .8 volume isopropanol to precipitate the DNA. Pelletized the DNA at 10,000rpm for 10 minutes and the pellet was saved and washed with 70% ethanol twice. Dried it on oven for 5-10 minutes to allow ethanol to vaporize and added TE buffer (Appendix II) and stored at -20°C refrigerator. All the samples were checked for DNA in .8 % agarose gel and confirmed.

3.1.7.2 **Using DNeasy Plant Mini Kit**

Young leaves collected were weighed of about 100-200mg.Grind it in pestle and mortar using liquid nitrogen, added 400ul buffer AP1 and 4ul RNaseA. Vortexed and incubated for 10 minutes at 65°C.Inverted the tubes 2-3 times during incubation. Added 130ul Buffer P3. Mixed and incubated for 5 minutes on ice. Centrifuged the lysate for 5 minutes at 20,000□g (14,000 rpm).Pipetted the lysate into a QIAshredder spin column placed in a 2ml collection tube. Centrifuged for 2 minutes at20,000□g.Tranferred the flow-through into a new tube without disturbing the pellet. Added 1.5 volumes of Buffer AW1 and mixed by pipetting. Transferred 650ul of the mixture into a DNeasy Mini spin column placed in a 2ml collection tube. Centrifuged it for 1 minute at >- 6000□g(>-8000 rpm).Discarded the flow-through. Repeated this step with the remaining sample. Placed the spin column into a new 2ml collection tube. Added 500ul Buffer AW2, and centrifuged for 1 minute at >- 6000 □ g. Discarded the flow-through. Added another 500ul buffer AW2.Centrifuged for 2 minutes at 20,000□g. Removed the spin column from the collection tube carefully so that the column does not come into contact with the flow-through. Tranferred the spin column to a new 1.5ml or 2ml microcentrifuge tube. Added 100ul Buffer AE for elution. Incubated for 5 minutes at room temperature (15-25°C). Centrifuged for 1 minute at >- 6000□g. Repeated last step and kept the sample in -20°C refrigerator. All the samples were checked for DNA in .8 % agarose gel and confirmed. DNeasy Plant Mini Kit was comparatively less time consuming and DNA obtained using this method has high purity.

3.1.8 **Quantification of DNA**

Isolated DNA quantified using spectrophotometer at 260 and 280nm. It helped to assess its yield and purity. Sterile distilled water was used to calibrate the spectrophotometer (Systronics, India) to blank i.e. zero absorbance at the above mentioned wave lengths. 10µl DNA sample was diluted to 2 ml using sterile distilled water and the OD values at 260nm and 280nm were recorded. Concentration of DNA present in the sample was quantified using the following formula:

Concentration (µg ml^{-1}) = Absorbance at 260 nm × 50 × dilution factor, where, the value 50 corresponds to the concentration of DNA in µg/ml of unit absorbance.

Dilution factor = Total volume/ Volume of sample taken.

The purity was determined by measuring the OD at 260nm/OD at 280 nm ratio. According to the better absorbance value/OD value samples are selected.

3.1.9 **Dilution of Samples**

Samples were diluted using Rnase/Dnase free water.

3.1.10 **Primer dilution**

Primers like ISSR, SSR and RAPD were selected from literatures based on their ability to show high polymorphism. 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 DNAase/RNAase free water. Initially freezer stock was made at 100 micromolar 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. Working stock of 10 micro molar are made by taking 10ul from the main stock and diluted it with 90ul of DNAase/RNAase free water and stored in -20°C refrigerator.

3.1.11 PCR amplification

The diluted samples were amplified in thermal cycler using different primers of ISSR, SSR and RAPD at different conditions, temperature gradients .Screened the best primers and optimum amplifying conditions were standardized for all primers of ISSR, SSR and RAPD.

Table 6. ISSR reaction mixture

Components	STOCK conc.	Required Conc.	Volume for one reaction (20µl)
Buffer with 15mM (Finzyme)	10 X	1 X	2µL
$Mgcl_2$ (Thermo Scientific)	25Mm	1Mm	0.8µL
dNTP (Gene i)	2mM	2mM each	0.5µL
Primer (from DOGR)	10µM	0.3µM	0.6µL
DNA	10ng/µL	20ng	2µL
Taq DNA Polymerase (Finzyme)	2U/µL	1U	0.5µL
dH_2O	-	-	13.6µL
Total			20 µL

PCR Conditions

PCR was carried out in Biorad Thermocycler. The program is as follows;

Lid - 105°C

94°C - 5 mins (initial denaturation)

94°C - 30 sec (denaturation)

56°C - 1 min (annealing) Cycles × 35

72°C - 1 min (extention)

72°C – 10 mins (final extension)

4°C - hold

The amplified products were separated on 2% agarose gel along with 1Kb and 100bp ladders to identify molecular weight of obtained bands and for polymorphism studies.

Table 7. SSR reaction mixture

Components	**STOCK conc.**	**Required Conc,**	**Volume for one reaction (10µl)**
Buffer with 15mM (Finzyme)	10 X	1 X	1µL
Mgcl$_2$ (Thermo Scientific)	25mM	0.5mM	0.2µL
dNTP (Gene i)	8mM	1mM each	1.25µL
Primer (F)	10mM	0.5mM	0.5uL
Primer (R)	10mM	0.5mM	0.5µL
DNA	10ng/uL	50ng	5µL
Taq DNA Polymerase (Finzyme)	2U	1U	0.5µL
dH$_2$O	-	-	1.05µL
Total			10 µL

PCR Conditions

PCR was carried out in Biorad Thermocycler. The program is as follows;

Lid - 105°C

94°C - 5 mins (initial denaturation)

94°C - 30 sec (denaturation)

51°C - 1 min (annealing) Cycles × 35

72°C - 1 min (extention)

72°C - 8 mins (final extension)

4°C - hold

Annealing temperature changed to 58°C and 59°C for primers YM 5, YM 15, YM 26, MT 10 and MT 13 respectively for better results using gradient study. The amplified products were separated on 2% agarose gel along with 1Kb and 100bp ladders to identify molecular weight of obtained bands and for polymorphism studies. SSR primers which showed bands that clearly discriminate between dwarf and tall genotypes were further confirmed through PAGE. After confirmation, particular band was eluted from AGE gel using UV transilluminator or UV torch and were taken for sequencing using genetic analyzer.

RAPD reaction mixture

Takara master mix emerald was used for fast and easy preparation of PCR mix for all the ISSR, SSR and RAPD primers. It consist of green dye, that helps in easy loading of sample for analysis without wasting additional time in adding dyes to amplicons.

Table 8. RAPD reaction mixture

Components	STOCK conc.	Required Conc.	Volume for one reaction (15µl)
Takara emerald master mix	2x	1x	7.5 µL
Primer	100µM	4µM	0.6 µL
DNA	10ng/µL	50ng	5µL
dH_2O	-	-	1.9 µL
Total			15µL

PCR Conditions

PCR was carried out in Biorad Thermocycler. The program is as follows;

Lid - 105°C

94°C - 5 mins (initial denaturation)

94°C - 1 min (denaturation)
36°C - 1 min (annealing)
72°C - 2 min (extention)
} Cycles × 35

72°C – 1min (final extension)

4°C - hold

The amplified products were separated on 2% agarose gel along with 1Kb ladders to identify molecular weight of obtained bands and for polymorphism studies.

3.1.12 Agarose Gel Electrophoresis (AGE)

Agarose gel electrophoresis is used to separate DNA molecules according to the size. The negatively charged DNA molecules might migrate in an electric field from cathode to anode. Smaller molecules migrates faster than bigger ones through the pore of the matrix. The pore of the matrix can be altered by changing the agarose concentration. Increasing the agarose concentration will also increase sieving effect. Inter-calating agents like ethidium bromide are added along with agarose solution for visualizing DNA bands on UV light under UV transilluminator or UV torch or Alpha imager.

Weighed 2g of agarose in 250ml conical flask, added 100ml 1X TBE buffer and gently boiled the solution in a microwave oven with occasional mixing until agarose completely dissolved in buffer. Allowed it to cool for 40°C and added 1ul/ml ethidium bromide carefully without spilling. Prepared the gel plates/moulds and kept combs in position. Poured the warm gel to plate and cool for 20 minutes. Filled the horizontal electrophoretic tank with 1X TBE buffer (Appendix III). After gel got solidified removed the comb and placed the plate with gel in to the tank. Loaded 7uLsamples along with the dye, bromophenol blue to wells and also added 5uL 1Kb or 100bp ladders for reference. Run the gel at 85V and 220mA for 1 to 1.5 hour. Visualized the band under UV transilluminator and documented the image on alpha imager. The images could be finally scored to detect polymorphism or to identify specific bands that can be linked to a particular trait.

3.1.13 **Denaturing Polyacrylamide Gel Electrophoresis**

7M urea denaturing PAGE (6% polyacrylamide) was performed using Biorad Sequi-Gen® Cell (38 × 50 cm) according to the biorad manual with heat denatured samples and detected by silver staining.

3.1.13.1 **INTEGRAL PLATE CHAMBER (IPC) UNIT ASSEMBLY**

IPC unit consisted of smaller and larger plates. Smaller plate and larger plates were first cleaned using liquid detergent (laboline) and tap water followed by rinsing with distilled water. The IPC could be cleaned using distilled water also. After placing the small and large plate on the work bench, 85per cent ethanol was used to wipe both the surfaces of the plates using kim wipes by carefully covering all the corners of the plates. After that, coat the small plate with bind silane (Appendix V) from top to bottom of the plate in vertical motions and to large plate coat labolin solution as repellent in similar way.

The vinyl spacers and combs were wiped using 85 per cent ethanol while allowing plates to dry. A 0.4 mm spacer was positioned along each long edge of the larger glass plate. Plugged two plates with clamps by adjusting the levers on the IPC panel. Ensuring that the siliconized surfaces faces down, the small glass plate was positioned on the larger one. Made this IPC-glass plate sandwitched upright vertically on the bench top in a precision caster base containing the precision caster gasket and fixed to the base by locking the cam pegs. The entire assembly was laid flat on the bench with the IPC panel (drain port) facing the user.

3.1.13.2 **GEL PREPARATION AND CASTING**

The acrylamide solution was prepared according to the (Appendix IV). 15ml of the prepared acrylamide solution was taken. 42g of urea was weighed and the urea

was transferred to a solution of 10ml 10Xbuffer and 15ml double distilled water. The mixture was then heated for 45seconds. The urea solution was then sieved and poured into a beaker containing 15ml acrylamide solution and then made upto 100ml. Simultaneously 0.1g of APS was weighed and dissolved in 1ml double distilled water by vortexing. Then 600µl of the APS solution and 60µl of TEMED was added to this mixture simultaneously without any time lag. Then the solution was injected into the injection port of the caster base using a syringe. As soon as the gel front reached the other end of the init, the vinyl comb was inserted between the plates. The gel was allowed to polymerase for 15 – 20 mins.

3.1.13.3 Gel Running

The IPC assembly was then dislodged from the precision caster base and fit vertically into the universal base using a stabilizer bar. The temperature indicator was adhered to the surface of the outer plate to monitor the temperature during the run. The upper and the lower buffer chambers were filled with the required volume of 1X TBE buffer. The gel was pre-run for 20 minutes at 100 W after positioning the top and bottom safety covers. Following completion of the pre-run, the power supply was stopped and the top safety cover removed. The wells were thoroughly rinsed using a pipette to remove any deposited urea. 6 µl of samples denatured at 95°C for 5 minutes were loaded along with 100bp ladder (Appendix XI). After replacing the safety cover, the samples were electrophoresed at 100 W for 35-40 minutes.

The power supply was turned off after the completion of the run and the upper buffer chamber was partially emptied by attaching the connector to the drain port on the IPC unit. The clamps were removed and the glass plate containing the gel was separated carefully from the IPC unit. Fragments of gel were removed from the plate by cleaning with lint free tissue.

3.1.13.5 **Silver Staining for PCR Product Detection**

The glass plate was transferred into a large trough containing fixer (Appendix VI) and placed on a shaker for 20 min, ensuring that the gel surface faced upwards. Similarly, staining was performed using silver stain (Appendix VII) after the gel was washed in another trough containing purified distilled water for 5 min. Subsequent to a further wash, the stained gel was developed by transferring into a trough containing the developer (Appendix VIII) and gently rocking the trough in a to and fro motion. A white surface was placed under the gel to enable visualization during development. After the bands had visibly developed, the plate was immediately transferred into the stopper. Following a final wash step, the gel was allowed to dry. Clear and reproducible bands were only selected for scoring.

3.1.14 **Gel Elution**

Elution of SSR primer **Dab2C05** showing distinct bands for dwarf yam was eluted using QIAquick Gel Extraction Kit. First of all, using this SSR primer Dab2C05, multiple tube PCR have been done for released dwarf yam variety Sreedhanya as DNA source and single tube for tall varieties Sreepriya and Sreesubhra for comparison. Amplicons were run on 2% gel and distinct band for Sreedhanya, the dwarf variety was sliced under UV transilluminator using clean and sharp scalpel. Distinct bands obtained from the multiple tube reaction of Sreedhanya were pooled to an eppendorf tube. Weighed it, before that took weight of empty eppendorf tube and difference between gel slice containing eppendorf and empty eppendorf gave the weight of gel slices.

After this, using QIAquick Gel Extraction Kit added 3 volumes Buffer QG to 1 volume gel (100mg~ 100ul).The maximum amount of gel per spin column was 400 mg. For>2% agarose gels, added 6 volumes Buffer QG. Incubated at 50°C for 10

minute or until the gel slice was completely dissolved. Vortexed the tube every 2 to 3 so as to help to dissolve gel. After the gel slice has been dissolved completely, checked whether the color of the mixture is yellow, similar to buffer QG. If the color of the mixture is orange or violet, added 10ul 3M sodium acetate .pH 5.0, and mixed. The mixture will turn yellow. Added 1 volume isopropanol to the sample and mixed. Placed a QIAquick spin column in a provided 2ml collection tube or into a vaccum manifold. To bind DNA, applied the sample to the QIAquick column and centrifuged for 1 minute or applied vaccum to the manifold until all the samples have passed through column. Discarded flow-through and placed the QIAquick column back into the same tube. For sample volumes of >800ul, loaded and spinned/applied vaccum again. Added 500ul Buffer QG to the QIAquick column and centrifuged for 1 minute/ applied vaccum. Disarded flow-through and placed the QIAquick column back into the same tube. To wash , added 750ul Buffer PE to QIAquick column and centrifuged for 1 minute. Discarded flow-through and placed the QIAquick column back into the same tube. Centrifuged the QIAquick column in the provided 2ml collection tube for 1 minute to remove residual wash buffer. Placed QIAquick column into a clean 1.5ml microcentrifuge tube. To elute DNA, add 50ul buffer EB(10mM TrisCl, pH 8.5) or water to the center of the QIAquick membrane and centrifuged the column for 1 minute. After the addition of Buffer EB to the QIAquick membrane, increasing the incubation time to up to 4 minutes can increase the yield of purified DNA. Purified DNA was analyzed on a gel, by adding 1 volume of Loading Dye to 5 volumes of purified DNA. Mixed the solution by pipetting up and down before loading the gel. Gel image was documented and confirmed that eluted DNA band was present on the gel. Eluted samples were stored on -20°C refrigerator for sequencing.

3.1.15 **DNA sequencing in Genetic Analyzer**

Eluted DNA samples were used for cycle sequencing reaction that included 3µl template or eluted sample, primer(SSR-Dab2C05) of 2µl, Ready reaction mix (Big dye) of 0.5µl,5x dilution buffer of 1.75µl and remaining DNAase or RNAase free water for each 10µl reaction.

Sequencing reaction was carried out in Biorad thermocycler. The program was follows;

Lid - 105°C

96°C - 1 mins (initial denaturation)

96°C - 10 sec (denaturation)

50°C - 5 sec (annealing) } Cycles × 25

60°C - 4 mins (extention)

4°C - hold

After this reaction, transferred the reaction product into a 1.5ml eppendorf tube and prepared it for clean up. Add 12µl of master mix I (Appendix XII) to each reaction containing 10ul of reaction. Ensured that the contents were mixed. Added 52µl of master mix II(Appendix XII) to each reaction. Mix the contents and incubated at room temperature for 15 minutes. Spinned at a speed of 12000g for 20 minutes at room temperature. Decanted the supernatant. Added 250µl of 70% ethanol and spinned at 12000g for 10 minutes at room temperature. Decanted the supernatant. Added 12- 15µl of Hi-Di formamide, transferred to sample tubes covered with septa, denatured, snapchilled proceeded for electrophoresis.

3.1.16 **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. Estimation of genetic diversity parameters results in an overview of the genetic variability and can be used as a criterion for comparing both marker efficiency and groups from different studies (Laurentin, 2009).

The average number of alleles per locus (n) was calculated as following

$$N=(1/K)\sum n_i$$

Where, n_i is the number of alleles per locus and K is the number of loci.

Parameters for calculating the marker efficiency and genetic characteristics were used. Polymorphic information content (PIC) was calculated using the formula

$$PIC = 2fi(1 - fi)$$

where fi is the frequency of the amplified allele and $1 - fi$ is the frequency of null allele.

Heterozygosity per locus was calculated according the formula:

$$He = 1 - p2 - q2$$

Where, p2 =fi.

Average heterozygosity per marker was calculated based on:

$Hav = \Sigma (He / L)$, where L = total of detected bands.

3.1.17 Cluster Analysis

A binary matrix of presence/absence was obtained from gels for each marker. Jaccard's similarity coefficient was calculated for use in clustering analysis by Unweighted Pair-group Method with Arithmetic Average (UPGMA). 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's test.

3.2 Standardization of *in vitro* propagation protocol

3.2.1 Glasswares and Instruments in Tissue Culture Lab

Glasswares like test tubes, erlenmeyer's flask, jambottles, glass petriplates, beakers, measuring cylinders were washed in detergent solution of 10% Teepol and rinsed in tap water and kept in hot air oven for drying. The dried testubes, jambottles, glass petriplates were wrapped and autoclaved. Forceps, needles, sterile blades (21,11),blade holders (3, 4),foil paper rolls are the other requirements in tissue culture experiments. Forceps were washed in detergent solution of 10% teepol and rinsed in tap water and kept in hot air oven for drying and then autoclaved along with glassware's. All these materials should be wiped using ethanol before usage inside Laminar Air Flow. Autoclaved double distilled water was used for reducing

contamination risk on washing. Neat and clean plastic bags, gloves and scissors are other essentials used for explant collection from fields.

3.2.2 **Instruments**

- Laminar Air Flow Chamber
- Stereo zoom microscope with illuminator for meristem excision filter
- Shaker
- Autoclave

3.2.3 **Nutrient media formulation**

The formulation of Murashige and Skoog's (1962) basal medium (MS) is used in the propagation studies of dwarf yams. In different studies the formulation of the medium was modified. For the ease of use and due to limited time readymade MS basal salt mixture with vitamins and without calcium chloride was used.On preparation, this readymade pack of 1L took from refrigerator and mixed thoroughly along with sucrose (30g/l)which was weighed separately. After that pH of the medium was checked and confirmed between 5.6 to 5.8.Then added 8 g agar to the medium with activated charcoal and heated it till bubble formation. Filtered it and poured to sterile test tubes, capped it using foil paper and stored for 2 days to analyze any contamination and then used for inoculation.

Table 9. Media composition

SL. No	Media Code	Media Composition
1	MS	MS+ Sucrose 30g^{L-1} + Agar 8g^{L-1}+ Charcoal 1g^{L-1}
2	MSK1	MS+ Sucrose 30g^{L-1} + Agar 8g^{L-1}+ Charcoal 1g^{L-1} + 1mg kinetin
3	MSK2N	MS+ Sucrose 30g^{L-1} + Agar 8g^{L-1}+ Charcoal 1g^{L-1} + 2mg kinetin +.5mg NAA
4	MSK2	MS+ Sucrose 30g^{L-1} + Agar 8g^{L-1}+ Charcoal 1g^{L-1}+ 2mg kinetin
5	MSBN	MS+ Sucrose 30g^{L-1} + Agar 8g^{L-1}+ Charcoal 1g^{L-1} + 2mg BAP + .5mg NAA

3.2.4 Sterilization of Nutrient media, Materials, Glasswares and Instruments

The test tubes containing media were steam sterilized in autoclave for 15 to 20 minutes at 121°C (15 lbs). After sterilization, the culture tubes containing medium was transferred to baskets and allowed them to solidify. Media was left at 20°C prior to use under sterile conditions .In between, they were screened for microbial contamination visually under tube lights. Glass wares were also autoclaved along with media. Instruments like Laminar air flow cabinet before usage should be completely wiped with 70% ethanol, turn on UV knob for15 to 20 minutes for microbial free working area in air hood and allowed the air hood to run about half an hour before work. Kept forceps, blades, petri dishes, foil papers, bottles wiped with ethanol inside airhood before work.

3.2.5 Explant Preparation

Explants used were nodal segments, shoot tips and tuber bud sprouts of dwarf yam. Nodal segments and shoot tips were collected from field gene bank. Tuber sprouts were collected from harvested tubers stored in yam storage room CTCRI, Trivandrum. Collected nodal segments and shoot tips were initially washed

thoroughly under tap water 3 to 4 times. After washing, cut the leaves of nodal segments carefully without damaging the node and tips. After cutting off the leaves, transferred nodal segments and shoot tips to bottles having autoclaved water. Washed it once and transferred to another bottle having laboline solution, which have antibacterial action. Kept the bottle in shaker for 30 minutes. After this step rinsed the explants with autoclaved distilled water 3 to 4 times to remove soapy solution from the explants. Transferred the explants using autoclaved forceps to another bottle having fungicide solution called bavistin. Kept the bottle in shaker again for 30 minutes. Rinsed it thoroughly using autoclaved distilled water till the bavistin solution diminishes. Took the clean and clear explants inside laminar air flow hood for inoculation

Tuber bud sprouts, after collecting it needed 5-6 times of rinsing with tap water and careful excised outer layers of bud without damaging the meristem..After collecting buds rinsed thoroughly with autoclaved distilled water 2 to 3 times and transferred to bottles having laboline solution and kept bottles at shaker for 1 hour to reduce chance for bacterial contamination. After that rinsed thoroughly with autoclaved distilled water till soapy solution disappears and transferred the buds to bottles having bavistin solution and kept it in shaker for 1.5 hr for the initiation of fungus free cultures. Rinsed it using autoclaved distilled water till bavistin was washed out. Took the neat and clear buds inside laminar air flow chamber for inoculation.

3.2.6 **Surface sterilization**

Mainly done using mercuric chloride and sodium dichloroisocyanurate solution. Mercuric chloride was conventionally used and it has lot of side effects including neurotoxicity and due to this we had gone for less toxic or least toxic chemical like sodium dichlor isocyanurate solution.

For standardization, different percentage and treatment time were carried out for sodium dichloroisocyanurate as shown in (Table 10).

Table 10. Treatments of surface sterilizing chemical

SL. No	Conc of sodium dichloroisocyanurate	Treatment time (minutes)
1	2%	5,10,15,20
2	1%	5,10,15,20
3	0.5%	5,10,15,20

From literature, standardization of treatment time in mercuric chloride also was standardized by giving 0.1% mercuric chloride treatment time of 5, 8, 10, 12, 15 minutes and the comparative study of both the chemicals in surface sterilization was analyzed.

3.2.7 Inoculation

Surface sterilized explants were inoculated in autoclaved medium using sterilized forceps and cultured tubes were capped using alcohol treated foil papers.

3.2.8 Culture conditions

All the cultures were kept at 25+-1^{0}C under 12 hour of photoperiod and at 70% to 80% relative humidity. Culture tubes were examined daily and data collected.

3.2.9 Thiourea treatment for breaking bud dormancy

Thiourea treatment was used for decreasing dormancy period in tubers.

Thiourea solution was prepared at concentration of 20g/l and stored in amber colored bottles. Tubers of *Dioscorea rotundata* after harvest were collected. From this collection few tubers of dwarf variety Sreedhanya was selected and washed under tap

water for several times to remove soil and dust from the tubers. Kept it for drying and after that cut the tubers in to head, middle and tail portions carefully. Labeled each portion with tag and treated each part of tubers according to the tests listed on (Table 11).Took cut tubers with forceps and completely immersed in 100ml thiourea solution of various concentration. After the listed time in different tests transferred the treated tubers to soil filled pots in the net house .Six treated tuber parts were planted on each pot and pots were labeled with date, test number and sample name. Recorded days taken for sprouting of tubers due to dormancy breakage and documented. Control was kept for comparison.

Table 11. Thiourea treatment of Tubers

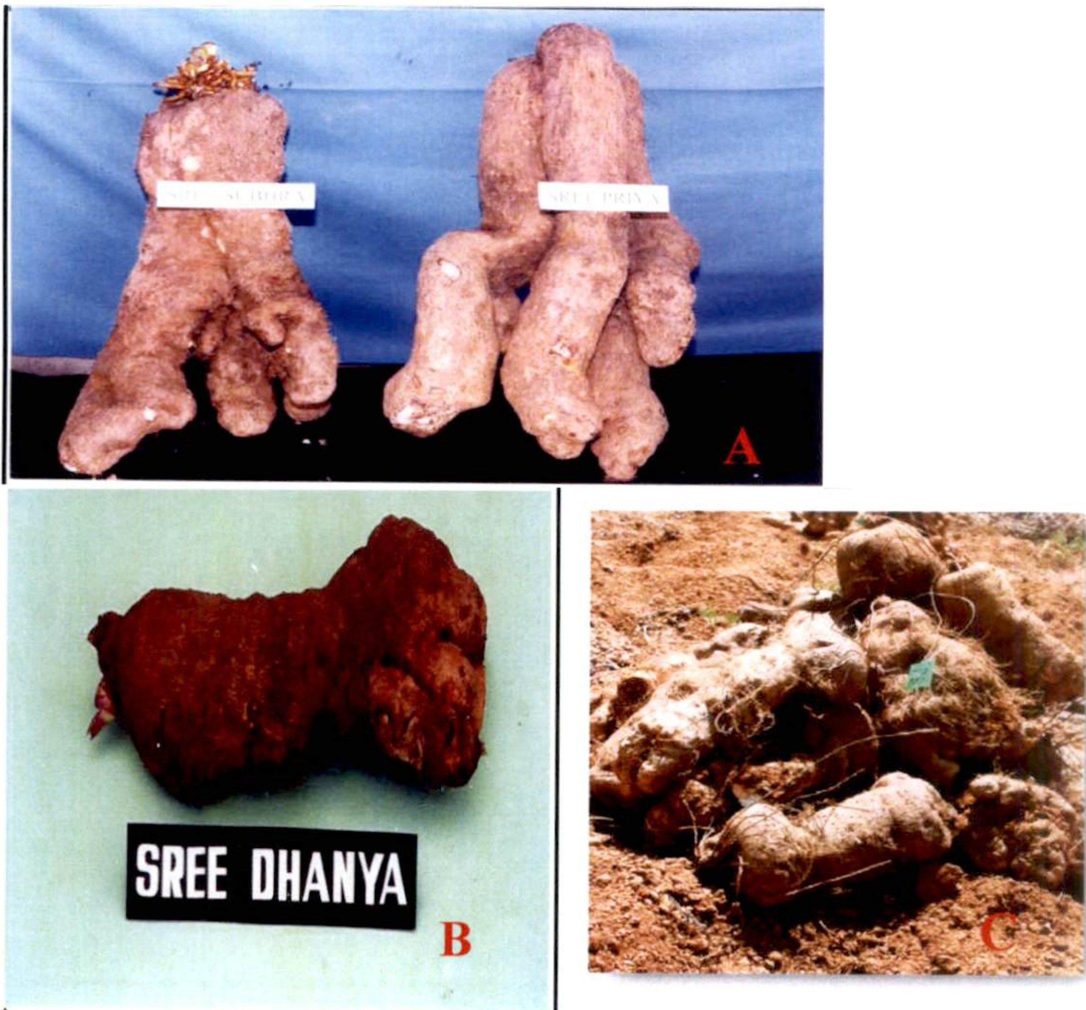
No of Tests	Conc. of thiourea	Amt of thiourea sol	Distl water	Time of incubation
T_1	20g/l	100ml	0	60 min
T_2	20g/l	100ml	0	30 min
T_3	10g/l	50ml	50ml	60 min
T_4	10g/l	50ml	50ml	30 min
T_5	5g/l	25ml	75ml	60 min
T_6	5g/l	25ml	75ml	30 min
T_7	1g/l	5 ml	95ml	60 min
T_8	1g/l	5 ml	95 ml	30 min
T_9	Not used	0	100ml	60 min
T_{10}	Not used	0	100ml	30 min
Control	Not used	0	0	0

RESULTS

4. RESULTS

The results of the study entitled "*In vitro* propagation and identification of molecular markers linked to dwarfness in white yam (Dioscorea rotundata Poir.)"was carried out at the Division of Crop Improvement, ICAR- Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram during 2014-2015 are presented in this chapter.

4.1 MORPHOLOGICAL DATA ANALYSIS



White Yam tubers: A-Sree Priya & Sree Shubra, B- Sree Dhanya, C-DRS 657

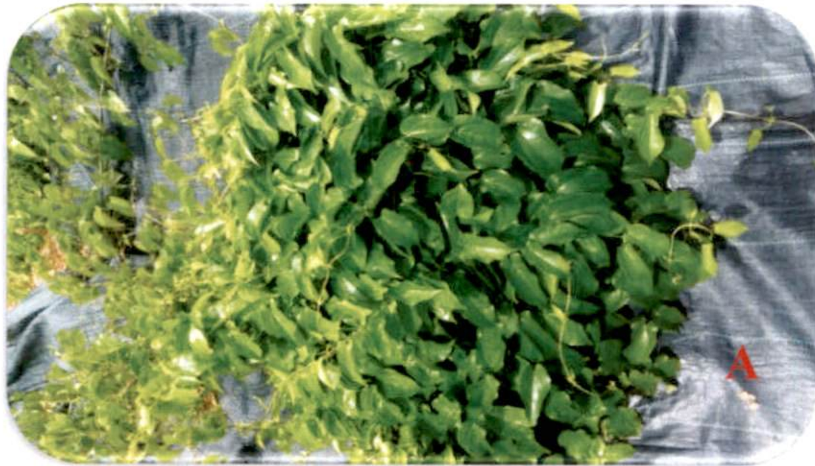


Plate 1 : A represents dwarf genotype and B represents semi dwarf and C represents tall genotype.

4.2 EVALUATION OF WHITE YAM GENOTYPES

Table 12. Analysis of Morphological Data

SL. No.	Accessions	Plant height (cm)	Plant Spread (cm)	Tuber Yield (Kg/Plant)
1.	SREE DHANYA	62.50	71.2	3.5
2.	DRD 1110	83.00	84.0	4.5
3.	DRD 495	50.80	66.04	4.0
4.	DRD 1118	74.32	68.21	3 .0
5.	DRD 1835	68.28	75.20	3 .0
6.	DRD 1060	66.04	67.31	2 .0
7.	DRD 1033	53.34	81.28	3.0
8.	DRD 920	76.20	63.54	3.0
9.	DRD 1142	45.72	83.82	4.0
10.	DRD 949	55.88	63.51	4..0
11.	DRD 1157	83.82	78.74	3.0
12.	DRD 1068	58.42	68.54	2.0
13.	DRD 835	55.88	96.52	3.0
14.	SD 15	71.12	76.2	4.0
15.	DR 2	76.21	71.12	3.0
16.	DR 29	60.96	96.52	6.0
17.	DRS 47	420.0	120.3	5.0
18.	DRS 45	386..0	115.0	3.8
19.	DR 130	510.8	114.0	5.0
20.	DR 17	420.0	120.0	8.0
21.	DR 73	375.8	115.0	5.0
22.	DRS 652	425.0	125.0	4.2
23.	DR VIOLET	378.5	136.0	4.5
24.	DRS 36	560.3	112.0	5.5
25.	DRS 1155	490.0	120.0	4.8
26.	DRH 1047	525.5	116.0	6.2
27.	DRH 657	480.0	132.0	5.5
28.	DRH 657 A	466.0	125.0	4.0
29.	SREEPRIYA	515.0	120.0	4.5
30.	SREESUBHRA	525.0	135.0	5.2

4.3 STANDARDIZATION OF DNA ISOLATION PROTOCOL

Isolation of DNA on *Dioscorea rotundata* were tried by using both manual and kit based method to identify optimum method to extract quality DNA with better concentration. Upon modified method of Doyle and Doyle (1987), DNA obtained was of poor quality contaminated with RNA and proteins with sheared bands as shown in Plate 2. This method gave aberrant readings on spectrophotometer. DNA isolated from kit method (DNeasy Plant Mini Kit) was of good quality compared to manual one. This is rapid and easy to use method. However, concentration of DNA was less. Plate 2 and 3 shows the DNA profiles of both methods on agarose gel (0.8%).Table 13 shows the spectrophotometric readings of the DNA isolated from kit method that is used for the study. The absorbance values of DNA using kit method from spectrophotometer revealed quantity of DNA with purity more or less between the range of 1.3 to 2.4.

Table 13. Absorbance value and Concentration of DNA used for the study

Accession code	Absorbance (A_{260} nm)	Absorbance (A_{280} nm)	A_{260}/A_{280}	DNA Yield (ng μl^{-1})
V1	0.082	0.045	1.8	410
V2	0.008	0.005	1.6	40
V3	0.004	0.002	2.0	20
V4	0.009	0.004	2.3	45
V5	0.007	0.003	2.3	35
V6	0.008	0.005	1.6	40
V7	0.009	0.004	2.3	45
V8	0.008	0.004	2.0	40
V9	0.009	0.004	2.3	45
V10	0.008	0.004	2.0	40
V11	0.018	0.008	2.3	90
V12	0.009	0.004	2.3	45
V13	0.008	0.004	2.0	40
V14	0.006	0.003	2.0	30
V15	0.010	0.006	1.7	50
V16	0.010	0.006	1.7	50
V17	0.019	0.014	1.4	95
V18	0.009	0.007	1.3	45
V19	0.014	0.007	2.0	70
V20	0.015	0.008	1.9	75
V21	0.019	0.014	1.4	95
V22	0.017	0.008	2.1	85
V23	0.012	0.005	2.4	60
V24	0.013	0.008	1.6	65
V25	0.008	0.005	1.6	40
V26	0.014	0.006	2.3	70
V27	0.012	0.006	2.0	60
V28	0.057	0.035	1.6	285
V29	0.019	0.011	1.7	95
V30	0.008	0.005	1.6	40

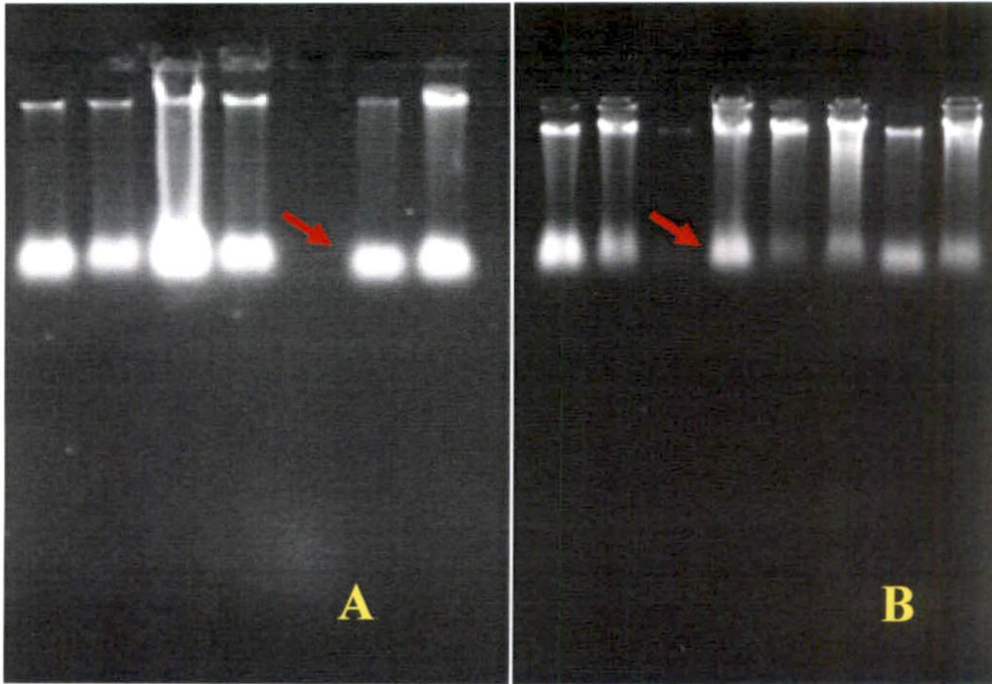


Plate 2. (A and B) shows low purity DNA with sheared bands

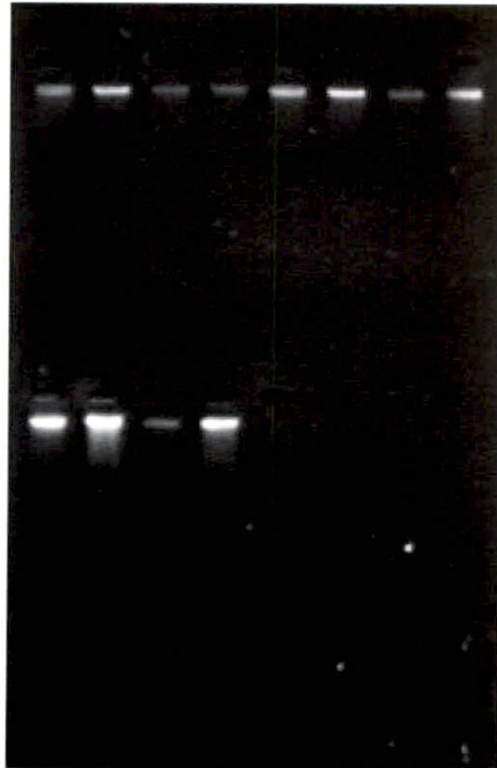


Plate 3. shows DNA obtained using DNeasy Plant Mini Kit method having high quality DNA with distinct bands

4.3 PCR STANDARDIZATION

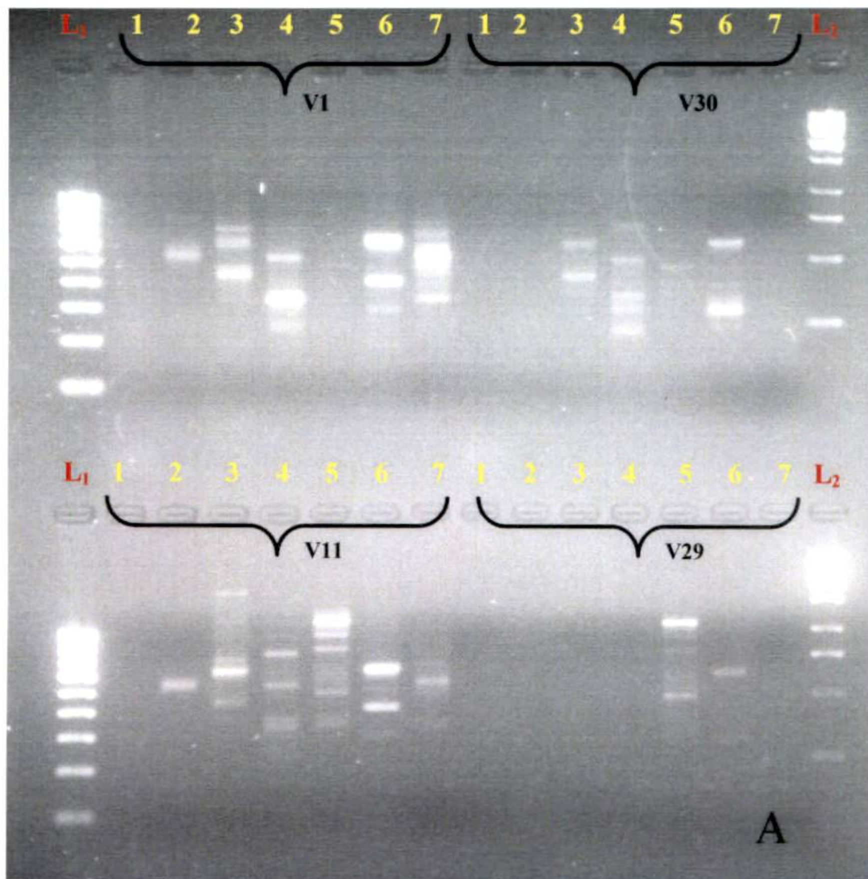
Standardization of PCR conditions was attempted for few RAPD and SSR markers to obtain clear and consistent amplicons under reduced cycle duration. Changing annealing temperature for certain SSR and RAPD primers gave better results. Usage of Takara emerald master mix reduced preparation time for PCR.

4.4 PRIMER SCREENING

For preliminary screening of ISSR, 19 ISSR primers were used of which, only 15 were selected based on their polymorphism and repeatability. Plates 4 (A and B) shows the gel profiles of the ISSR used at annealing temperature of 56°C. ISSR UBC 824, UBC 849, UBC 851, UBC 11 were eliminated after preliminary screening due to poor amplification profile. ISSR $(GA)_9AT$ showed good amplification pattern, but did not show any polymorphism.

For preliminary screening of SSR, 14 SSR were used of which, only 10 were selected based on their polymorphism and repeatability. Plate 5 A shows the gel profiles of the SSR primers used at annealing temperature of 51°C. SSR primers like Dab2Do8, DalF08, Dpr3D06, DA3G04 were eliminated after preliminary screening due to poor amplification profile. Plate 5 B shows gel profiles of the SSR primers used at annealing temperature of 58°C. Primer MT 13 did not show any amplification, hence done a gradient analysis and optimized annealing temperature at 59°C.

For preliminary screening of RAPD, 19 RAPD primers were used of which, only 8 were selected based on their polymorphism and repeatability. Plates 6 (A and B) shows the gel profiles of the RAPD primers used at annealing temperature of 36°C. 11 RAPD primers were eliminated after preliminary screening due to poor amplification profile in tall genotypes.



Lane I

L_1-100bp; 1 to 7 sample used is V1 and V30 as two sets screened by primers: 1-UBC 11;2-UBC 14; 3-UBC 818; 4-UBC 836;5-$(GA)_9AC$; 6-$(GA)_9AT$;7-$(ACC)_6Y$;L_2-1kb

Lane II

L_1-100bp;1 to 7 sample used is V11 and V30 as two sets screened by primers : 1-UBC 11;2-UBC 14; 3-UBC 818; 4-UBC 836;5-$(GA)_9AC$; 6-$(GA)_9AT$;7-$(ACC)_6Y$;L_2-1kb

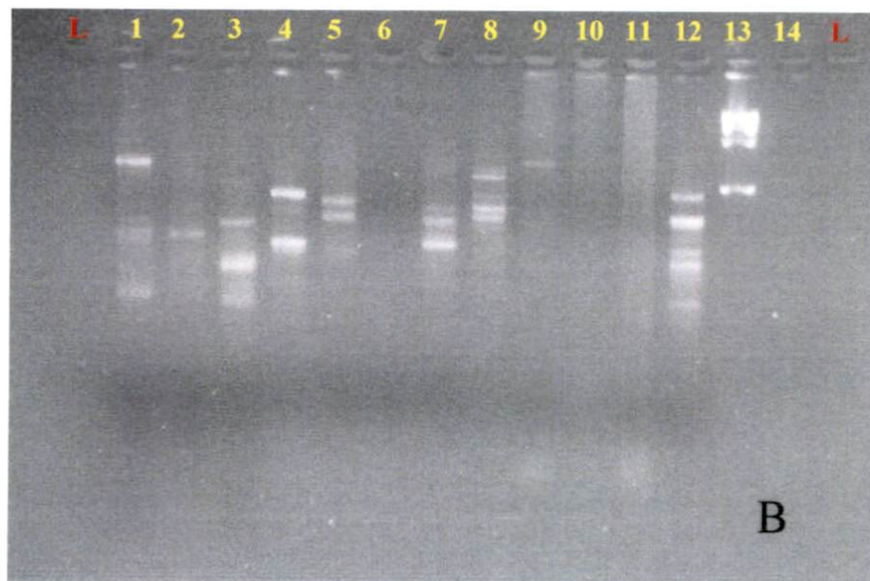
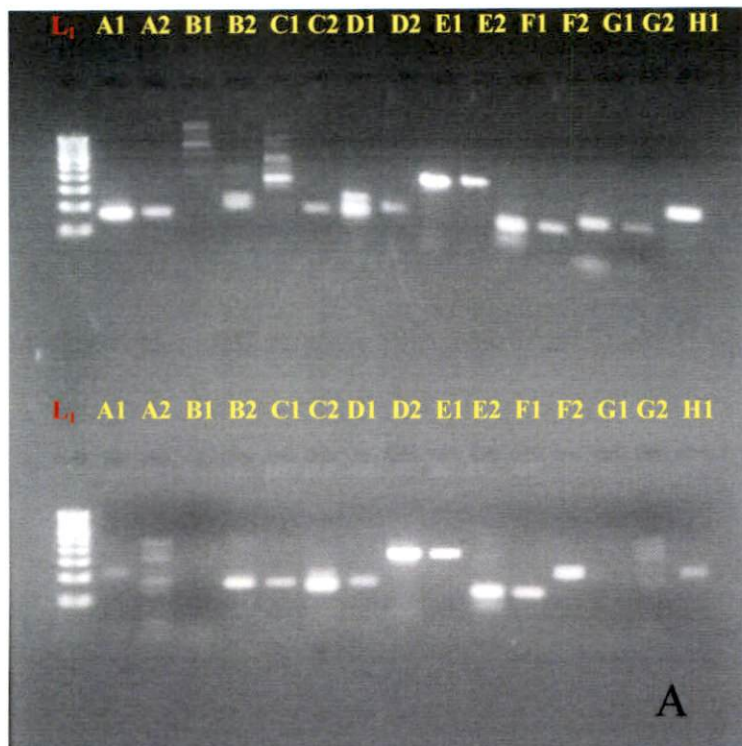


Plate 4. A and B shows preliminary ISSR primer screening test at 56°C in tall and dwarf genotypes

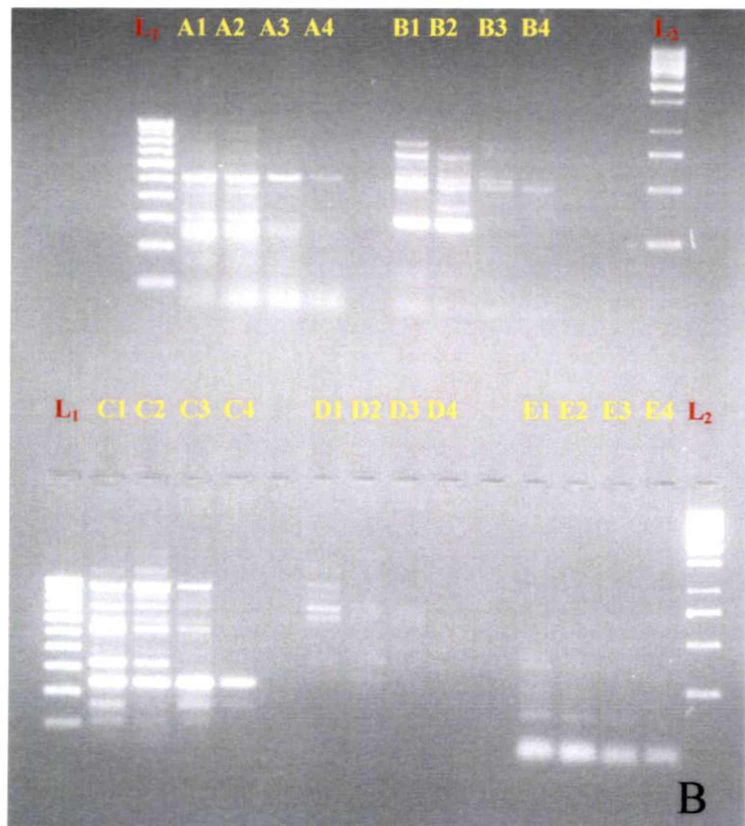


Lane I

L_1-100bp ladder; 1 and 2 represents sample V1 and V29 respectively. Primers used for screening : A-Da1F08; B-Dab2CO5; C- Dab2D0G; D- Dab2E07; E-Dab2D08; F- Dpr3F04; G- Dpr3D06; H-Da1A01

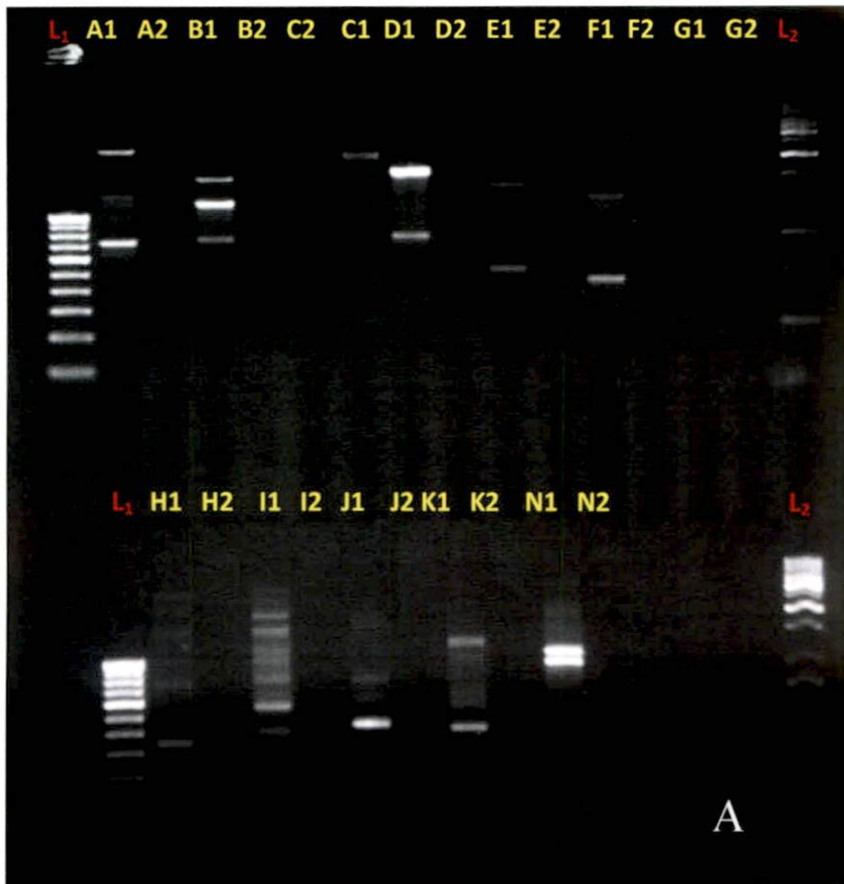
Lane II

L_1-100bp ladder; 3 and 4 represents sample V11 and V30 respectively. Primers used for screening : I- Da3G04; C-Dab2D0G; D- Dab2E07; E-Dab2D08; F-Dpr3F04; G- Dpr3D06; H- Da1A01

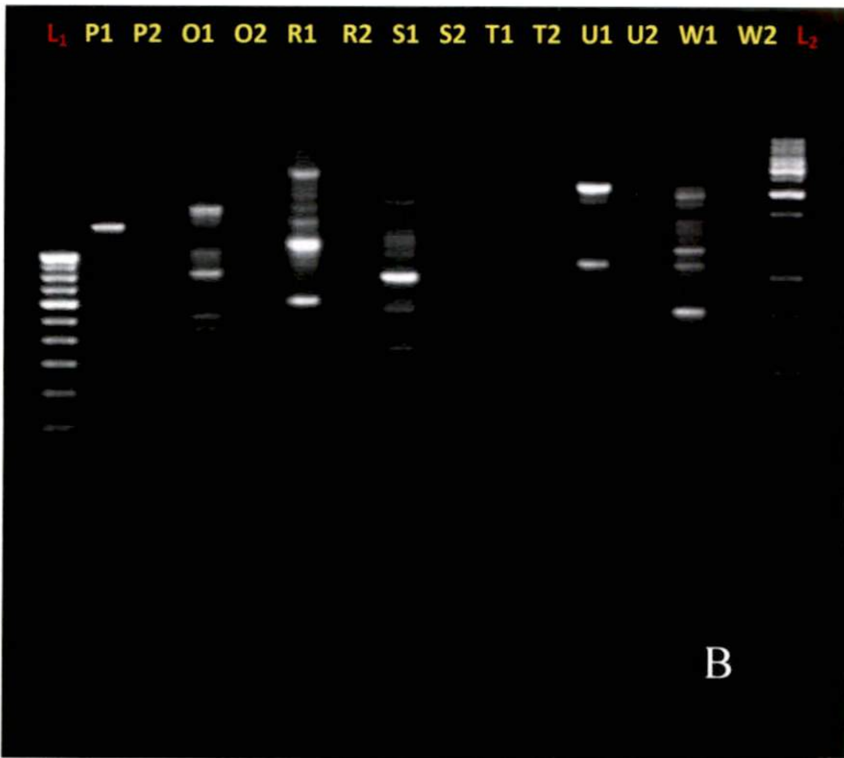


L_1- 100bp ladder, samples representing 1,2,3 and 4 areV1,V11,V29 and V30.Primers used for screening A-YM 5; B- YM 15; C-YM 26; D- MT 13; E-MT 10; L_2- 1kb ladder

Plate 5. A shows SSR primer screening at annealing temperature at 51°C and B shows SSR primer screening at 58°C



L_1- 100bp; Sample 1 and 2 represents V1 and V30 respectively; Primers used for screening : A-OPW-1;B-OPW-2;C- OPW-5;D-OPW-6;E-OPW-8;F-OPW-12;G-OPW-14;H-OPW-15;I- OPW-16;J-OPW-17;K-OPW-18;N-OPQ-4; L_2- 1kb



L_1- 100bp ladder; Sample 1 and 2 represents V1 and V30 respectively; Primers used for screening: P-OPD-03;O-OPG-02;R-OPG-03;S-OPG-05;T-OPG-06;U-OPG-08;W-OPG-13;L_2-1kb ladder

Plate 6. A shows RAPD primers screening at $36^{o}C$ and B shows RAPD primers screening at $37^{o}C$

4.5 **ISSR ANALYSIS OF WHITE YAM ACCESSIONS**

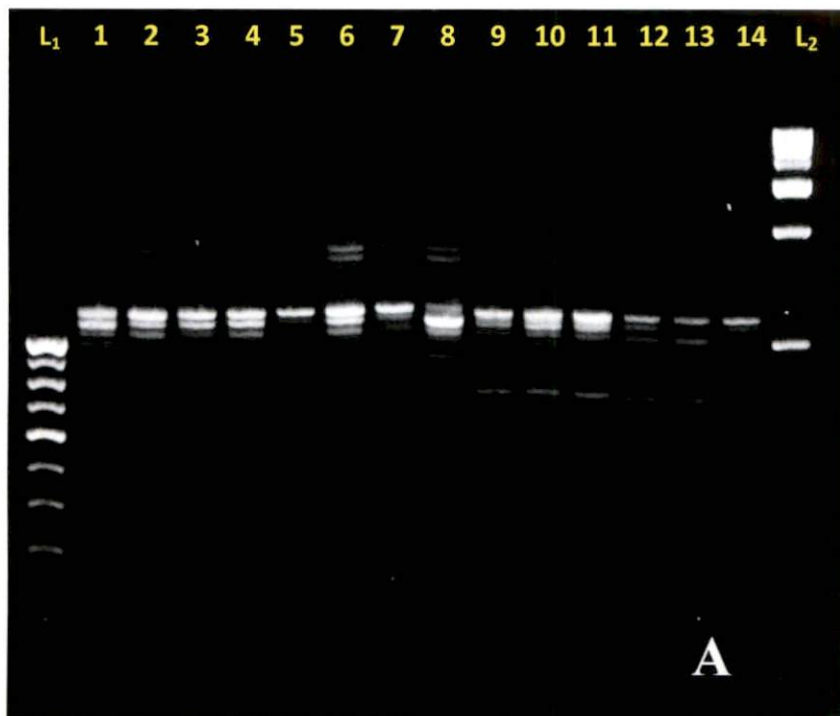
The amplicons obtained after the ISSR analysis of 30 accessions using the selected primers were initially resolved in two per cent agarose gel (Plates 7 to 16). Genetic characteristics of the fifteen ISSR markers used to analyze the white yam accessions is given in Table 14.Total number of bands per ISSR primer ranged from 1 ($(ACC)_6Y$) to 10 (UBC 825).The primers UBC 809, UBC 827 and UBC 864 recorded nine bands followed by UBC 810 (8 bands). The primers UBC 808, UBC 811, $(GA)_9AC$ and UBC 817 recorded seven bands. The lowest number of bands was recorded by $(ACC)_6Y$ (1) followed by $(GA)_9AT$, UBC 818 and UBC 14 with three bands.UBC 825 that recorded the highest number of bands also recorded the highest number of polymorphic bands (10) followed by UBC 827 with UBC 864 with nine bands. In the case of primer UBC 810, all the eight bands were found to be polymorphic. Bands obtained in the product size as mentioned in Table14.

The polymorphism of the ISSR primers studied ranged from 77.78% to 100%. UBC 809 recorded the lowest polymorphism (77.78%) among the primers followed by UBC 811 (85.71%). The Observed heterozygosity value (Hobs) of the ISSR primers ranged between 0 ($(ACC)_6Y$) to 0.88(UBC 825 and UBC 864) . For most of the ISSR primers studied Hobs was found to be >0.8. Among the primers, the $(GA)_9AT$ and UBC 818 recorded lower Hobs values of 0.5954 and 0.5448 respectively.

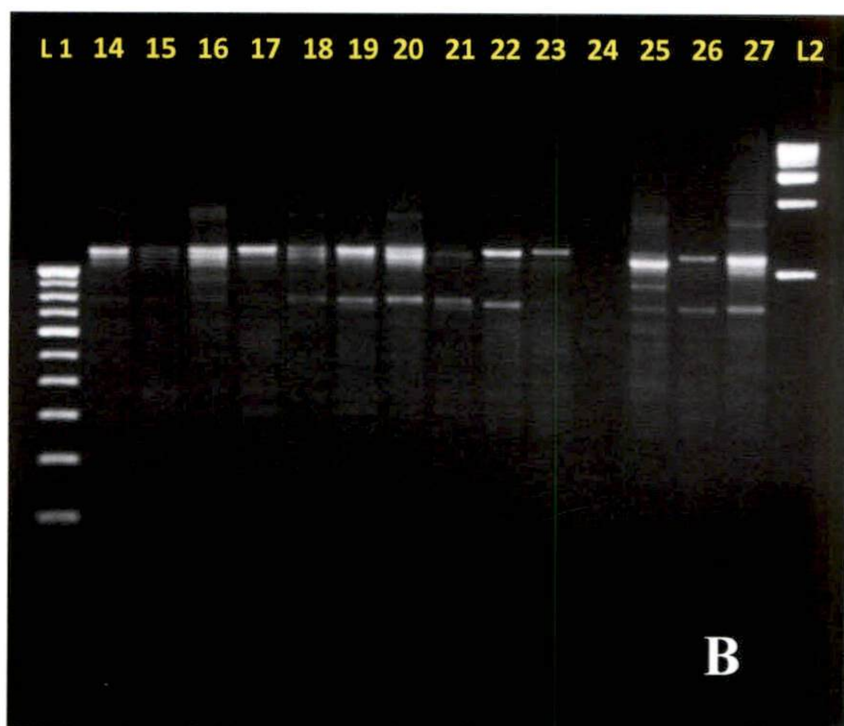
The polymorphism Information content (PIC) of the primers ranged from 0.8719 (UBC 864) followed by UBC 825(0.868), UBC 827 (0.8641) and UBC 809 (0.8601). The primers, UBC 818, $(GA)_9AT$, UBC 14 and $(ACC)_6Y$ recorded lower PIC content of <0.6. The PIC and Hobs values of the ISSR primers studied were given in Fig 2.

In the present study, the ISSR primers produced an average of six polymorphic alleles with mean Hobs and PIC values of 0.738 and 0.707 respectively.

Molecular Characterisation using ISSR primers



L_1	100bp Ladder
1.	SreeDhanya
2.	DRD1142
3.	DRD1110
4.	DRD 1033
5.	DRD 835
6.	DRD 1118
7.	DRD 920
8.	SD 15
9.	SREESUBHRA
10.	SREEPRIYA
11.	DRH 1047
12.	DRH 657
13.	DRH 657 A
14.	DR 130
L_2	1 Kb Ladder

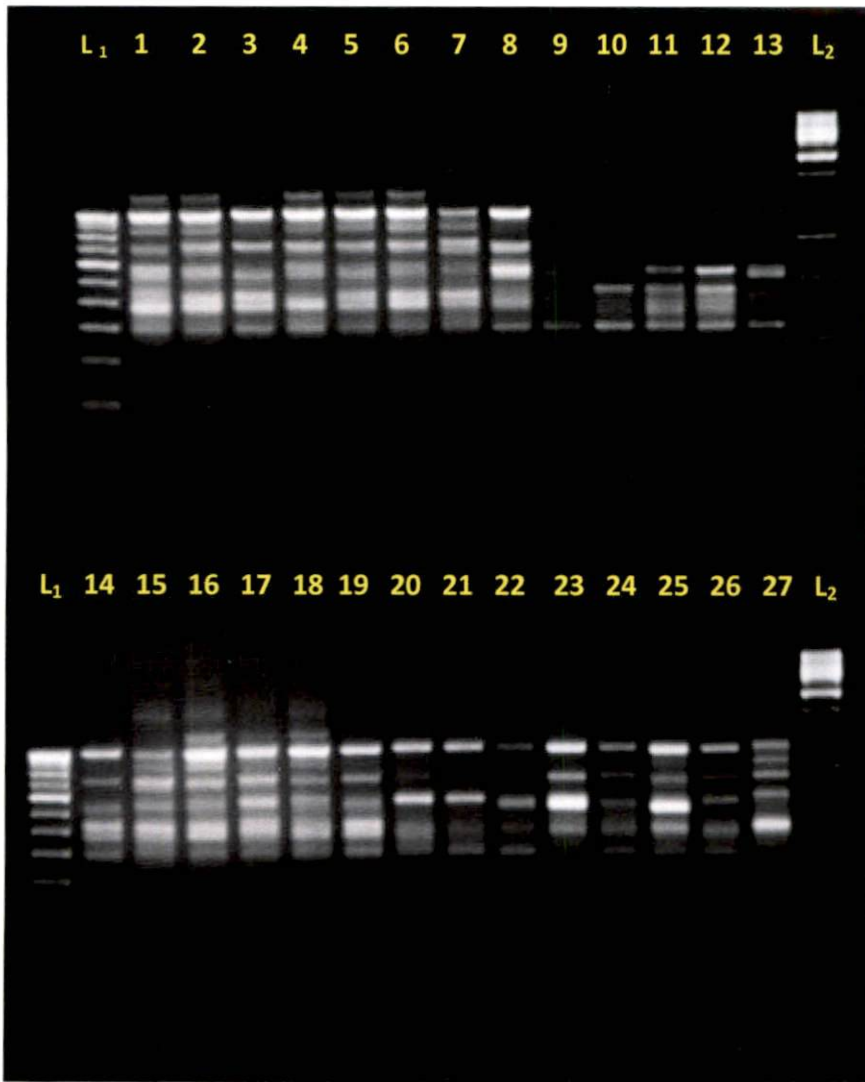


L_1	100bp Ladder
14	DRD 495
15	DRD 1835
16	DRD 1157
17	DRD 1060
18	DRD 1068
19	DR73
20	DRS 47
21	DRS 652
22	DR 36
23	DRVIOLET
24	DR 17
25	DRD 949
26	DRS 45
27	DRS 1155
L_2	1 Kb Ladder

Plate 7. (A and B) shows agarose gel (2%) profile of the ISSR primer UBC 808 for 30 accessions

Lane I

L_1	100bp Ladder
1	SreeDhanya
2	DRD1142
3	DRD495
4	DRD 1835
5	DRD 920
6	DRD 1118
7	SD 15
8	SREEPRIYA
9	SREESUBHRA
10	DRH 1047
11	DRH 657
12	DRH 657 A
13	DR 73
L_2	1 Kb Ladder



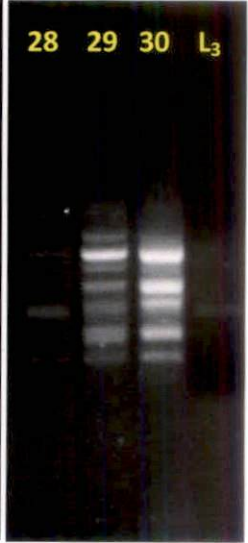
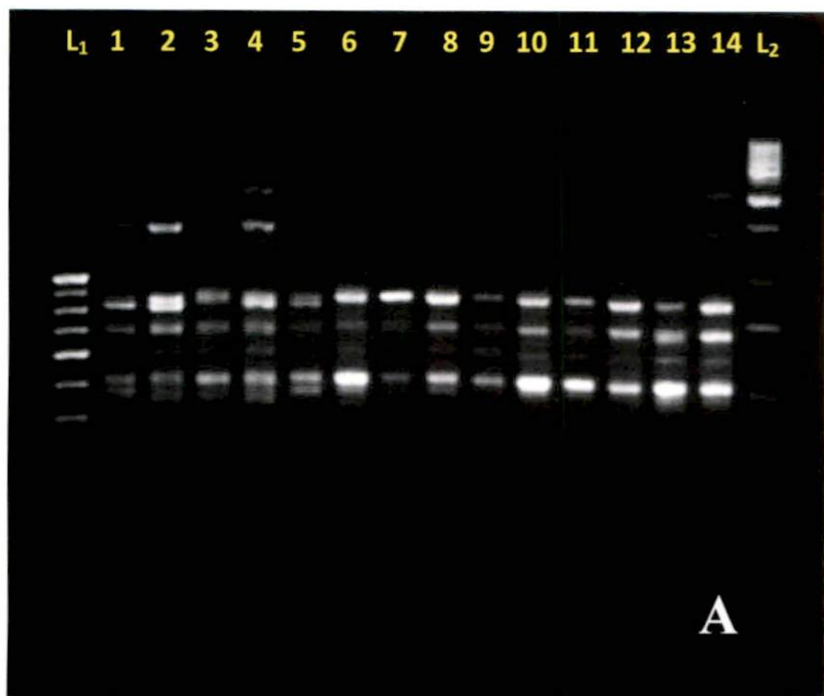


Plate 8. shows agarose gel (2%) profile of the ISSR primer UBC 809 for 30 accessions

Lane II

L_1	100bp Ladder
14	DRD 835
15	DRD 949
16	DRD 1033
17	DRD 1060
18	DRD 1110
19	DRD 1068
20	DR17
21	DR130
22	DR VIOLET
23	DR2
24	DRS45
25	DRS 652
26	DRS 36
27	DRS 47
L_2	1 Kb Ladder
28	DR 73
29	DRD 1157
30	DRS 1155
L_3	100 bp Ladder



L_1	100bp Ladder
1	DRD 835
2	DRD 1033
3	DRD 1060
4	DRD 1110
5	DRD 1068
6	DRH 657 A
7	DRVIOLET
8	DR2
9	DRS 45
10	DR 17
11	DR 130
12	DRS 47
13	DRS 652
14	DR 29
L_2	1 Kb Ladder

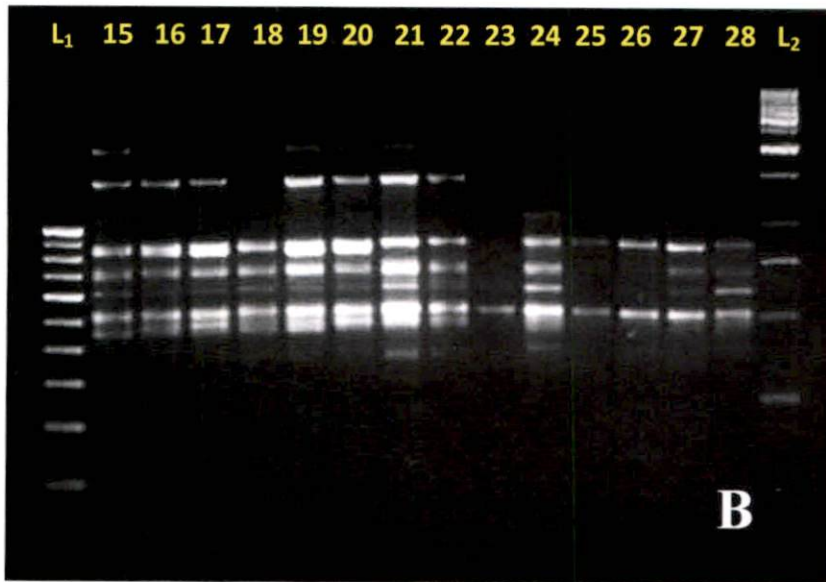
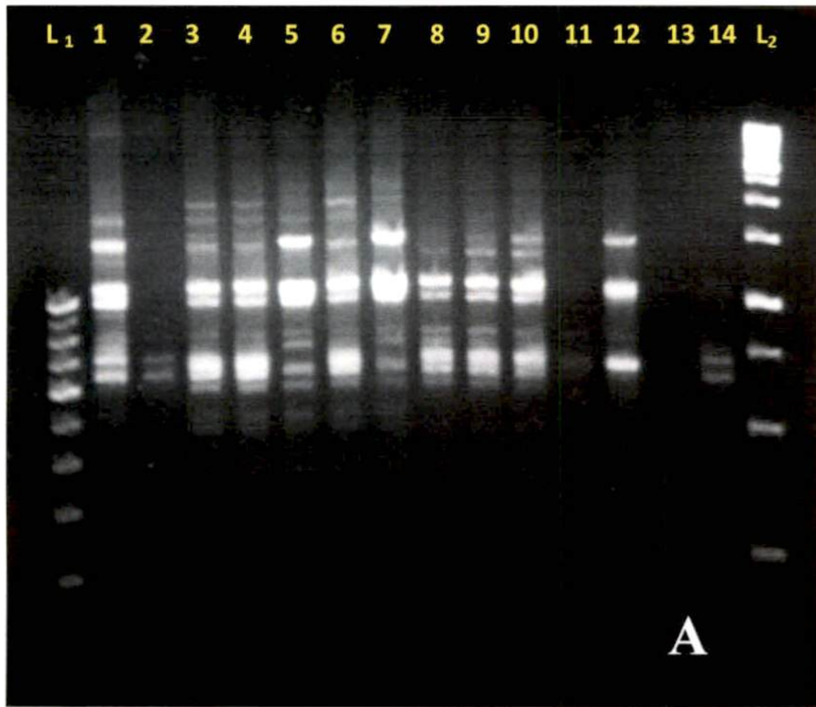


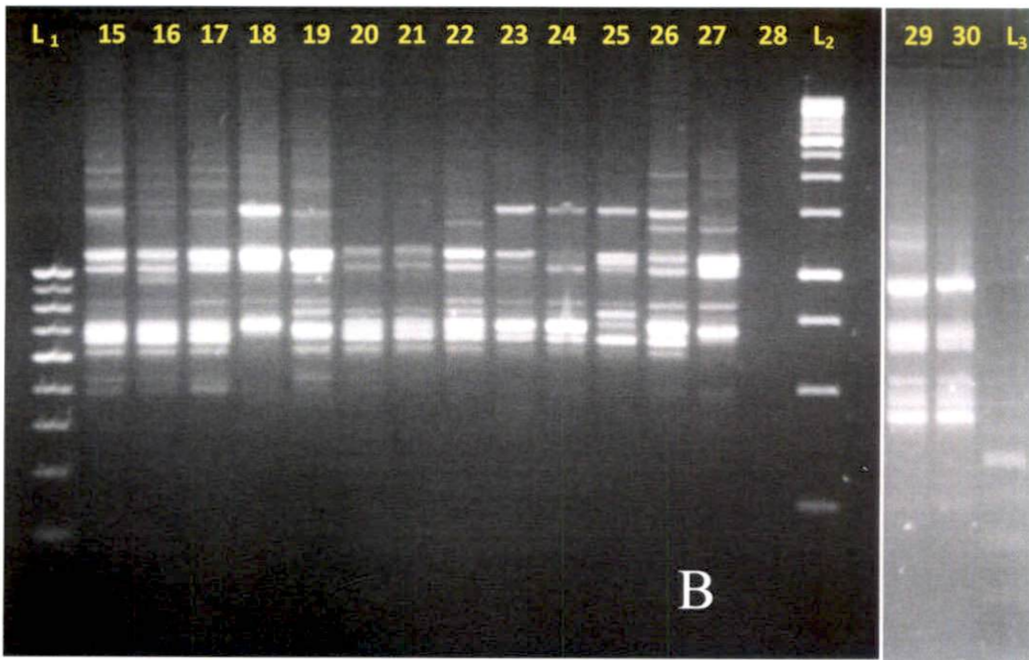
Plate 9. (A and B) shows agarose gel (2%) profile of the ISSR primer UBC 811 for 30 accessions

L_1	100bp Ladder
15	SreeDhanya
16	DRD1142
17	DRD495
18	DRD 1835
19	DRD 1118
20	DRD 920
21	DRD 949
22	SD 15
23	SREESUBHRA
24	SREEPRIYA
25	DRH 1047
26	DRH 657
27	DRS 36
28	DR 73
L_2	1 Kb Ladder

L_3	100bp Ladder
29	DRD 1157
30	DRS 1155
L_4	100bp Ladder

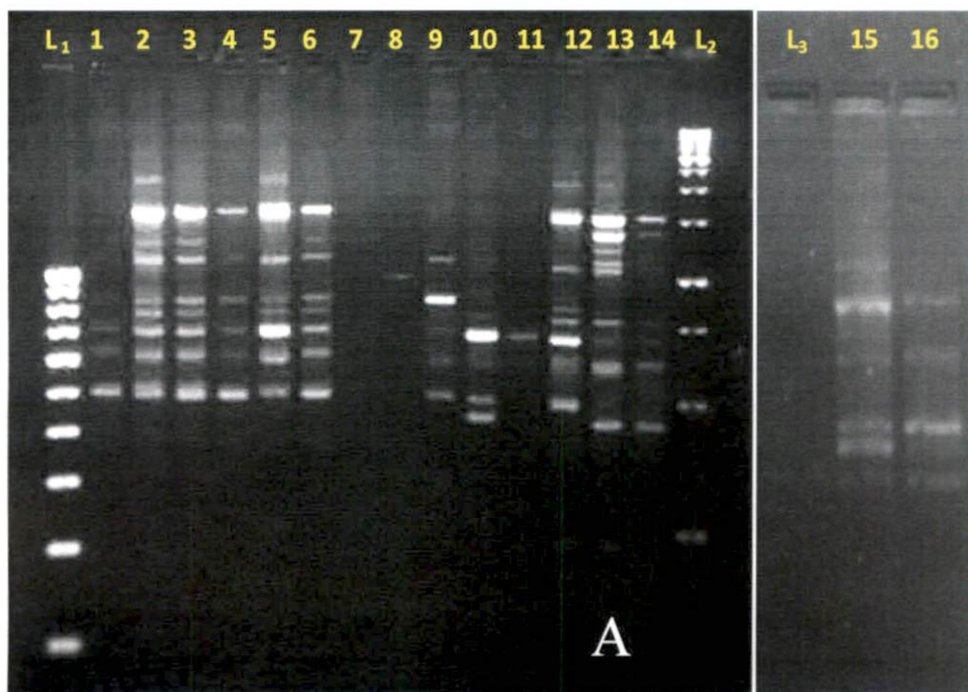


L_1	100bp Ladder
1	SreeDhanya
2	DRD1110
3	DRD1118
4	DRD920
5	DRD 949
6	DRD 1033
7	SD 15
8	SREESUBHRA
9	SREEPRIYA
10	DRH 1047
11	DRH 657
12	DRH 657 A
13	DR 130
14	DR VIOLET
L_2	1 Kb Ladder

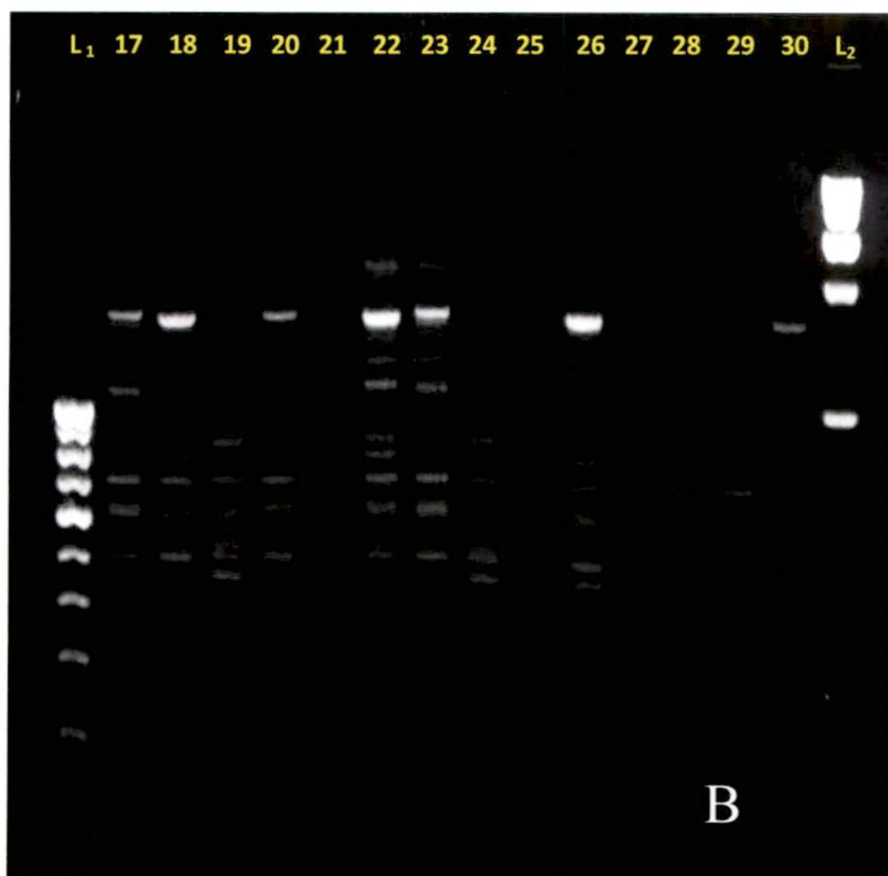


L_1	100bp Ladder
15	DRD 1157
16	DRD 495
17	DRD 1142
18	DRD 1060
19	DRD 1835
20	DRD835
21	DRD 1068
22	DR 73
23	DRS652
24	DRS36
25	DRS45
26	DRS1155
27	DR47
28	DR 17
L_2	1Kb Ladder
29	DR29
30	DR2
L_3	100bp Ladder

Plate 10 . (A and B) shows agarose gel (2%) profile of the ISSR primer UBC 827 for 30 accessions

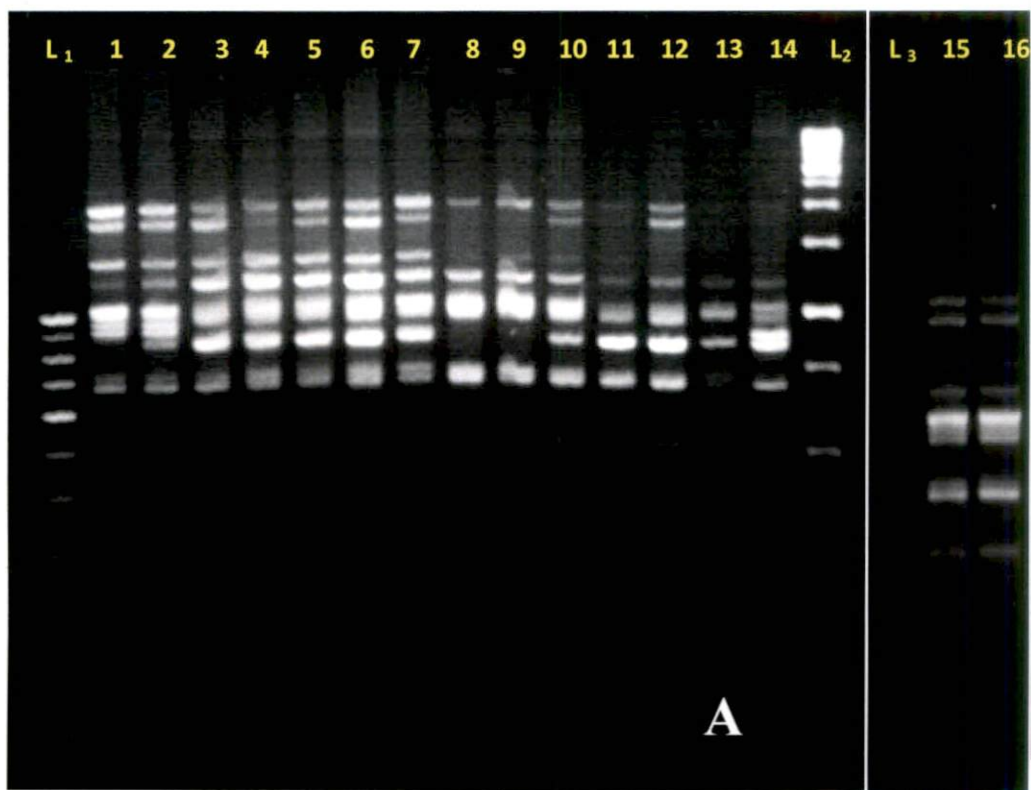


L_1	100bp Ladder
1	DRD 1033
2	DRD 920
3	DRD 495
4	DRD 835
5	DRD 1060
6	DRD 1068
7	DRVIOLET
8	DRS 17
9	DRS 652
10	DRS 45
11	DRS 36
12	DRS 47
13	DR 2
14	DR 29
L_2	1 Kb Ladder
L_3	100bp ladder
15	DRS 1155
16	DR 130

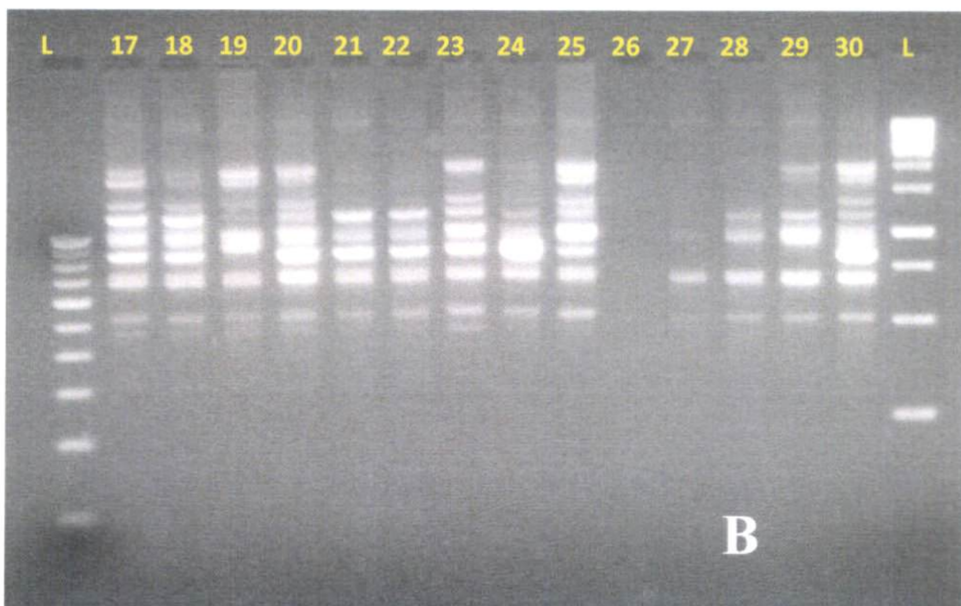


L_1	100bp Ladder
17	SreeDhanya
18	DRD1835
19	DRD1157
20	DRD 1142
21	DRD1118
22	DRD 1110
23	SD 15
24	DRD 949
25	SREE SUBHRA
26	SREEPRIYA
27	DRH 1047
28	DRH 657
29	DRH 657 A
30	DR 73
L_2	1 Kb Ladder

Plate 11 . (A and B) shows agarose gel (2%) profile of the ISSR primer UBC 825 for 30 accessions



L1 100bp Ladder
1. SreeDhanya
2. DRD1110
3. DRD1033
4. DRD1157
5. DRD1142
6. DRD 1118
7. SD 15
8. SREESUBHRA
9. SREEPRIYA
10. DRH 1047
11. DRH 657
12. DRH 657 A
13. DRS 36
14. DR 130
L2 1 Kb Ladder
L3 100bp Ladder
15 DR 29
16 DR 2



L1 100bp Ladder
17 DRD 920
18 DRD 495
19 DRD 1835
20 DRD 835
21 DRD 1060
22 DRD 1068
23 DRD949
24 DRS 652
25 DRS1155
26 DR 17
27 DR VIOLET
28 DRS45
29 DR73
30 DRS 47
L2 1 Kb Ladder

Plate 12 . (A and B) shows agarose gel (2%) profile of the ISSR primer UBC 864 for 30 accessions

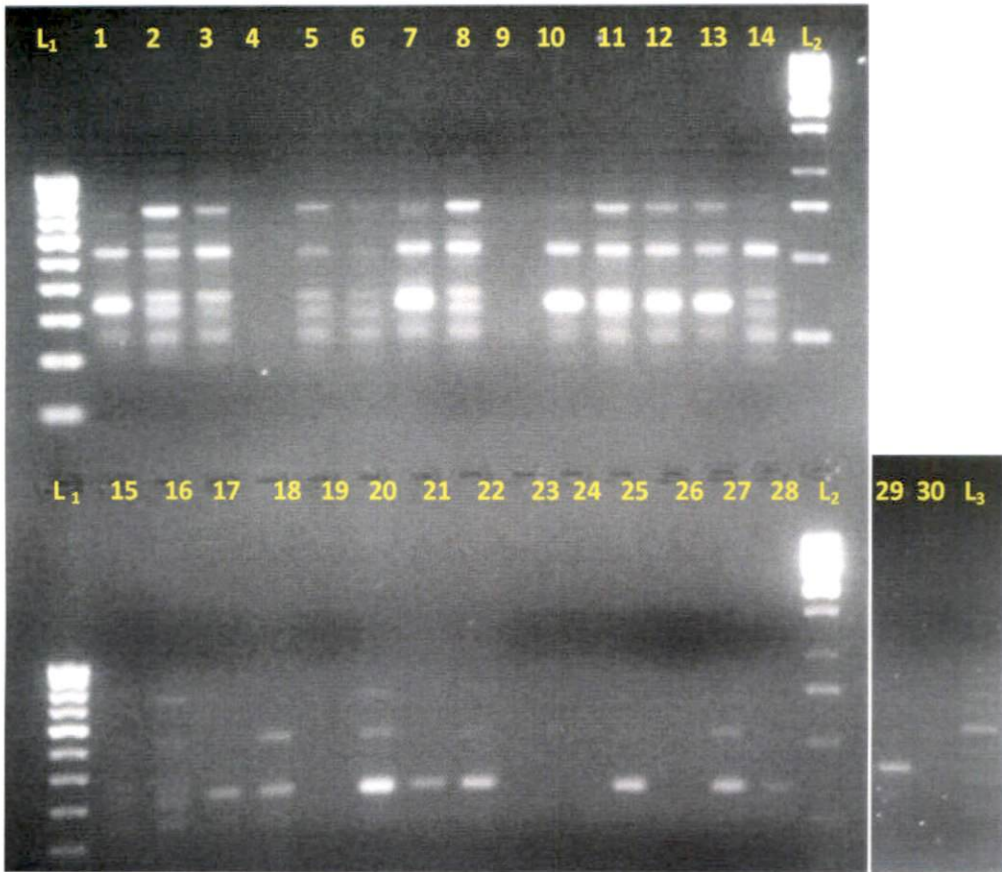


Plate 13 . shows agarose gel (2%) profile of the ISSR primer UBC 836 for 30 accessions

Lane I

L_1 100bp Ladder
1. SreeDhanya
2. DRD 1033
3. DRD 920
4. DRD 949
5. DRD 495
6. DRD 1068
7. DRD 1110
8. DRD 1118
9. DRD 835
10. DRD 1060
11. DRD 1157
12. DRD 1142
13. DRD 1835
14. SD 15

L_2 1 Kb Ladder

Lane II

L_1 100bp Ladder
15 SREESUBHRA
16 SREEPRIYA
17 DRH 657
18 DRH 657 A
19 DRH 1047
20 DR 29
21 DR 17
22 DR 2
23 DR 1150
24 DR VIOLET
25 DRS 652
26 DRS 45
27 DRS 47
28 DRS 36
L_2 1 Kb Ladder
29 DR 130
30 DR 73
L_3 100bp Ladder

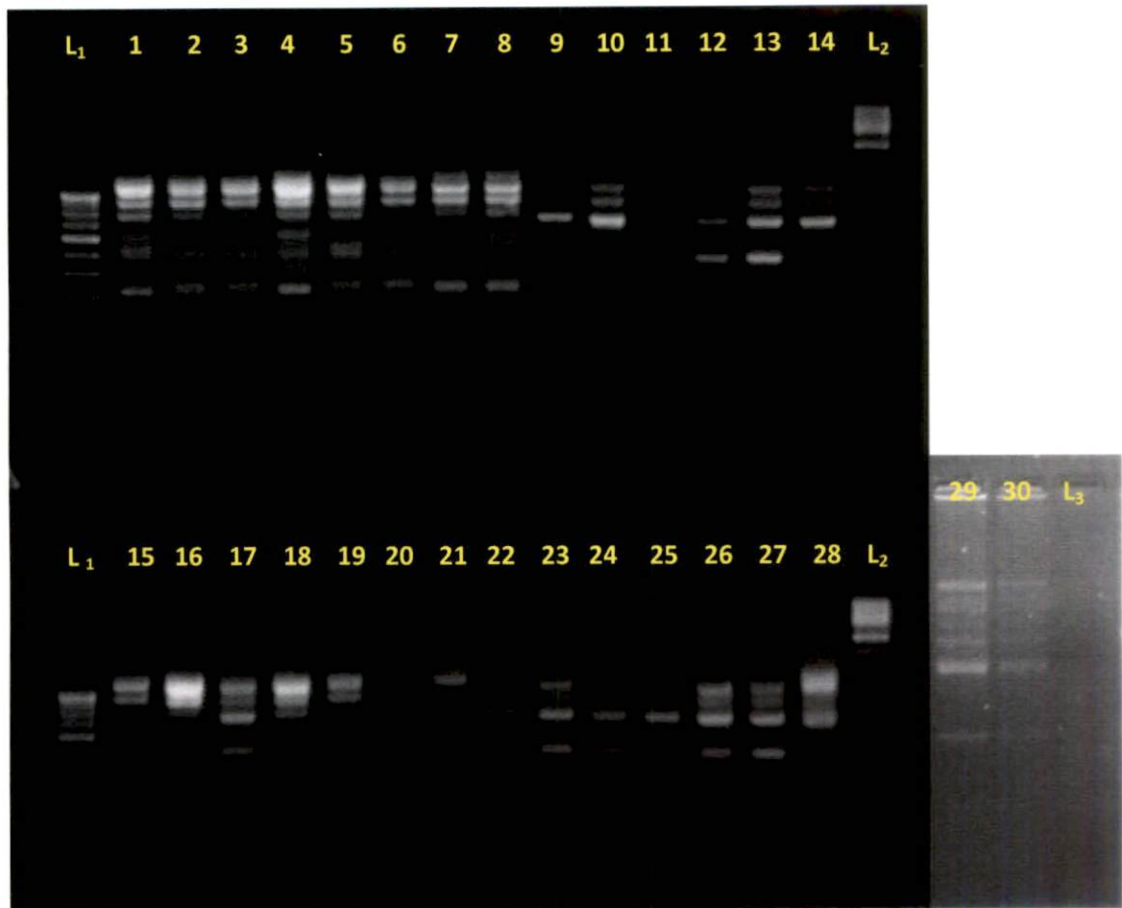


Plate 14. shows agarose gel (2%) profile of the ISSR primer $(GA)_9AC$ for 30 accessions

Lane I

L_1 100bp Ladder
1. SreeDhanya
2. DRD1142
3. DRD495
4. DRD 1835
5. DRD 1118
6. DRD 920
7. DRD 949
8. SD 15
9. SREESUBHRA
10. SREEPRIYA
11. DRH 1047
12. DRH 657
13. DRH 657 A
14. DR 73

L_2 1 Kb Ladder

Lane II

L_1 100bp Ladder
15 DRD 835
16 DRD 1033
17 DRD 1060
18 DRD 1110
19 DRD 1068
20 DRVIOLET
21 DR2
22 DRS 45
23 DR17
24 DR130
25 DRS 36
26 DRS 47
27 DRS 652
28 DR 29
L_2 1 Kb Ladder
29 DRD 1157
30 DRS 1155
L_3 100bp

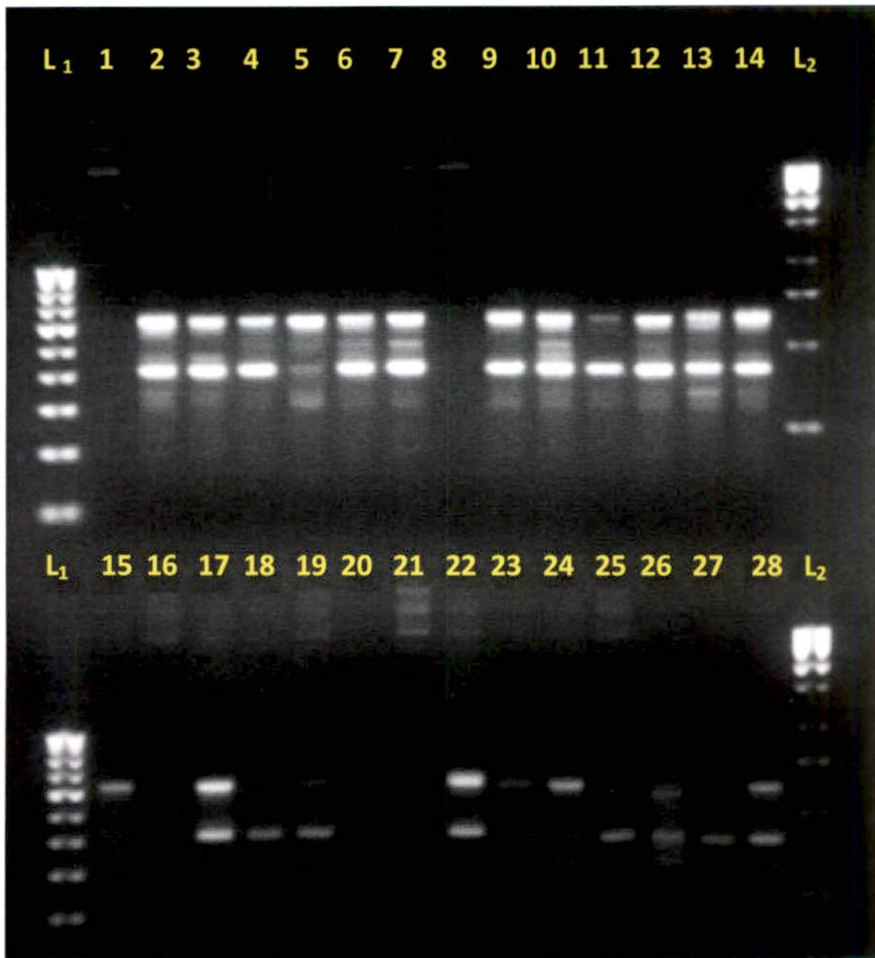


Plate 15 . (A and B) shows agarose gel (2%) profile of the ISSR primer $(GA)_9AT$ for 30 accessions

Lane I

L_1 100bp Ladder
1. DRD 835
2. DRD 1068
3. DRD1835
4. DRD 920
5. DRD 949
6. DRD 1118
7. DRD 1033
8. DRD 495
9. DRD 1110
10. DRD 1157
11. DRD 1060
12. DRD 1142
13.
14. SreeDhanya

L_2 1 Kb Ladder

Lane II

L_1 100bp Ladder
15 SD 15
16 DR VIOLET
17 DR 1150
18 DR 130
19 DR 2
20 DRH 1047
21 DR 73
22 DR 29
23 SREESUBHRA
24 SREEPRIYA
25 DRS 652
26 DRS 45
27 DRH 657
28 DRH 657 A
L_2 1 Kb Ladder

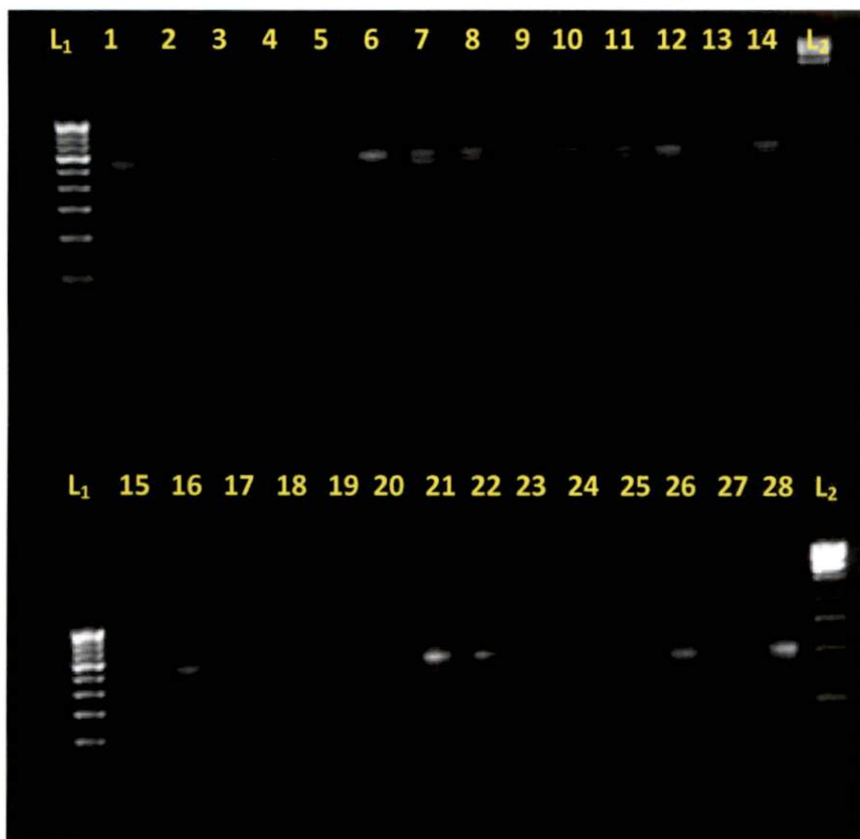


Plate 16. shows agarose gel (2%) profile of the ISSR primer UBC 14 for 30 accessions

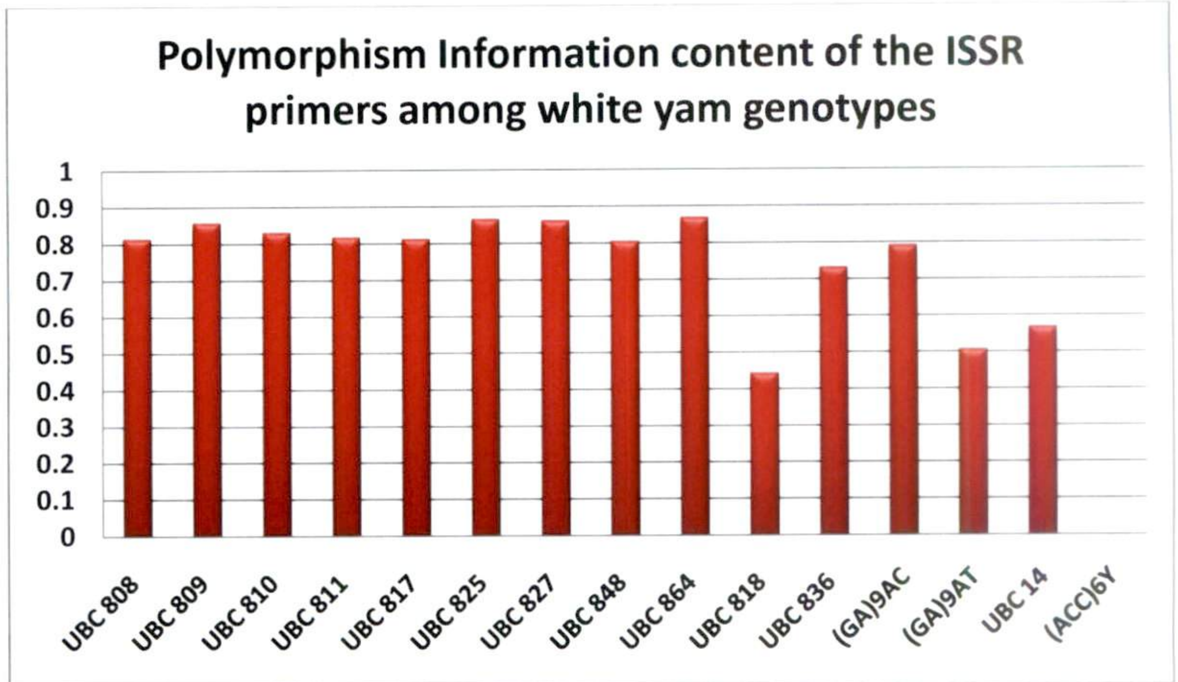
Lane I
L_1 100bp Ladder
1 SreeDhanya
2 DRD 1110
3 DRD 495
4 DRD 1118
5 DRD 1068
6 DRD 1060
7 DRD 1033
8 DRD 920
9 DRD 1142
10 DRD 949
11 DRD 1157
12 DRD 1835
13 DRD 835
14 SD 15
L_2 Kb Ladder

Lane II
L_1 100bp Ladder
15 Sreesubhra
16 SreePriya
17 DRH 657
18 DRH 657 A
19 DRH 1047
20 DR 17
21 DR 29
22 DR 2
23 DR 1150
24 DR VIOLET
25 DRS 45
26 DRS 47
27 DRS36
28 DRS 1155
L_2 1 Kb Ladder

Table 14. Genetic characteristics of the ISSR markers used to analyze the white yam accessions

Primer Code	Size Range (bp)	N_B	N_{PB}	P (%)	Hobs	PIC
UBC 808	732-2031	7	7	100%	0.8376	0.8163
UBC 809	274 - 1799	9	7	77.78%	0.8736	0.8601
UBC 810	158 - 543	8	8	100%	0.8514	0.8335
UBC 811	467 - 1908	7	6	85.71%	0.8403	0.8197
UBC 817	439 - 848	7	7	100%	0.8359	0.8141
UBC 825	408 - 1831	10	10	100%	0.88	0.868
UBC 827	618 - 1463	9	9	100%	0.8771	0.8641
UBC 848	659 - 1048	6	6	100%	0.8309	0.8073
UBC 864	549 - 1778	9	9	100%	0.8837	0.8719
UBC 818	443 - 1056	3	3	100%	0.5448	0.4452
UBC 836	240 - 810	5	5	100%	0.7694	0.733
$(GA)_9AC$	302 - 1165	7	7	100%	0.8199	0.794
$(GA)_9AT$	413 - 674	3	3	100%	0.5954	0.5084
UBC 14	338 - 709	3	3	100%	0.6428	0.5705
$(ACC)_6Y$	573	1	1	100%	0	0

Figure 1. PIC content of the ISSR Primers



4.6 SSR ANALYSIS OF WHITE YAM ACCESSIONS

The amplicons obtained after the SSR analysis of 30 accessions using the selected primers were initially resolved in two per cent agarose gel (Plates 17 to 22). Band obtained in the product size as mentioned in Table15.

Among the ten SSR markers studied, the number of alleles per marker ranged from two to five while the number of polymorphic alleles ranged from one to five. YM15 recorded the maximum number of polymorphic alleles (5) followed by Dab2D06 and YM26 with four alleles. The percentage of polymorphism ranged from 50 (Dab2C05, Dab2E07) to 100 (YM 5,YM 15, YM 26,MT 13 ,MT 10 and Da1A01). The observed heterozygosity values ranged from 0.4339 (Dab2C05) to 0.775 (YM 15). The SSR makers viz. Dab2D06, YM15 and YM 26 recorded high Hobs values (>0.7). The polymorphism information content ranged from 0.3398 (Dab2C05) to 0.7388 (Dab2D06). YM15 also recorded high PIC value of 0.7377. On average the SSR markers recorded Hobs value of 0.5715 and PIC of 0.4866. The study of Hobs and PIC value is given in Fig 2.

Molecular characterisation using SSR primers

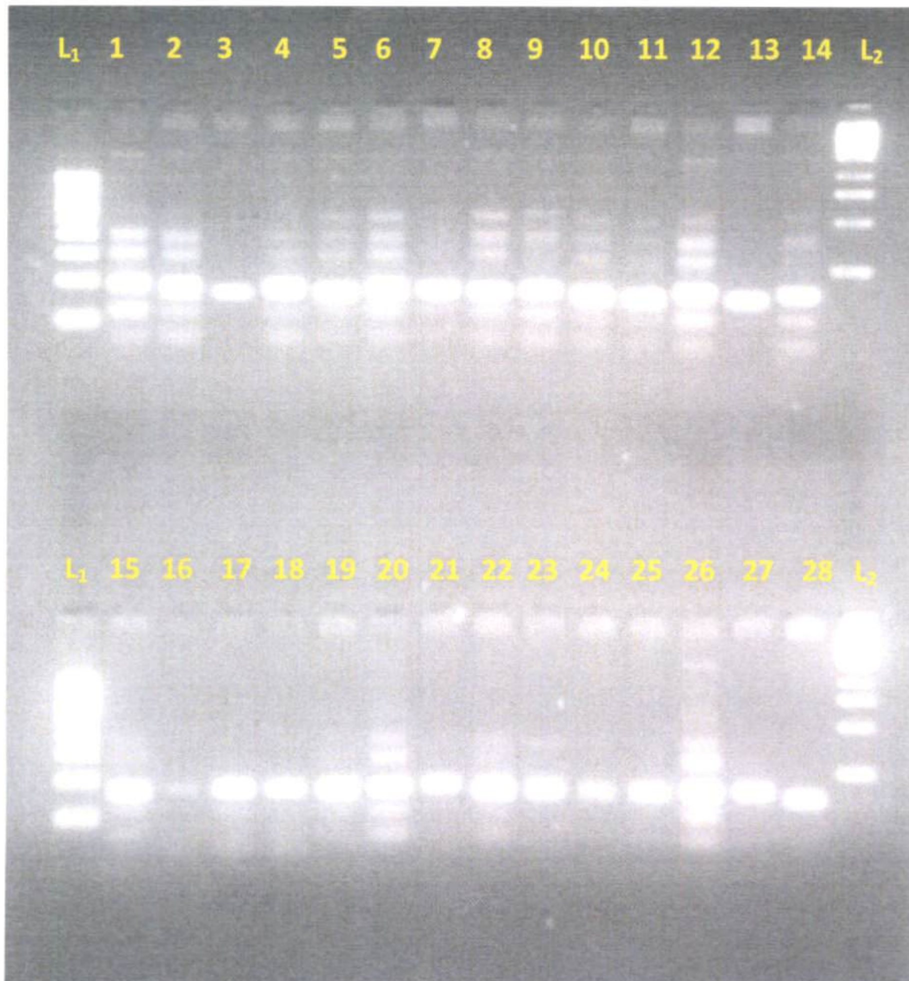


Plate 17. shows agarose gel (2%) profile of the SSR primer Dab2DO6 For 30 accessions

Lane I

L_1	100bp Ladder
1	SREE DHANYA
2	DRD 1835
3	DRD 1068
4	DRD 1060
5	DRD 1033
6	DRD 1060
7	DRD1142
8	DRD920
9	DRD 1118
11	DRD 949
12	DRD 1157
13	DRD495
14	DRD1110
15	SD 15
L_2	1 KB

Lane II

L1	100bp Ladder
15	SreePriya
16	DRH 1047
17	DRH 657 A
18	DRH 657
19	DRS 652
20	DRS 47
21	DRS 45
22	DRS 1155
23	DRS 36
24	DR VIOLET
25	DR 130
26	DR 29
27	DR 2
28	DR 73
L2	1 Kb

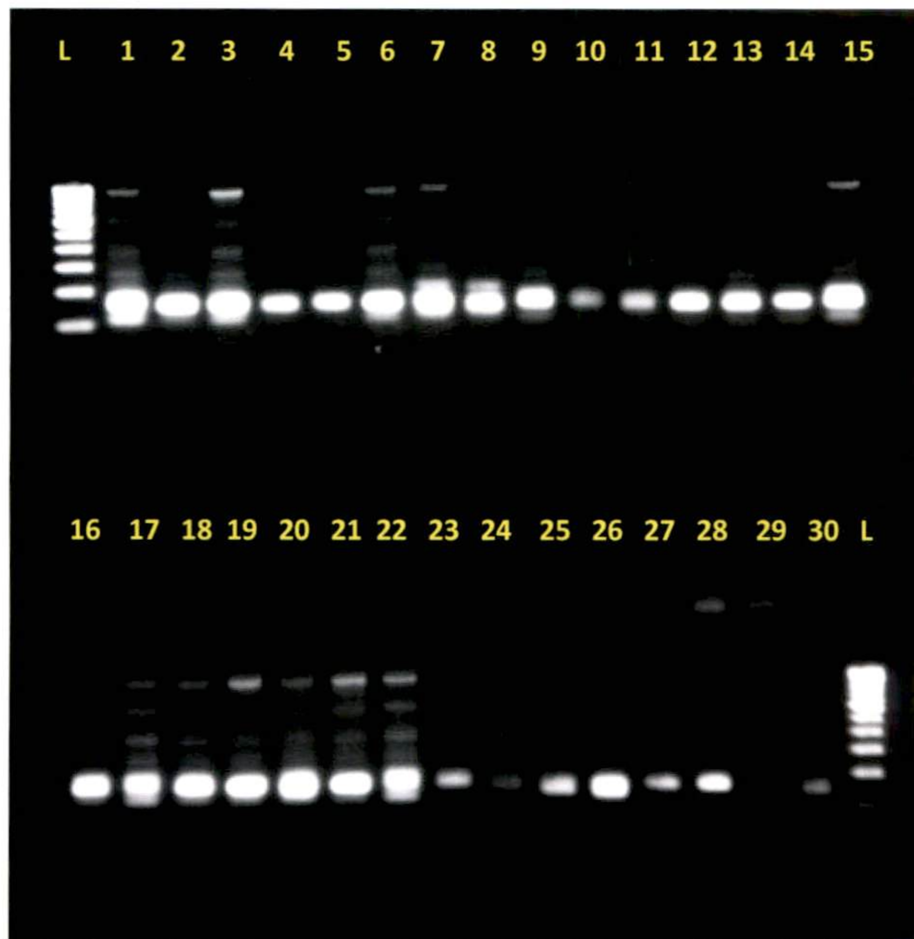


Plate 18. shows agarose gel (2%) profile of the SSR primer Dab2E07 for 30 accessions

Lane I

L_1	100bp Ladder
1	SREE DHANYA
2	DRD 495
3	DRD 1157
4	DRD 1068
5	DRD 835
6	DRD 920
7	DR 29
8	DR 2
9	DR 130
11	DR 17
12	Sreepriya
13	Sreesubhra
14	DRS 1155
15	DRS 36

Lane II

16	DRD 1835
17	DRD 949
18	DRD 1142
19	DRD 1033
20	DRD 1060
21	DRD 1118
22	SD 15
23	DRS 45
24	DRH 1047
25	DRH 657
26	DRH 657 A
27	DRS 652
28	DR 73
29	DR
30	DR VIOLET
L1	100bp

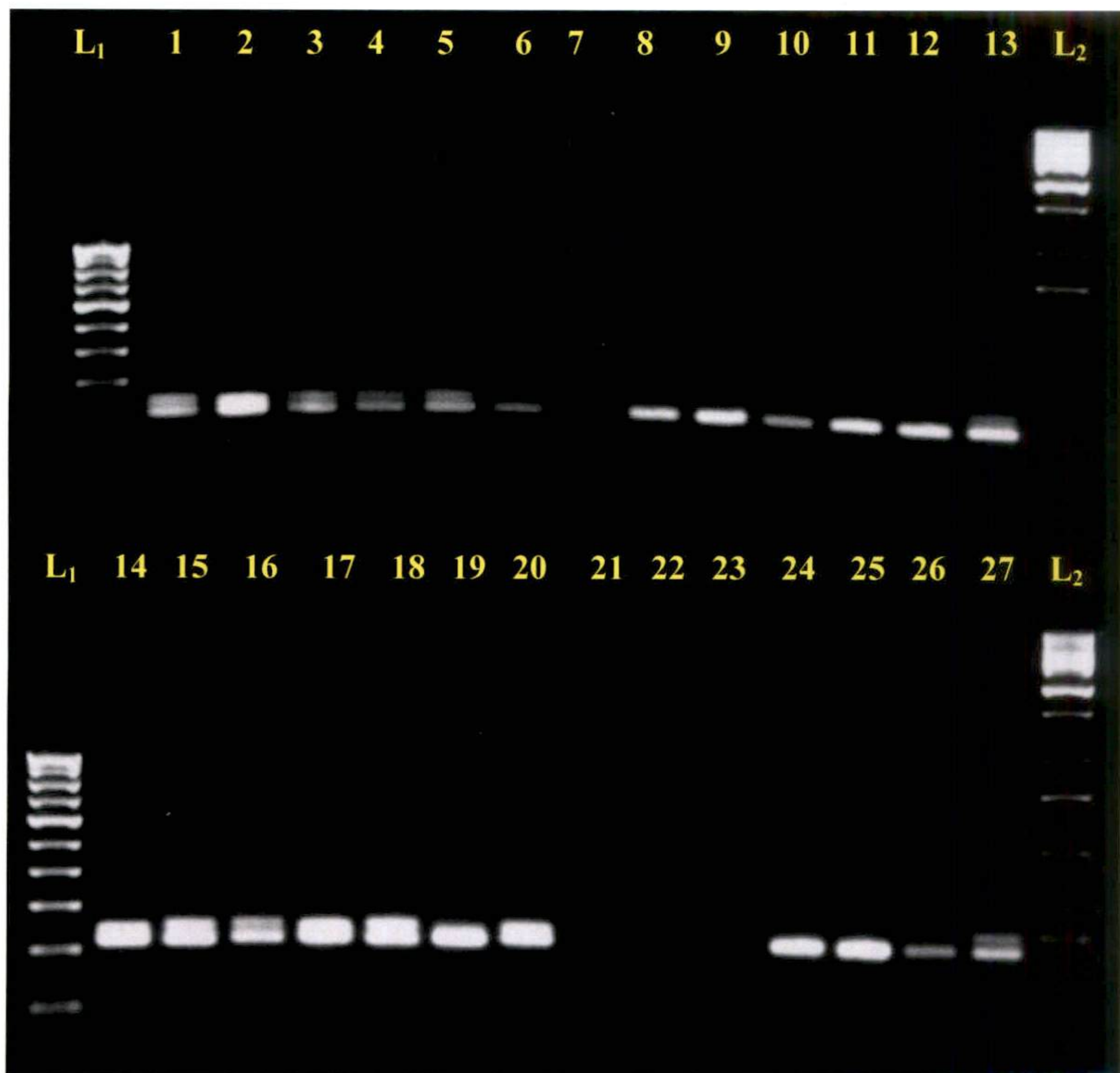


Plate 19. (A and B) shows agarose gel (2%) profile of the SSR primer Da1A01 for 30 accessions

Lane I

L_1	100bp Ladder
1	DRD 1118
2	DRD 1110
3	DRD 495
4	DRD 1068
5	DRD 835
6	DR VIOLET
7	DR 1150
8	DR 130
9	DRS 652
10	DRS 36
11	DR 2
12	DR 29
13	DRS 1155
L_2	1kb Ladder

Lane II

L_1	100bp Ladder
14	SREE DHANYA
15	DRD 920
16	DRD 1033
17	DRD 1835
18	DRD 1157
19	DRD 1060
20	SD 15
21	Sreesubhra
22	Sreepriya
23	DRH 1047
24	DRH 657
25	DRH 657 A
26	DR 17
27	DR 73
L_2	1kb

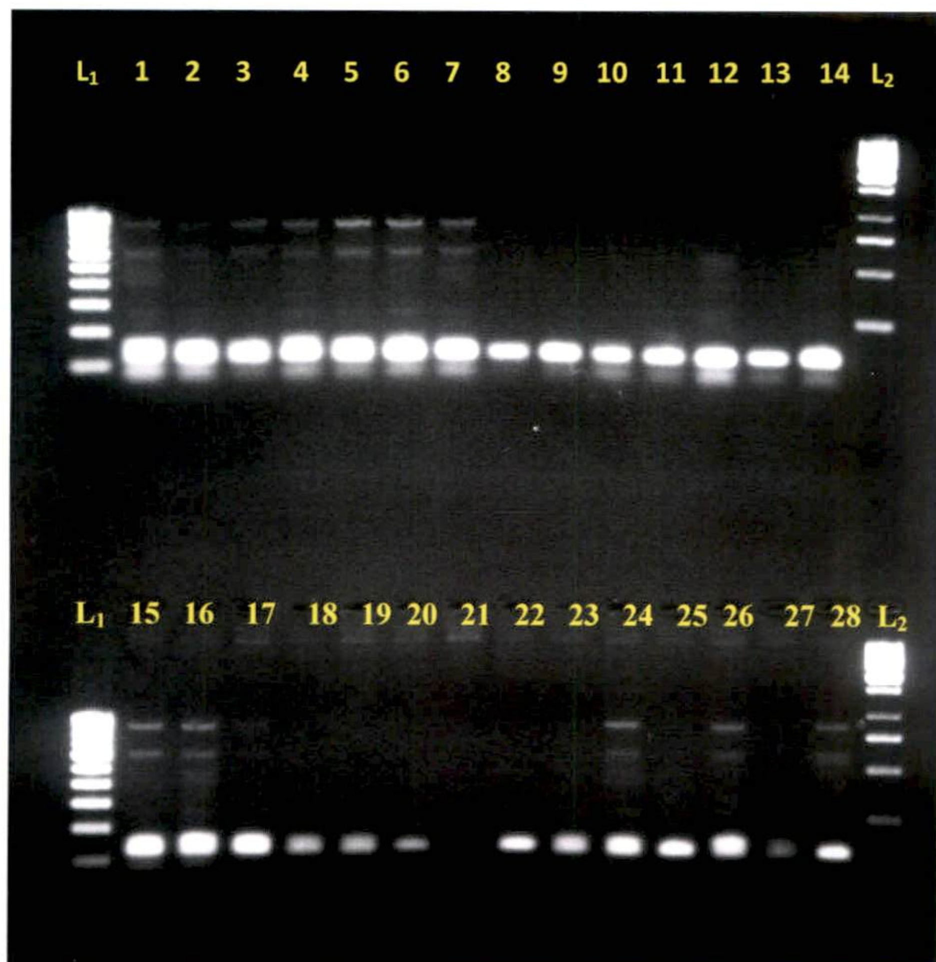


Plate 20. shows agarose gel (2%) profile of the SSR primer Dpr_3F04 for 30 accessions

Lane I	
L_1	100bp Ladder
1	SREE DHANYA
2	DRD 920
3	DRD 1033
4	DRD 1835
5	DRD 1157
6	DRD 1060
7	SD 15
8	Sreesubhra
9	Sreepriya
10	DRH 1047
11	DRH 657
12	DRH 657 A
13	DR 17
14	DRD 949
L_2	1kb Ladder

Lane II	
L_1	100bp Ladder
15	DRD 1118
16	DRD 1110
17	DRD 495
18	DRD 1068
19	DRD 835
20	DR VIOLET
21	DR 1150
22	DR 130
23	DR 2
24	DR 29
25	DRS 652
26	DRS 1155
27	DRS 45
28	DRS 47
L_2	1kb Ladder

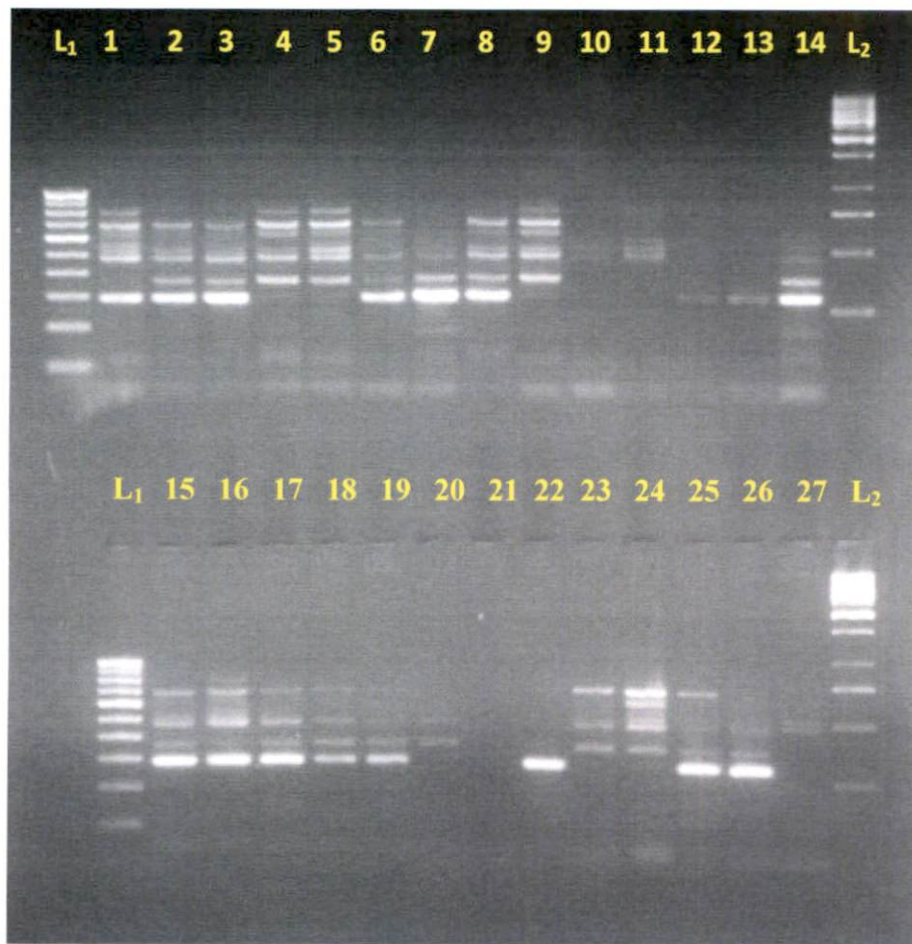


Plate 21.shows agarose gel (2%) profile of the SSR primer YM 15 for 30 accessions

Lane I	
L_1	100bp Ladder
1	SREE DHANYA
2	DRD 920
3	DRD 1033
4	DRD 949
5	DRD 1835
6	DRD 1157
7	DRD 1060
8	DRD 1142
9	SD 15
10	SREESUBHRA
11	SREEPRIYA
12	DRH 1047
13	DRH 657
14	DRH 657 A
L_2	1kb Ladder

Lane II	
L_1	100bp Ladder
15	DRD 1118
16	DRD 1110
17	DRD 495
18	DRD 1068
19	DRD 835
20	DR VIOLET
21	DR 1150
22	DR 130
23	DR 2
24	DR 29
25	DRS 652
26	DRS 1155
27	DRS 45
L_2	1kb Ladder

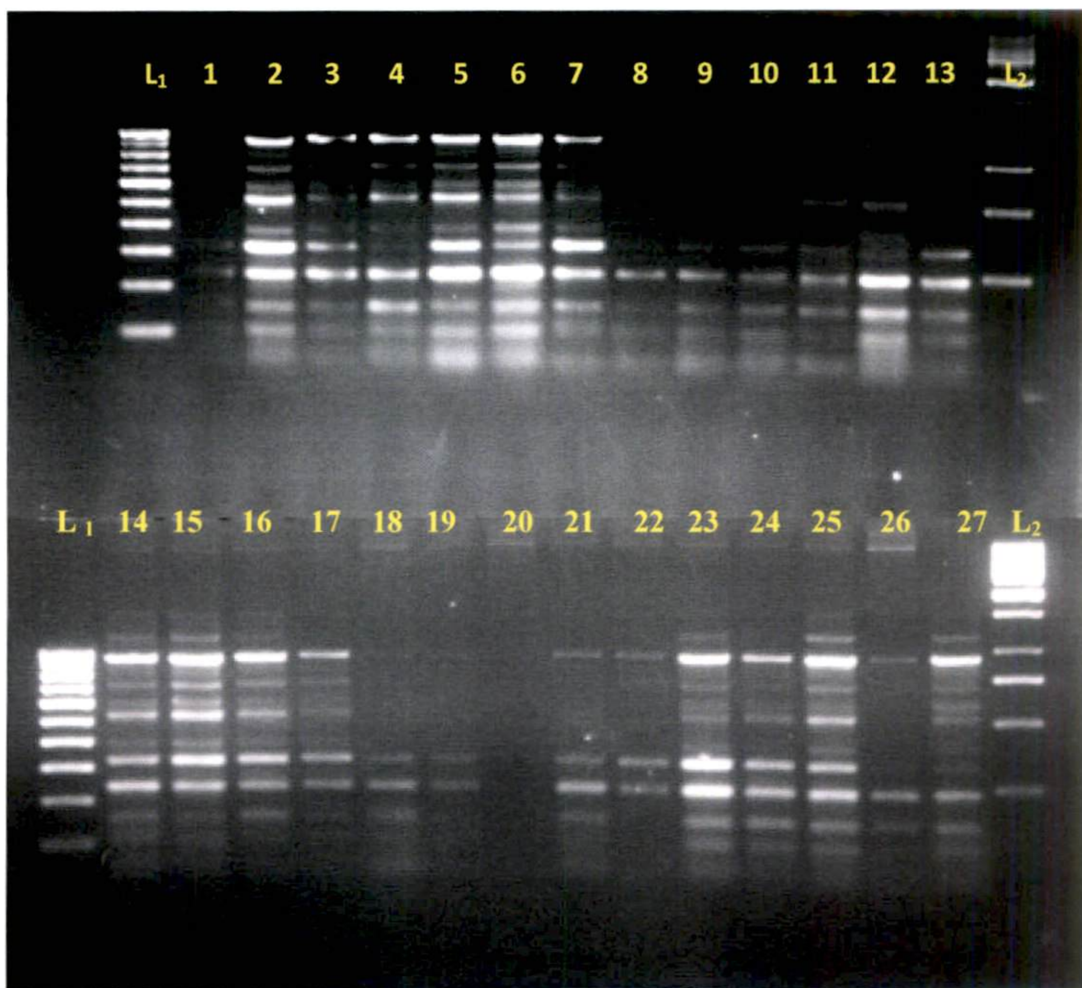


Plate 22 . shows agarose gel (2%) profile of the SSR primer YM 26 for 30 accessions

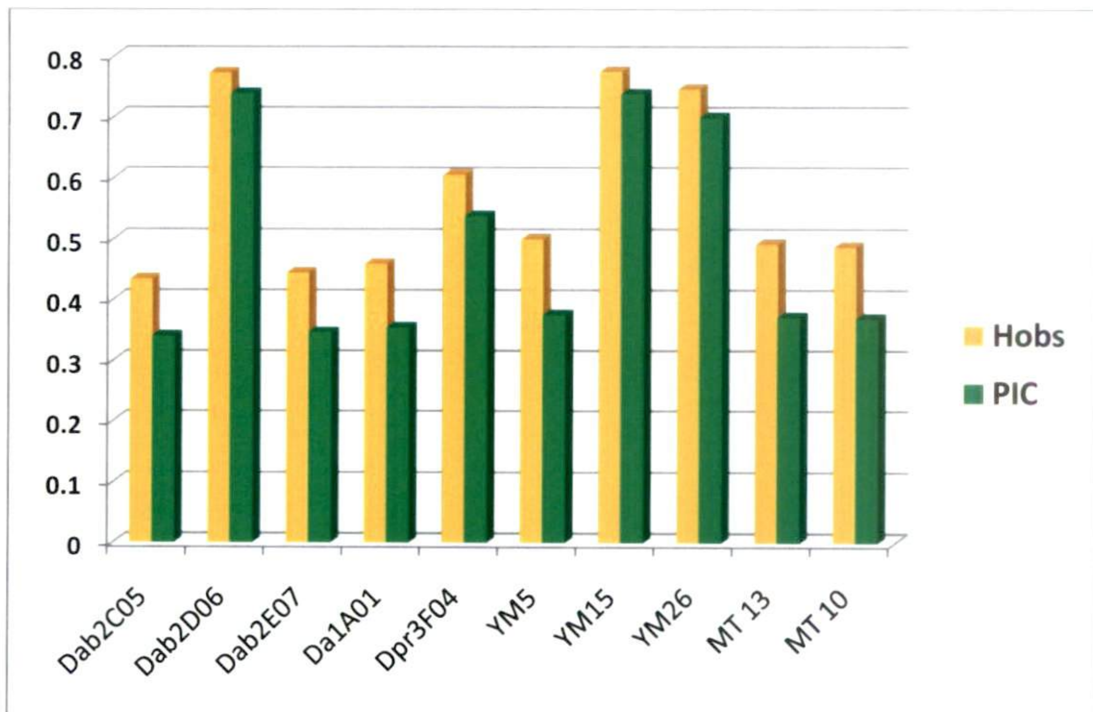
Lane I	
L_1	100bp Ladder
1	SREE DHANYA
2	DRD 920
3	DRD 1033
4	DRD 1835
5	DRD 1157
6	DRD 1060
7	SD 15
8	SREESUBRA
9	SREEPRIYA
10	DRH 1047
11	DRH 657
12	DRH 657 A
13	DR17
L_2	1kb Ladder

Lane II	
L_1	100bp Ladder
14	DRD 1118
15	DRD 1110
16	DRD 495
17	DRD 1068
18	DRD 835
19	DR VIOLET
20	DR 1150
21	DR 130
22	DR 2
23	DR 29
24	DRS 652
25	DRS 1155
26	DRS 45
27	DRS 47
L_2	1kb Ladder

Table 15. Genetic characteristics of the SSR markers used to analyze the white yam accessions

Primer Code	Size Range (bp)	N_B	N_{PB}	P (%)	Hobs	PIC
Dab2C05	217-1478	2	1	50%	0.4339	0.3398
Dab2D06	83-394	5	4	80 %	0.7732	0.7388
Dab2E07	157-948	2	1	50%	0.4444	0.3457
Da1A01	213-248	2	2	100%	0.4592	0.3538
Dpr3F04	141-951	3	2	66.66 %	0.6052	0.5376
YM5	391-586	2	2	100%	0.4998	0.3749
YM15	277-868	5	5	100%	0.775	0.7377
YM26	216-961	4	4	100%	0.7459	0.6986
MT 13	215-673	2	2	100%	0.4918	0.3709
MT 10	108-196	2	2	100%	0.4872	0.3685

Figure 2. The Hobs and PIC values of SSR markers evaluated in white yam



4.7 DENATURING PAGE FOR SSR ANALYSIS FOR SELECTED PRIMERS

For obtaining better resolution of DNA bands for separation of the PCR products, denaturing PAGE was done for SSR primers Dpr3F04 and Dab2C05 that showed specific bands linked to dwarf genotypes during agarose gel electrophoresis. Plates 23 and 24 represents the denaturing PAGE profiles of the amplicons analyzed by silver staining.The number of bands obtained was higher compared to two percent agarose gel analysis. This shows the average number of alleles computed for each primer from the PAGE analyzed data. All the primers used for PAGE analysis showed polymorphism and null alleles were also not detected for any primer.

PAGE analysis of selected SSR Primers

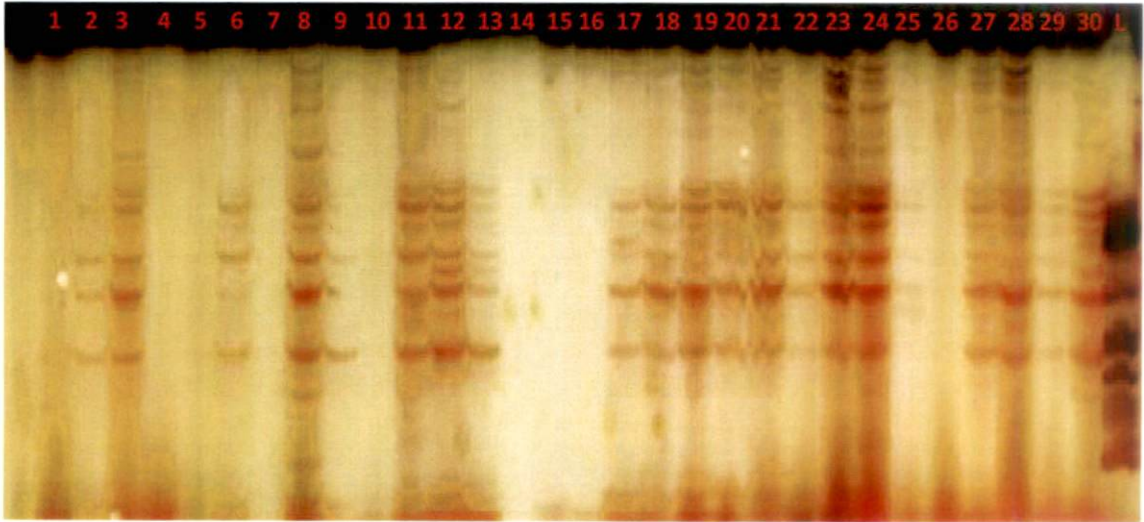
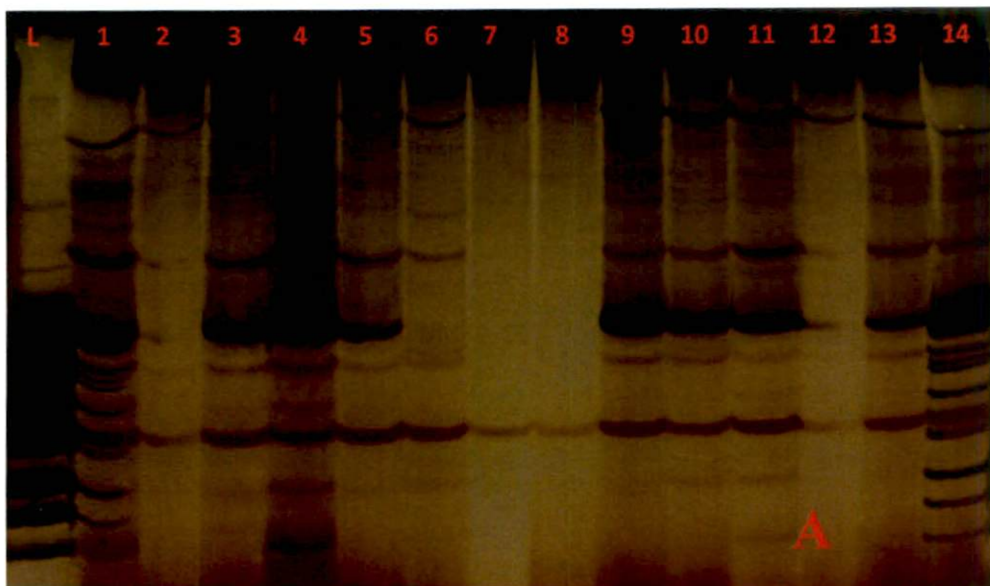


Plate 23. shows PAGE profile of the SSR primer Dpr3F04 for 30 accessions

1. 1.SreeDhanya
2. DRD 1110
3. DRD 495
4. DRD 1118
5. DRD 1835
6. DRD 1060
7. DRD 1033
8. DRD 920
9. DRD 1142
10. DRD 949
11. DRD 1157
12. DRD 1068
13. DRD 835
14. SD 15

15\. DR 2
16\. DR 29
1\. DRS 47
2\. DRS 45
3\. DR 130
4\. DR 17
5\. DR 73
6\. DRS 652
7\. DR VIOLET
8\. DRS 36
9\. DRS 1155
10\. DRH 1047
11\. DRH 657
12\. DRH 657 A
13\. SREEPRIYA
14\. SREESUBHRA
L 100bp



L 100bp
1. SreeDhanya
2. DRD 1110
3. DRD 495
4. DRD 1118
5. DRD 1835
6. DRD 1060
7. DRD 1033
8. DRD 920
9. DRD 1142
10. DRD 949
11. DRD 1157
12. DRD 1068
13. DRD 835
14 . SD 15

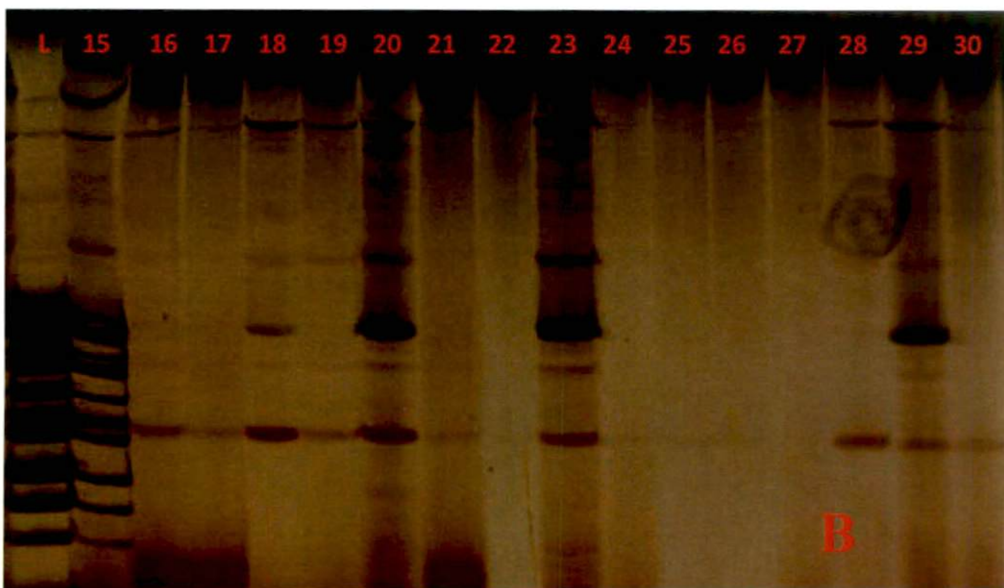


Plate 24. (A and B) shows PAGE profile of the SSR primer Dab2C05 for 30 accessions

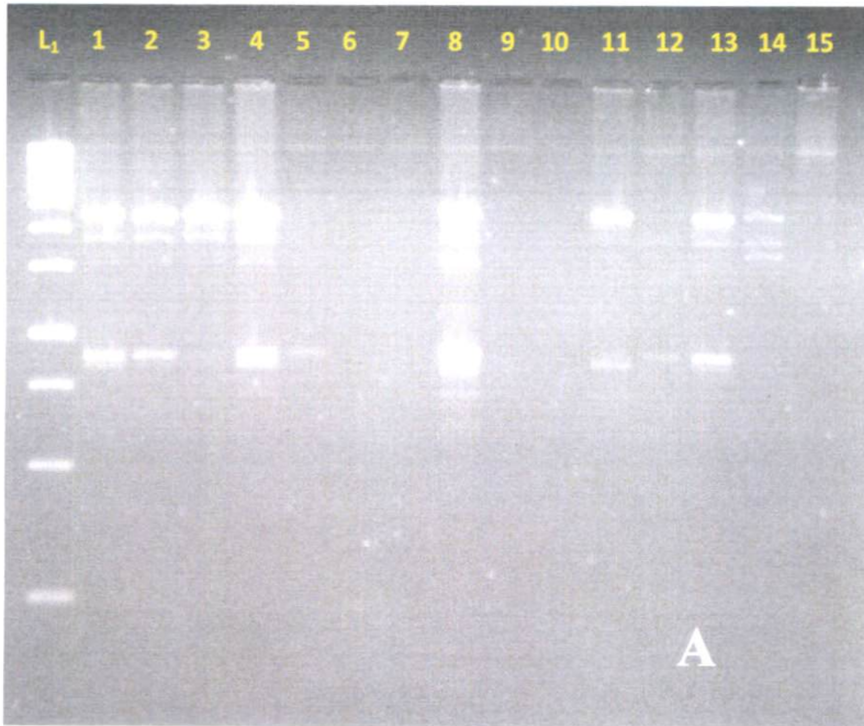
15. DR 2
16. DR 29
17. DRS 47
18. DRS 45
19. DR 130
20. DR 17
21. DR 73
22. DRS 652
23. DR VIOLET
24. DRS 36
25. DRS 1155
26. DRH 1047
27. DRH 657
28. DRH 657 A
29. SREEPRIYA
30. SREESUBHRA
L 100bp

4.8 RAPD ANALYSIS OF WHITE YAM ACCESSIONS

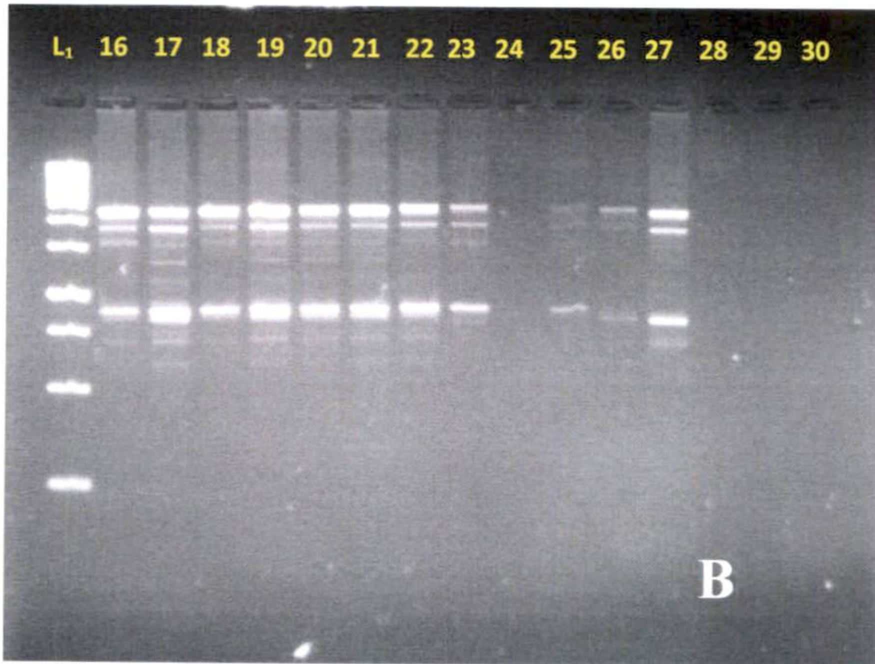
The amplicons obtained after the RAPD analysis of 30 accessions using the selected primers were initially resolved in two per cent agarose gel (Plates 25, 26 and 27). Band obtained in the product size as mentioned in Table 16.

Among the eight RAPD primers studied in white yam, OPW -16 was found be the best that produced ten polymorphic bands followed by OPG-02 and OPG-13 with six polymorphic bands. All the primers evaluated resulted in 100% polymorphism. The Hobs values ranged between 0.7377 (OPW1) to 0.8937 (OPW-16). OPG-13 and OPG-16 recorded high observed heterozygosity (>0.8). The polymorphism information content ranged from 0.6997 (OPW-1) to 0.8838 (OPW-16). All the primers recorded high PIC value of >0.6. The genetic characteristics of the RAPD primers evaluated is given in Fig 3.

Molecular characterisation using RAPD primers

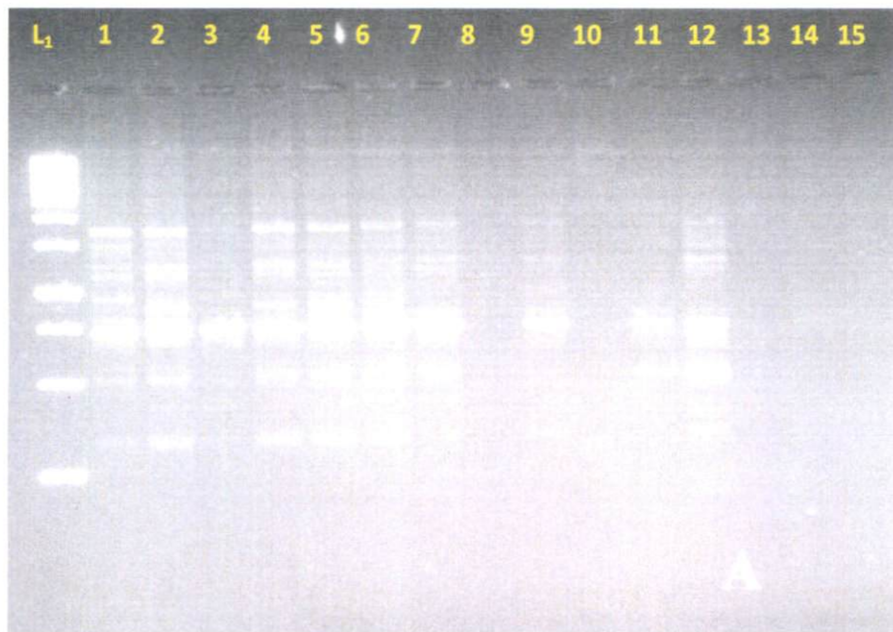


L_1	1Kbp
1	DRD 920
2	DRD 949
3	DRD 1835
4	DRD 1060
5	DRD 835
6	DRD 1068
7	DR 130
8	DRS 1155
9	DRS
10	DR 17
11	DRS 47
12	DR 73
13	DR D 495
14	DR 2
15	DRS 29

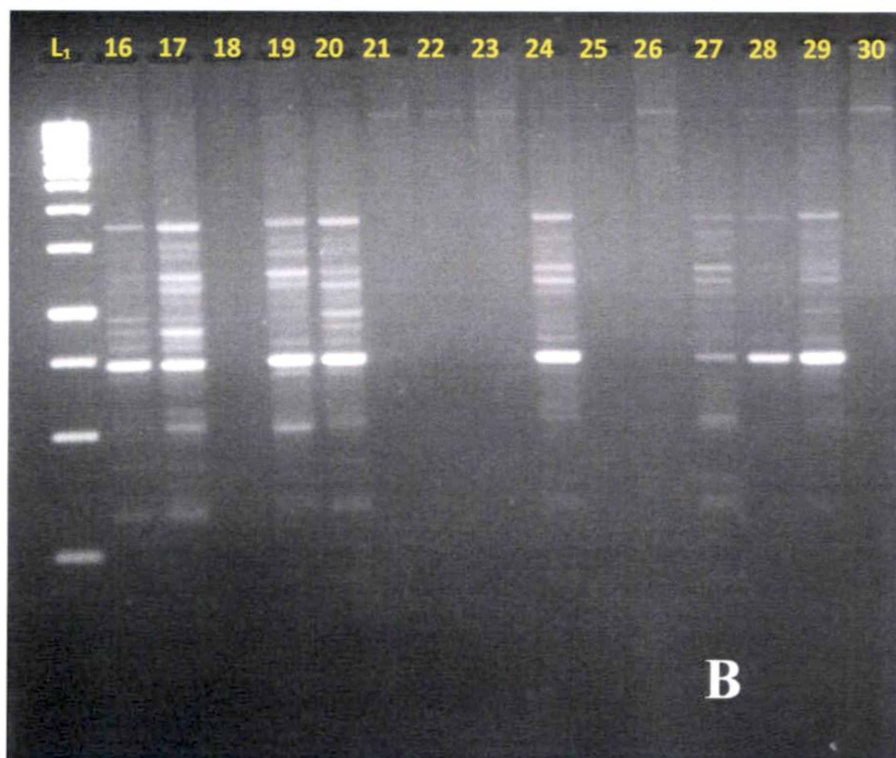


L_1	1Kbp Ladder
16	SREE DHANYA
17	DRD 1157
18	DRD 1110
19	DRD 1033
20	DRD 1118
21	DRD 1142
22	SD 15
23	SP
24	SS
25	DRH 1047
26	DRH 657
27	DRH 657 A
28	DRS 652
29	DR VIO
30	DRS 36

Plate 25. (A and B) shows agarose gel (2%) profile of the RAPD primer OPG-08 for 30 accessions

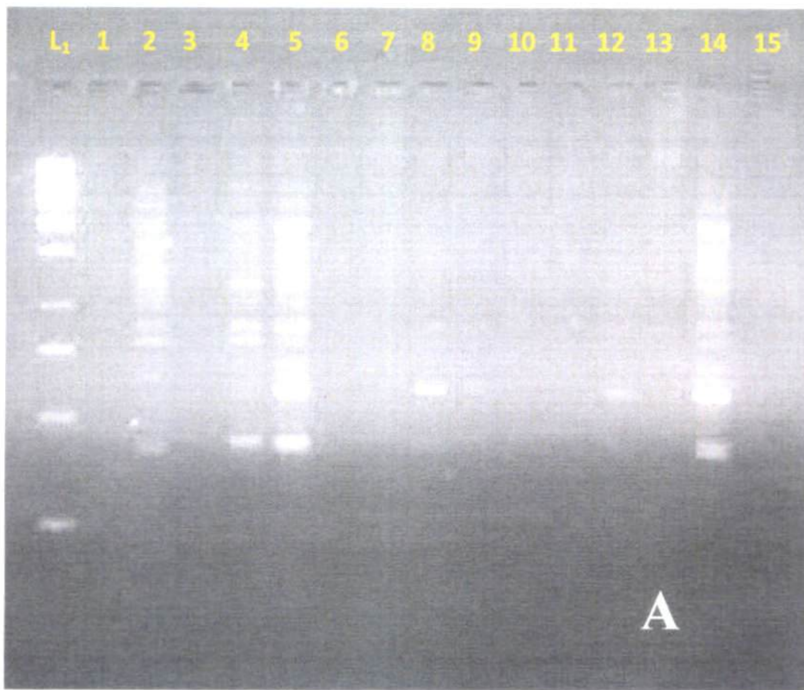


L1	1Kbp
1	SREE DHANYA
2	DRD 1033
3	DRD 1110
4	DRD 1142
5	DRD 920
6	DRD 1118
7	SD 15
8	SS
9	SP
10	DRH 1047
11	DRH 657
12	DRH 657 A
13	DR VIO
14	DR 130
15	DRS 45

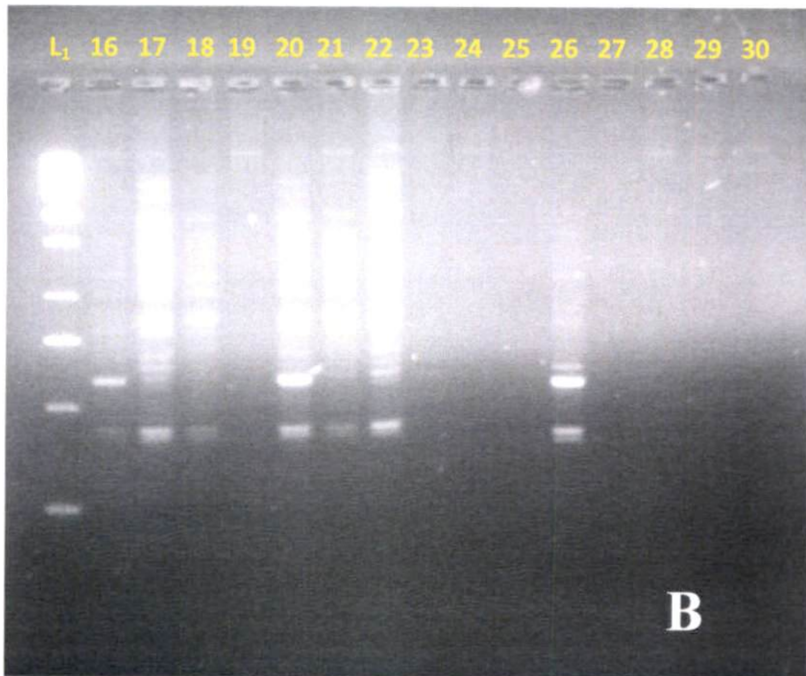


L1	1 Kbp
16	DRD 495
17	DRD 1157
18	DRD 949
19	DRD 1060
20	DRD 1835
21	DRD 835
22	DRD 1068
23	DRS 652
24	DRS 47
25	DRS 36
26	DR 73
27	DR 17
28	DR 2
29	DRS 1155
30	DRS 29

Plate 26. (A and B) shows agarose gel (2%) profile of the RAPD primer OPG-05 for 30 accessions



L_1 Kbp Ladder
1. DRD 1060
2. DRD 1157
3. DRD 1142
4. DRD 949
5. DRD 1835
6. DR 835
7. DR 2
8. DR 73
9. DRS 652
10. DRS 36
11. DR 29
12. DRS 45
13. DRS 1155
14.
15.



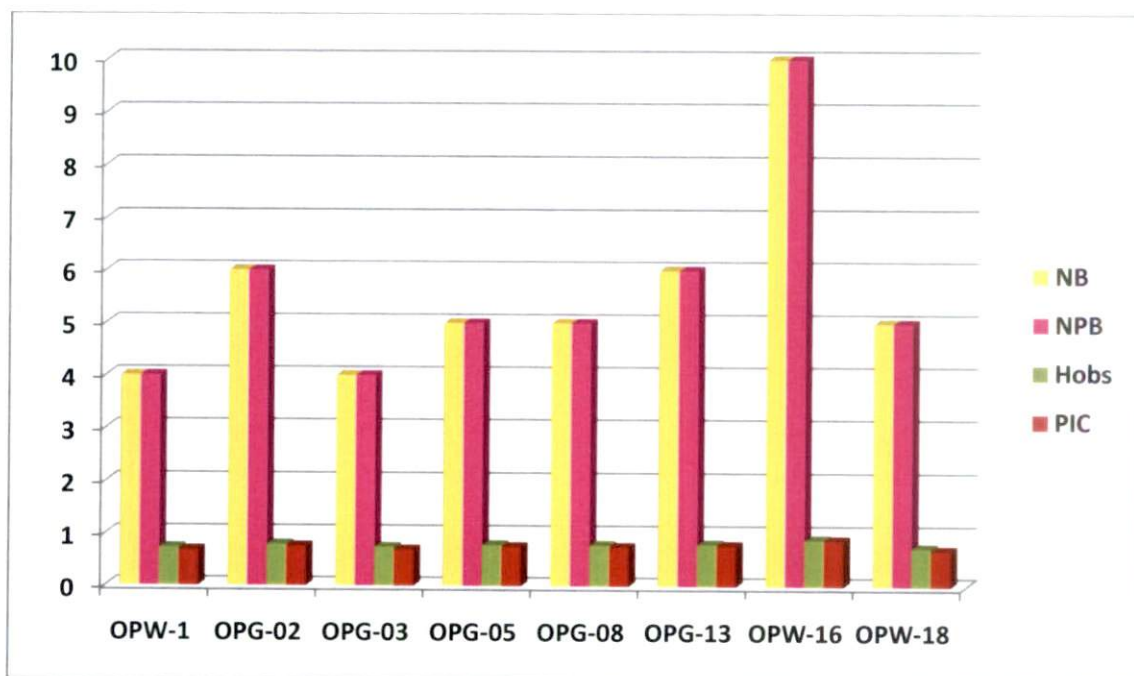
L_1 1 Kbp Ladder
16 SREE DHANYA
17 DRD 1033
18 DRD 920
19 DRD 1118
20 DRD 1110
21 DRD 495
22 SD 15
23 SS
24 SP
25 DRH 657
26 DRH 657 A
27 DR 17
28 DR VIO
29 DR 130
30 DRS 47

Plate 27. (A and B) shows agarose gel (2%) profile of the RAPD primer OPW-16 for 30 accessions

Table 16. Genetic characteristics of the RAPD markers used to analyze the white yam accessions

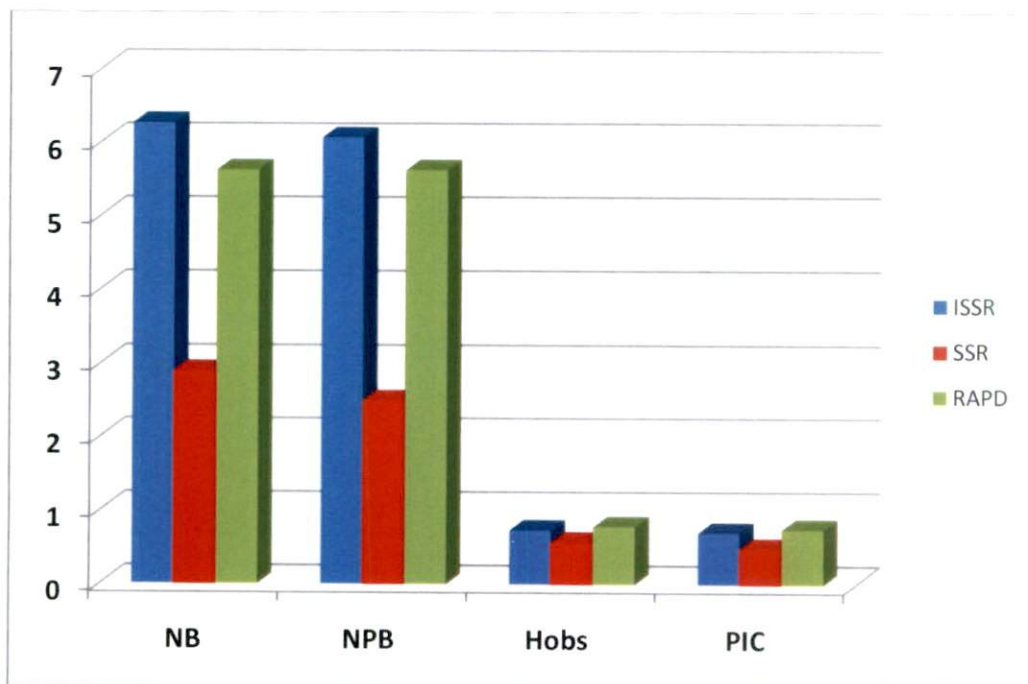
Primer Code	Size Range (Kbp)	N_B	N_{PB}	P (%)	Hobs	PIC
OPW-1	1.7 – 4.5	4	4	100%	0.7377	0.6894
OPG-02	1.9 -5.8	6	6	100%	0.7988	0.7676
OPG-03	0.6 - 2.5	4	4	100%	0.7469	0.6997
OPG-05	2.0 - 5.8	5	5	100%	0.7899	0.7562
OPG-08	2.3 - 6.6	5	5	100%	0.7876	0.7533
OPG-13	2.2 - 7.4	6	6	100%	0.8078	0.7789
OPW-16	1.6 - 6.1	10	10	100%	0.8937	0.8838
OPW-18	1.8 – 7.3	5	5	100%	0.738	0.6939

Figure 3. Genetic characteristics of RAPD primers evaluated in white yam



The comparison of the different type of primers (ISSR, SSR, RAPD) studied in white yam is depicted in Figure 4. It shows that even though the number of polymorphic bands are higher for ISSR and RAPD, the average polymorphic information content was almost same for the different primers evaluated.

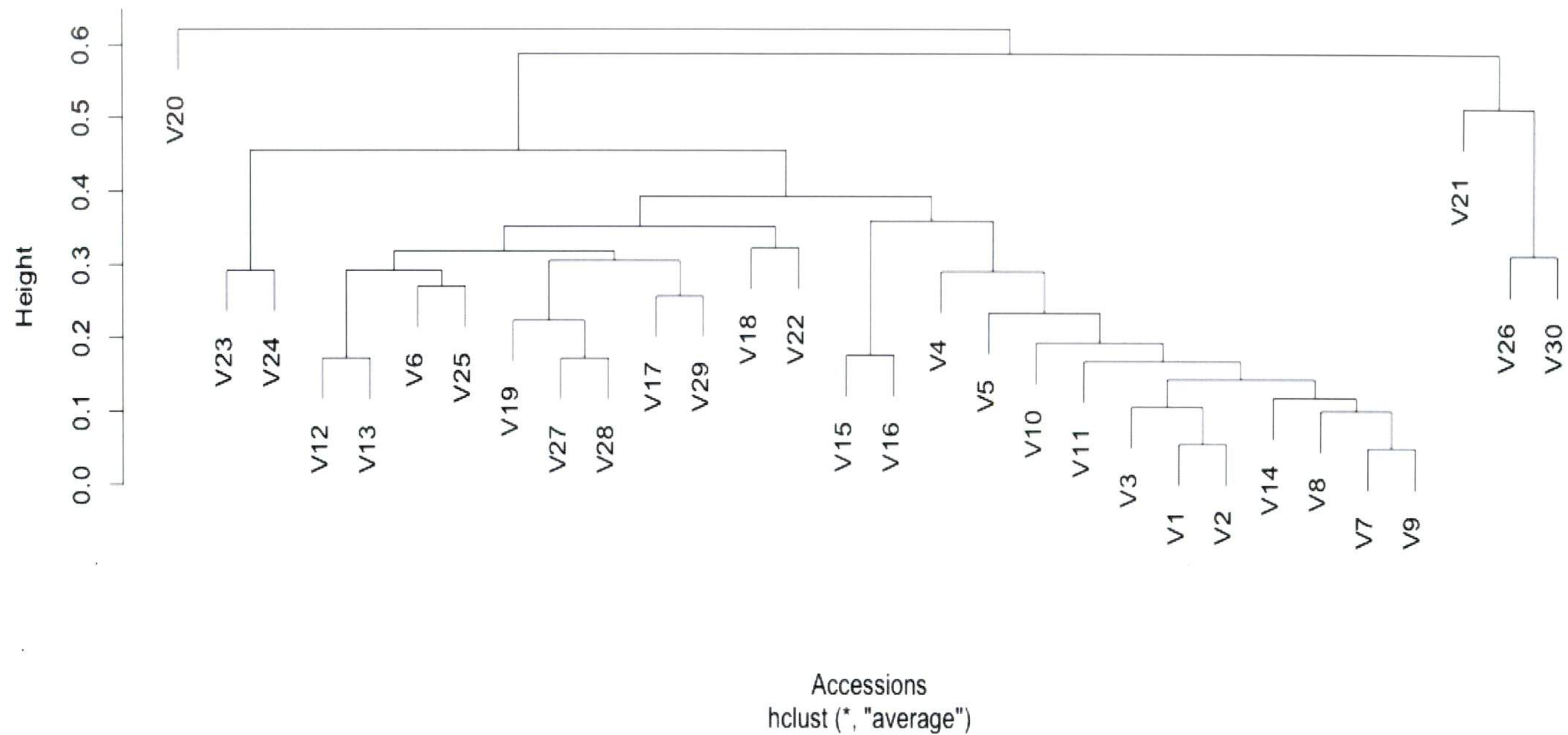
Figure 4. Comparison between ISSR, SSR and RAPD markers used for analysis



4.9 **Cluster Analysis**

Cluster analysis was done based on Euclidean distance for 30 accessions for ISSR, SSR and RAPD. Dendrogram shows the partition of most of the dwarf and tall genotypes in to two different clusters. Semi dwarf comes under the cluster of dwarf genotype on analysis.

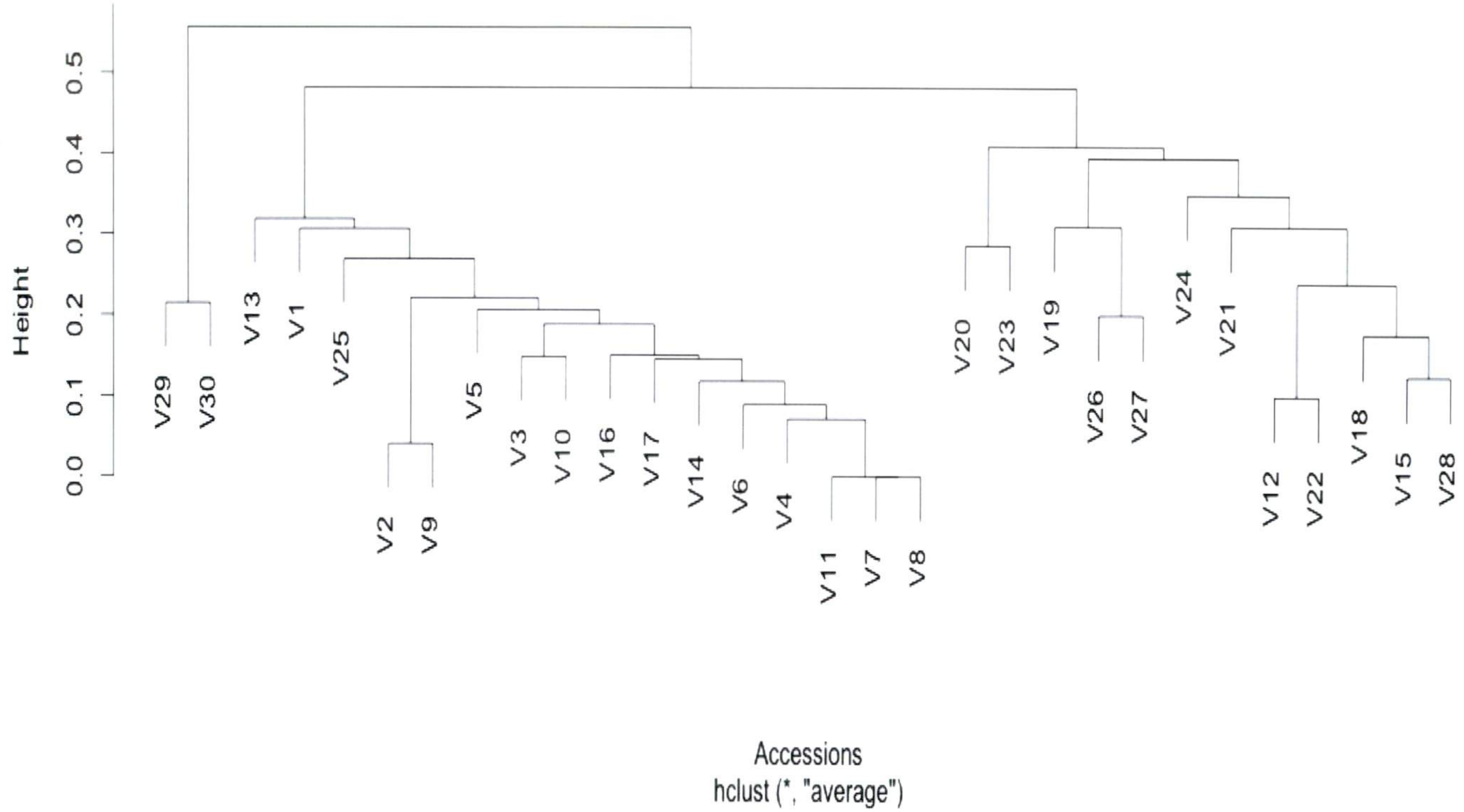
Based on ISSR data, the genotypes formed four clusters at 0.4 dissimilarity index. Cluster 1 has only one genotype i.e.V20 (DR17) and was found to be highly divergent than all other genotypes, formed as an outlier. The cluster 2 has two genotypes V23 & V24. Cluster 3 is the biggest one with twenty four genotypes and they were grouped in to two sub clusters 3a &3b. The cluster 4 has three genotypes and was sub clustered in to two groups. The clustering pattern based on ISSR markers is given in fig 5.



Cluster dendrogram based on ISSR
Height
0.0
0.1
0.2
0.3
0.4
0.5
0.6
V20
V23
V24
V12
V13
V6
V25
V19
V27
V28
V17
V29
V18
V22
V15
V16
V4
V5
V10
V11
V3
V1
V2
V14
V8
V7
V9
V21
V26
V30
Accessions
hclust (*, "average")

In the present study, the SSR markers gave unique results. Based on SSR markers The white yam genotypes were grouped in to three clusters. The cluster 1 with two genotypes (V29 and V30) is the smallest one. Cluster 2 comprise of 16 accessions of which V2 and V9 were found to be closely related. The dwarf genotypes (12) clustered together in Cluster 2 and tall genotypes (11) clustered together in cluster 3.

The clustering of the white yam genotypes based on Jaccard and Dice coefficients are given in Fig 6 and 7. The semi dwarf genotype SD15 was grouped together with other dwarf genotypes.

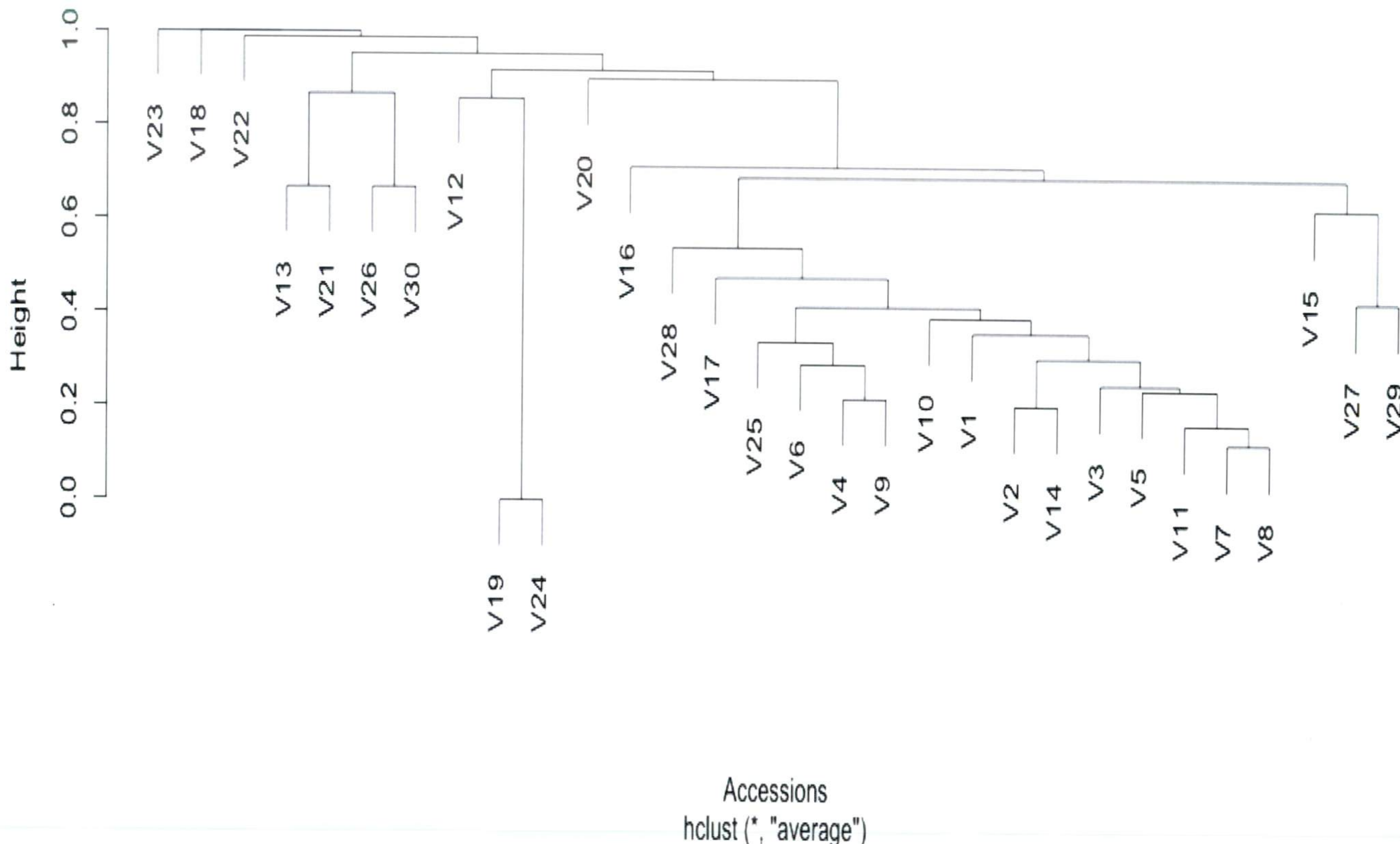


Cluster dendrogram based on SSR
Height
0.0
0.1
0.2
0.3
0.4
0.5
V29
V30
V13
V1
V25
V2
V9
V5
V3
V10
V16
V17
V14
V6
V4
V11
V7
V8
V20
V23
V19
V26
V27
V24
V21
V12
V22
V18
V15
V28
Accessions
hclust (*, "average")

DRD 1157
DRD 920
DRD 1033
DRD 1060
DRD 1118
SD 15
DRS 47
DR 29
DRD 949
DRD 495
DRD 1835
DRD 1142
DRD 1110
DRS 1155
SREE DHANYA
DRD 835
DRH 657 A
DR 2
DRS 45
DRS 652
DRD 1068
DR 73
DRS 36
DRH 657
DRH 1047
DR 130
DR VIOLET
DR 17
SREE SUBHRA
SREE PRIYA

CLUSTER DENDROGAM BASED ON SSR

More clusters were resulted in dendrogram formed based on RAPD markers as in Figure 8. At 0.8 dissimilarity level, it formed nine clusters. Cluster 1, 2 and 3 has only one accession *viz.* V23, V18 and V22. The fourth cluster has four genotypes and was again subdivided in to two sub clusters. The fifth cluster has three genotypes and also subdivided into two sub clusters 5a and 5b. The two genotypes included in 5b i.e. V19 and V24 were found to be similar. Sixth cluster has one genotype V20. The seventh cluster is the largest one with 16 genotypes which consist of mostly dwarf genotypes (11) and is divided into several sub clusters. The eighth cluster has one genotype (V15) and ninth cluster has two genotypes (V27 and V29). The (Figure 9.) depicts the results of the combined molecular data. It also shows the high divergence of the genotype V20 (DR17) and the clustering pattern indicates the grouping of the dwarf genotypes together.


Cluster dendrogram based on RAPD
Height
1.0
0.8
0.6
0.4
0.2
0.0
V23
V18
V22
V13
V21
V26
V30
V12
V19
V24
V20
V16
V28
V17
V25
V6
V4
V9
V10
V1
V2
V14
V3
V5
V11
V7
V8
V15
V27
V29
Accessions
hclust (*, "average")

DR 17

DR VIOLET

DRH 1047
DR 73
SREE SUBHRA

DRS 36
DRS 45
DRS 652
DR 130
DRD 1068
DRD 835
SREE PRIYA
DRS 47

DRD 1457
DRD 1118
SD 15
SREE DHANYA
DRD 949

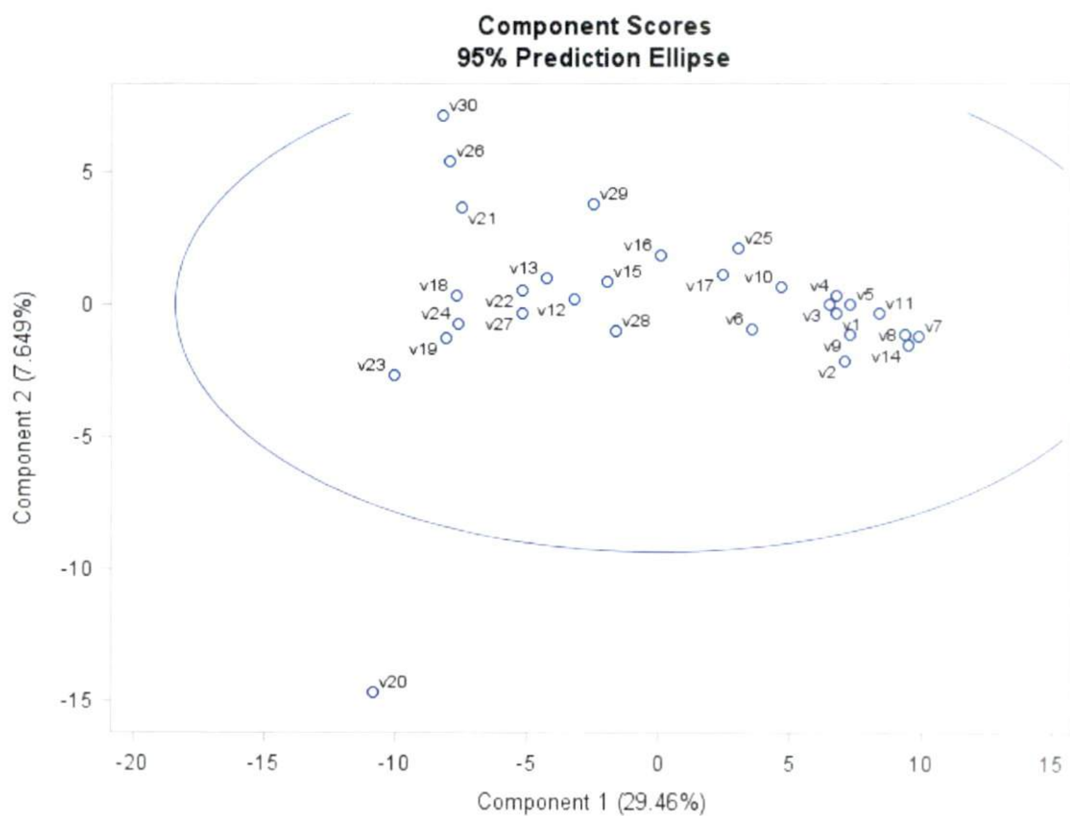
Clustering of white yam genotypes based on Dice coefficient

4.10 **Correlation of Molecular Data**

Mantel's test was done to assess correlation between the markers used. The analysis established that there is high correlation existing between these data. The mantel's test revealed significant correlation between the results of different type of markers used in white yam.

4.11 **Principal Component analysis**

Principal coordinate analysis (PCA) revealed that the first, second and third principal coordinate axes account for 47.4, 12.3 and 9.8 % of the total variation respectively. The Eigen values of the Correlation Matrix is given in Table 17 .A plot based on combined molecular data showing the first and second axes (Figure 10.) also recorded grouping of the dwarf varieties together. The 3D plot based on first, second and third principal coordinates clearly revealed V20 (DR 17 as an outlier which is highly divergent from all other genotypes) in (Figure 11).


Component Scores
95% Prediction Ellipse
Component 2 (7.649%)
Component 1 (29.46%)
v30
v26
v21
v29
v16
v25
v13
v15
v17
v10
v4
v5
v11
v18
v22
v12
v3
v24
v27
v28
v6
v1
v8
v7
v19
v9
v14
v23
v2
v20
5
0
-5
-10
-15
-20
-15
-10
-5
0
5
10
15

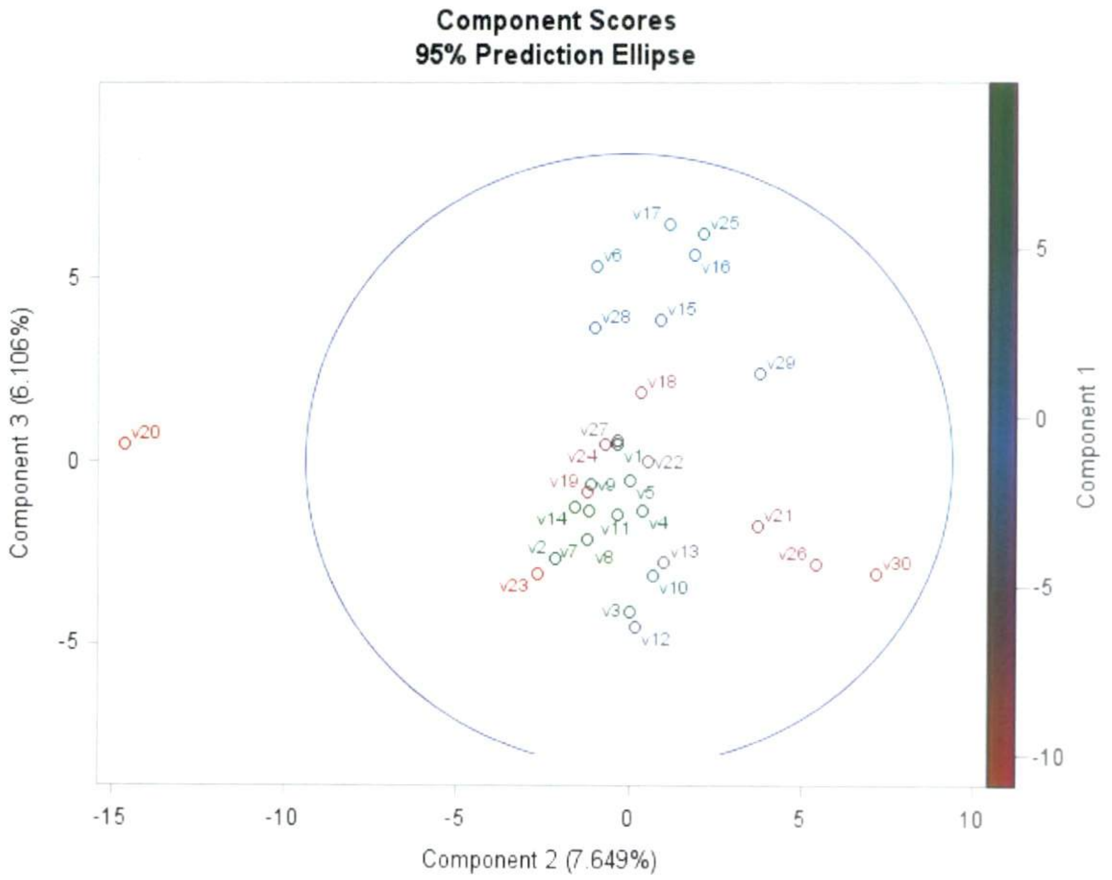


Figure 10. 3D plot based on first, second and third principal coordinates depicting variability among white yam genotypes

Table 17. **Eigen values of the Correlation Matrix**

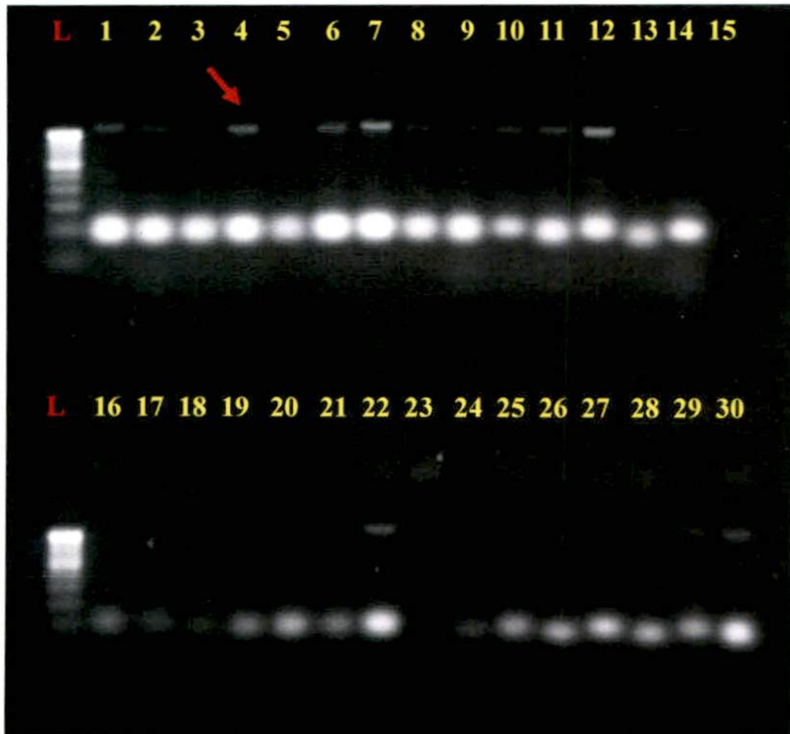
	Eigen value	Difference	Proportion	Cumulative
1	47.4246336	35.1089444	0.2946	0.2946
2	12.3156892	2.4856484	0.0765	0.3711
3	9.8300408	1.1269296	0.0611	0.4321
4	8.7031113	0.1124362	0.0541	0.4862
5	8.5906750	1.4215660	0.0534	0.5395
6	7.1691090	0.9961998	0.0445	0.5841
7	6.1729093	0.6197617	0.0383	0.6224
8	5.5531476	0.6871255	0.0345	0.6569
9	4.8660221	0.3093088	0.0302	0.6871
10	4.5567133	0.2818794	0.0283	0.7154
11	4.2748339	0.2379827	0.0266	0.7420
12	4.0368512	0.3035762	0.0251	0.7670
13	3.7332750	0.1606611	0.0232	0.7902
14	3.5726139	0.0384463	0.0222	0.8124
15	3.5341677	0.4790187	0.0220	0.8344
16	3.0551490	0.2090204	0.0190	0.8533
17	2.8461285	0.0185241	0.0177	0.8710
18	2.8276044	0.2932203	0.0176	0.8886
19	2.5343842	0.1861562	0.0157	0.9043
20	2.3482279	0.0726378	0.0146	0.9189
21	2.2755901	0.4231800	0.0141	0.9330
22	1.8524102	0.2197118	0.0115	0.9446
23	1.6326983	0.0487291	0.0101	0.9547
24	1.5839692	0.0588961	0.0098	0.9645
25	1.5250732	0.2215919	0.0095	0.9740

4.12 **Identification of Molecular markers**

The results indicate the association of markers with dwarf gene shown by 3 SSR primers Dab2C05, Dpr3F04 and Dab2E07.This primers shows specific bands for most of the dwarf varieties and therefore it can be used as effective markers for identifying dwarf gene in *Dioscorea rotundata* Poir. SSR marker finds ideal for analysis because of easy evalution of variability due to limited number of bands.ISSR primer UBC 836 found as a better marker among ISSR primers used, it shows specific band for dwarf genotypes.So it can be also used for identifying dwarf gene.

4.12.1 Sequence Data

For the SSR primer Dab2C05, showing band of 1478bp as shown in plate 28 specific for dwarf genotypes was eluted using QIAquick Gel Extraction kit. Eluted DNA then checked on 0.8% agarose gel and showed very less concentration with faint bands as in plate 29. The sequence got from the eluted DNA showed electropherogram with mixed trace as in figure 12. However, sequence obtained were used for similarity search in BLASTn and got 90% similarity with *Cocos nucifera* cultivar Chowghat Green Dwarf glycosyl hydrolases family 31 protein mRNA as in figure 13 (A and B).



L-100bp ladder;1-V1;2-V2;3-V3;4-V4;6-V12;7-V6;8-V7;9-V8;10-V9;11-V10;12-V11;13-V5;14-V13;15-V14;16-V29; 17-V30;18-V26;19-V27;20-V28;21-V20;22-V16;23-;24-V23;25-V15;26-V18;27-V22;28-V24;29-1-V17;30-V25

Plate 28. SSR Primer "Dab2C05" is screened for all the samples and on analysis, most of the dwarf varieties shows a particular band of size 1.5Kbp

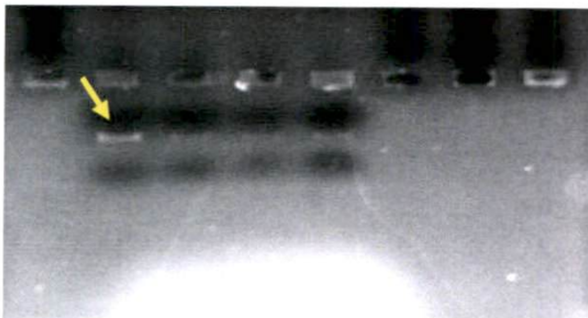
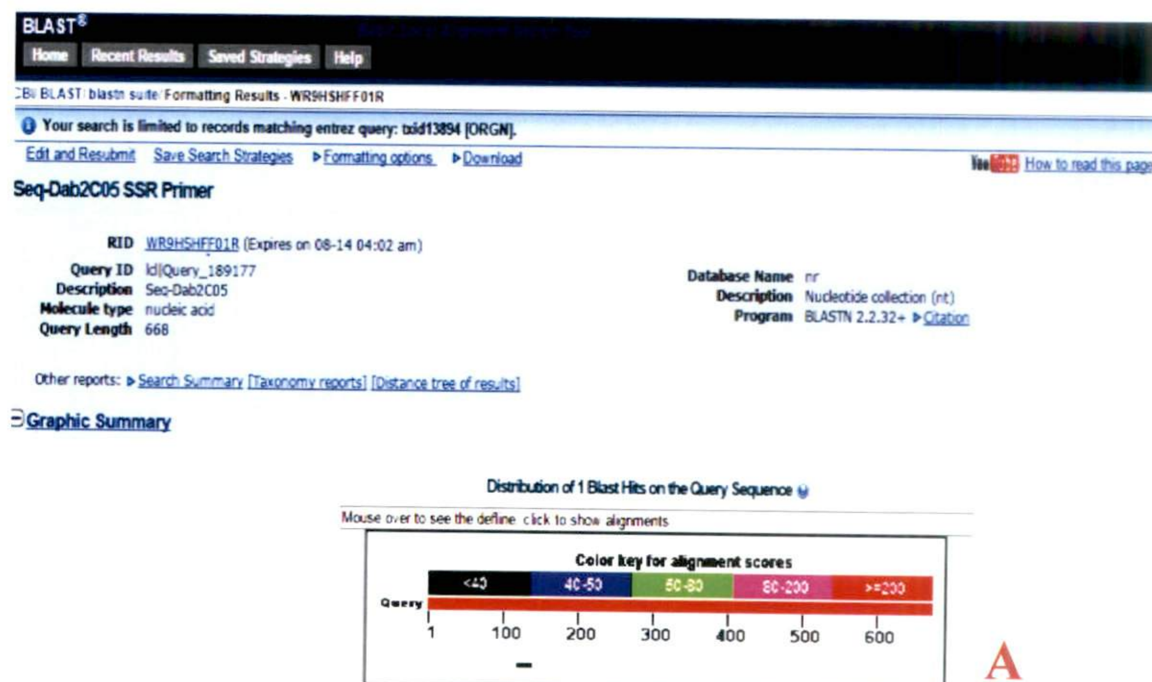


Plate 29. Eluted DNA showing less concentration

Figure 12 shows sequence obtained from Genetic analyzer

>Seq-Dab2C05
ACATTTGTCCGGGGATTGAMAAGGACTGGKASGCTATCAAATAAAATCTT
TTGGGAACCACTTATATACATAAWCTASGAGGTCTGCTCCATTGTAAACA
AGGGAATGCATWAWTGCTGTCAGGGRAACCCATGATGGWCCCATTTTCTA
CATGAACACGCATGTACTGCTAGCATAACTACATAAKCTTCCCTCATATG
CAACAATAAGTTGCCCCAATACACTTCATGCCTTAACCCCCCATTCCATA
CWACATGCCMTTTAKCACTTTTTTTCCTTTTTTTTCTTTTTCTTAACWAT
TATGTTTGGWCCACAAACACRCCTCTACTTCCWAAAATAATCCTTTTTCA
CAKSAATCATTGWCATAACATACGGYCTAAYATTCTCTAACATGTTTATA
ATCGGCTTACTTCTAGSATCTAAMACTRACCCATAAAATGTCTCACACAT
ATTGTTGTCWATAAGATCWCACTTCACTACWTCRRARAGAAGGCAASWTG
KCAACCTGTGATTGGCTAGTGCTCCAGTAAGTCCTTCACAGTTGCCTACC
CTCCATGAAAATTATACATCTGAYCTAGGYATTTTTGCATCTGGTGGTTG
ACTGGTAATCTGGCAASTTCCAKAATGCAATCTACAACCMCCTTCCCCKG
GGTGTTTTTTTCCCCWAC

Figure 13 (A and B) Shows similarity obtained by BLASTn analysis



⊖Descriptions

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

	Description	Max score	Total score	Query cover	E value	Ident	Accession
☐	Cocos nucifera cultivar Chowghat Green Dwarf glycosyl hydrolases family 31 protein mRNA, complete cds	30.1	30.1	2%	0.35	90%	KJ647170.1

⊖Alignments

Download GenBank Graphics ▼ Next ▲ Previous Descriptions

Cocos nucifera cultivar Chowghat Green Dwarf glycosyl hydrolases family 31 protein mRNA, complete cds

Sequence ID: gb|KJ647170.1| **Length:** 2694 **Number of Matches:** 1

Range 1: 1560 to 1579 GenBank Graphics

Score	Expect	Identities	Gaps	Strand
30.1 bits(32)	0.35	18/20(90%)	0/20(0%)	Plus/Plus

```
Query  121   CAGGGRAACCCATGATGGAC  140
             ||||| ||||||||||||| |
Sbjct  1560  CAGGGCAACCCATGATGGAC  1579
```

Related Information

B

4.13 *In Vitro* propagation studies

Five different media were used for the study and identified MS medium without hormones enhanced sprouting and shoot growth. MS medium with 2mg kinetin enhanced rooting compared to other media were shown in Table 18. Major problem faced during micropropagation is the delay of sprouting and contamination.

4.13.1 Surface sterilization standardization

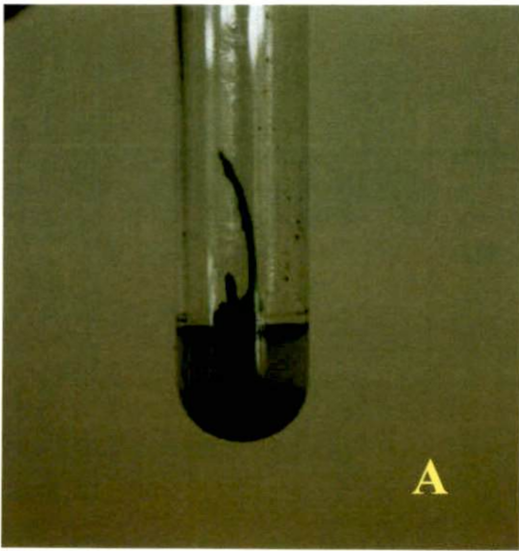
Sodium dichloroisocyanurate solution of 2 per cent for 15 minutes treatment found optimum for surface sterilization and got 70% recovery without any microbial contamination as shown in Table 19 .Other concentrations tried, lead to microbial contamination with treatment time less than 15 minutes. Increased treatment time with different concentration of sodium dichloroisocyanurate solution caused browning of explants, especially shoot tips. Mercuric chloride of 0.1 per cent for 8 minutes gave optimum surface sterilization results with 80 per cent recovery as shown in Table 19.Below 8 minutes treatment time caused increase in rate of contamination and low per cent recovery and more than 8 minutes treatment caused browning of shoot tips and tuber sprouts.

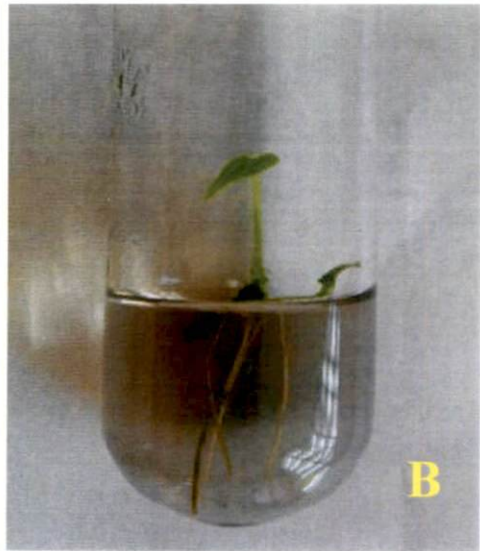
Table 18. Showing percentage recovery of plants after surface sterilization

Sample	Surface sterilization method	Explants used	No of plants treated initially	R_1	R_2	Days taken for contamination	No of plants recovered	Percentage recovery
V1	Sodium dichloroisocyanurate (2% for 15 minutes)	Nodal cuttings	20	20	20	6-8	46	76.66%
V1	Mercuric chloride (0.1% for 8 minutes)	Nodal cuttings	20	20	20	8-12	48	80.00%
V1	Sodium dichloroisocyanurate (2% for 15 minutes)	Shoot tips	10	10	10	6-8	21	70.00%
V1	Mercuric chloride (0.1% for 8 minutes)	Shoot tips	10	10	10	8-12	22	73.33%
V1	Mercuric chloride (0.1% for 8 minutes)	Tuber sprout	10	10	10	6-8	10	33.33%

Sample	Explants used	Media code	Surface sterilization method	No of days taken for sprouting	Shooting	Rooting
V1	Nodal cuttings	MS	Sodium dichloroisocyanurate (2% for 15 minutes)	115days	+ (3.8 cm)	+
V1	Nodal cuttings (from initial sprout)	MS	Sodium dichloroisocyanurate (2% for 15 minutes)	82days	+++++ (4.5 cm growth with 5-6 no of large dark green leaves)	+++
V1	Shoot tip	MSK2	Sodium dichloroisocyanurate (2% for 15 minutes)	112days	++ (2.1cm growth with one large light green leaf)	++++++
V1	Tuber sprout	MS	.1% mercuric Chloride for 8 minutes	30 days	Initial growth of Callus	

Table 19. Showing root and shoot growth in different media





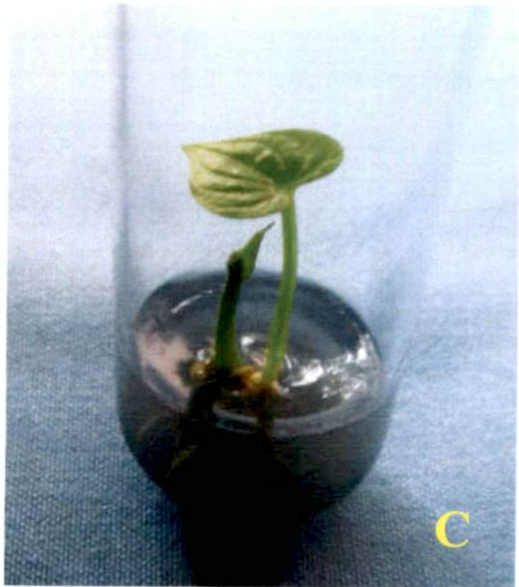


Plate 30. showing *in vitro* propagation of dwarf white yam

4.13.2 **Thiourea treatment**

On observation, tubers treated with thiourea did not break dormancy earlier as compared to control, as it takes only 78 days for sprouting. Sprouting on treatment with thiourea 20g/l observed was on 93 days after inoculation as shown in Table 20. But on further observation it was found that increasing concentration of thiourea progressively decline sprouting time as shown in graph.

Table 20. Showing different treatments and number of days taken for sprouting

Tests	Inoculation date	Sprouting observed date	No of Days taken for sprouting
T_1	13/1/14	17/4/15	93
T_2	13/1/14	28/4/15	104
T_3	14/1/14	10/5/15	116
T_4	14/1/14	10/5/15	116
T_5	14/1/14	12/5/15	118
T_6	14/1/14	14/5/15	120
T_7	16/1/14	21/5/15	127
T_8	16/1/14	21/5/15	127
T_9	14/1/14	2/5/15	108
T_{10}	14/1/14	2/5/15	108
Control	13/1/14	2/4/15	78

The application of PGRs could not break the dormancy of sprouting. Instead all the treatments enhanced the number of days to sprouting and hence are not useful for breaking dormancy in white yam. Among the treatments T7 and T8 too maximum days (127) for bud sprout.

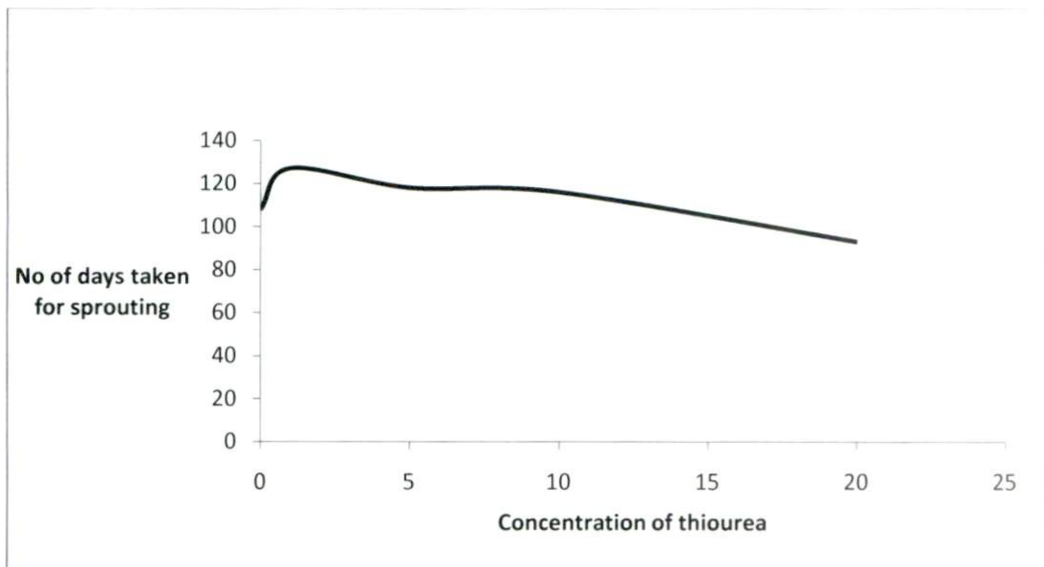


Figure 15. Thiourea treated plants in net house

DISCUSSION

5. DISCUSSION

Yam is an important food and nutritional security crop grown in tropical countries for its underground tubers. It is a source of income and also a part of socio-cultural events. They are plants that are grown for their modified, thickened roots or stems, which generally develop underground. They are rich in carbohydrates and are commonly used as a dietary staple, livestock feed, raw material for the production of industrial products such as starch and alcohol or processed into various food products.They represent the second most important set of food crops in developing countries, following the cereals. It contains higher value of protein (2.4%) and substantial amount of vitamins (thiamine, riboflavin and ascorbic acid) and minerals like calcium, phosphorous and iron. It also has medicinal properties such as the steroidal sapogenins which is used in the production of cortisone and synthetic steroid hormones. It comes under the family *Dioscoreacea* and genus *Dioscorea.*

White yam *(Dioscorea rotundata* Poir.) is emerging as an important tuber crop in Kerala and it is known for its yield potential. A massive white yam weighing 275 kg was harvested by a farmer in Trivandrum, has made it to the 2011 Limca Book of Records. Central Tuber Crops Research Institute (ICAR-CTCRI, India) holds a field genebank of 1100 *Dioscorea* accessions including 158 white yam genotypes. Use of stakes for trailing in white yam led to high cost of cultivation limiting the spread of the crop. Recent studies revealed that the most critical problems facing the farmers cultivating yams includes, non availability of adequate staking materials and its high cost that accounts for 30 per cent of the cost of cultivation. This leads to the development of a dwarf plant stature. Hence the present study aims at identifying molecular markers linked to genes affecting plant architecture (plant height/dwarfness) in white yam (*Dioscorea rotundata* Poir.), to assess variability and genetic diversity among white yam genotypes using molecular markers like RAPD,

ISSR and SSR and also to standardise *in vitro* protocol for the rapid propagation of dwarf/bushy yam.

A dwarf mutant, Sree Dhanya was released in 1993 as the first dwarf variety suitable for planting with closer spacing without any staking. This study helps to develop population for mapping of dwarfing gene in white yam to facilitate the transfer of this valuable trait among different yam species. Diversity study enables identification of divergent parents based on molecular markers to develop heterotic hybrids with high hybrid vigour. Also, standardisation of protocol for *in vitro* propagation, will help in mass propagation of bushy yam and enhance the availability of planting materials to farmers.

5.1 **Molecular marker Analysis**

Isolated DNA from 30 white yam (*Dioscorea rotundata* Poir.) genotypes having dwarf, semidwarf and tall plant type. 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 and RAPD primers gave better results.

Present study, molecular markers *viz.* ISSR, SSR and RAPD primers were selected based on previous reports in Yams (Alieu *et al.*, 2011; Narina *et al.*, 2011; Norman *et al.*, 2012; Sartie *et al.*, 2012; Nascimento *et al.*, 2013 and Scarcelli *et al.*, 2013). For preliminary screening of ISSR, 19 ISSR primers were used of which, only 15 were selected based on their polymorphism and repeatability. For preliminary screening of SSR, 14 SSR were used of which, only 10 were selected based on their polymorphism and repeatability. Nineteen RAPD primers were screened of which, only 8 were selected based on their polymorphism and repeatability. Eleven RAPD

primers were eliminated after preliminary screening due to poor amplification profile in tall genotypes.

The ISSR markers are useful in studying variation in microsatellite regions that are scattered particularly in nuclear genome. Ten polymorphic ISSR markers were used for the molecular characterization of selected white yam genotypes. The total number of bands per ISSR primer ranged from 1 ($(ACC)_6Y$) to 10 (UBC 825). The primers UBC 809, UBC 827 and UBC 864 recorded nine bands followed by UBC 810 (8 bands), UBC 817. UBC 825 that recorded the highest number of polymorphic bands (10) followed by UBC 827 with UBC 864 with nine bands. In the case of primer UBC 810 all the eight bands were found to be polymorphic.

The polymorphism of the ISSR primers studied ranged from 77.78% to 100%. UBC 809 recorded the lowest polymorphism (77.78%) among the primers followed by UBC 811 (85.71%). The Observed heterozygosity value (Hobs) of the ISSR primers ranged between 0 ($(ACC)_6Y$) to 0.88 (UBC 825 and UBC 864) . For most of the ISSR primers studied Hobs was found to be >0.8. Among the primers, the $(GA)_9AT$ and UBC 818 recorded lower Hobs values of 0.5954 and 0.5448 respectively.

The polymorphism Information content (PIC) of the primers ranged from 0.8719 (UBC 864) followed by UBC 825 (0.868), UBC 827 (0.8641) and UBC 809 (0.8601). The primers UBC 818, (GA)9AC, (GA)9AT, UBC 14 and (ACC)6Y recorded lower PIC content of <0.6. In the present study, the ISSR primers produced an average of six polymorphic alleles with a mean Hobs and PIC values of 0.738 and 0.707 respectively. However, Ramirez et al (2014) could only get moderate PIC estimate for the ISSR markers studied in *Dioscorea* germplasm in Mexico. The polymorphic information content measures the informativeness related to the expected heterozygousity (HE) and can also be estimated from allele frequencies. The higher PIC and Hobs values obtained in the present study for the ISSR markers

indicate the high variability of the population studied. It also indicated the usefulness of the ISSR markers identified, they can be used in elucidating genetic diversity among yams in future.

Among the ten SSR markers studied, the number of alleles per marker ranged from 2 to 5, while the number of polymorphic alleles ranged from 1 to 5. YM15 recorded the maximum number of polymorphic alleles (5) followed by Dab2D06 and YM26 with 4 alleles. The percentage of polymorphism ranged from 50 (Dab2C05, Dab2E07). The observed heterozygosity values ranged from 0.4339 (Dab2C05) to 0.775 (YM 15). The SSR makers *viz.* Dab2D06, YM15 and YM 26 recorded high Hobs values (>0.7). The polymorphism information content ranged from 0.3398 (Dab2C05) to 0.7388 (Dab2D06). YM15 also recorded high PIC value of 0.7377. On average the SSR markers recorded Hobs value of 0.5715 and polymorphism information content of 0.4866. Norman *et al* (2012) reported high polymorphic information content (PIC) of 0.8719 in loci Dab2C05. However, in the present study, Dab2C05 recorded lowest PIC value among the markers studied. This might be due to the difference in structure of the population studied. In the present study, the makers resulted in allele numbers of 2, 3, 4, 5 and 7 per locus, suggesting the presence of different ploidy levels of diploids, triploids, tetraploids, pentaploids in white yam studied. The results are in agreement with the findings of Alieu *et al* (2012) in tropical yams.

Among the eight RAPD primers studied in white yam, OPW -16 was found be the best that produced ten polymorphic bands followed by OPG-02 and OPG-13 with six polymorphic bands. All the primers evaluated resulted in 100% polymorphism. The Hobs values ranged between 0.7377 (OPW1) to 0.8937 (OPW-16). OPG-13 and OPG-16 recorded high observed heterozygosity (>0.8). The polymorphism information content ranged from 0.6997 (OPG-03) to 0.8838 (OPW-16). All the

primers recorded high PIC value of >0.6. However, Ramirez *et al* (2014) reported a PIC value of 0.43 for the RAPD markers in yams and was lesser than the high average PIC value (0.75) reported for RAPD markers studied in the present investigation.

The comparison of the different type of markers (ISSR,SSR,RAPD) studied showed that eventhough number of polymorphic bands are higher for ISSR and RAPD, the average polymorphism information content was not lesser for SSR as compared to RAPD and ISSR primers evaluated. The results implied that the all the different type of markers used were efficient in discriminating the genotypes studied.

5.2 Molecular marker identification of dwarf gene

Under the present study molecular marker analysis of different white yam accessions including dwarf, semi dwarf and tall genotypes was undertaken at ICAR-CTCRI, Sreekariyam, Thiruvananthapuram during 2014-2015.

The results indicated the association of three SSR markers **(Dab2C05, Dpr3F04, Dab2E07)** with dwarfness in white yam. One of the SSR marker Dab2C05 showed unique band of size 1.5Kbp for dwarf. The ISSR primer **UBC 836** was also identified as better marker that showed specific band for dwarf genotypes. So it can also be used for identifying dwarf gene. This finding is the first report of association of molecular markers with dwarfness in white yam. The molecular markers linked to plant height have been reported in other crops. In wheat, Daoura *et al* reported 3 SSR primers, BARC102, GWM533b and WMC623 on analyzing 21 pairs of SSR primers that showed a polymorphic band linked to dwarfness and the individuals lacking the polymorphic band performed as tall genotypes. Yuan *et al* (2015) reported the discovery of mendelian factor qPH6 controlled by a single Mendelian

factor, which is located on the long arm of chromosome 6. This information could be helpful for future identification and isolation of the candidate genes. Phenotypic evaluation of the near isogenic lines NIL (qPH6) and NIL (qph6) suggested that qPH6 has no pleiotropic effect on other traits. The discovery of qPH6 might be useful for the design and breeding of crops with high grain yield and quality. In coconut, Rajesh *et al* (2013) identified a SCAR marker for the dwarf gene derived from RAPD analysis. In the present study of white yam, the identified band can be used for the development of SCAR marker in future.

5.3 Genetic diversity studies

Cluster analysis done based on Euclidean distance of 30 accessions for ISSR, SSR and RAPD. Dendrogram showed the partition of most of the dwarf and tall genotypes in to two different clusters. Semi dwarf comes under the cluster of dwarf genotypes. Based on ISSR data, the genotypes formed four clusters at 0.4 dissimilarity index. Cluster 1 has only one genotype i.e. V20 (DR17) and was found to be highly divergent than all other genotypes. The cluster 2 has two genotypes V23 & V24. Cluster 3 is the biggest one with twenty four genotypes. They were grouped in to two sub clusters 3a & 3b. The cluster 4 has three genotypes and was sub clustered in to two groups.

In the present study, the SSR markers gave unique results. Based on SSR markers, white yam genotypes were grouped in to three clusters. The cluster 1 with two genotypes (V29 and V30) is the smallest one. Cluster 2 comprise of 16 accessions of which V2 and V9 were found to be closely related. The dwarf genotypes (12) clustered together in Cluster 3. The semi dwarf genotype SD15 was grouped together with other dwarf genotypes.

More clusters were resulted in dendrogram formed based on RAPD markers. At 0.8 dissimilarity level, it formed nine clusters. Cluster 1, 2 and 3 has only one accession *viz.* V23, V18 and V22 respectively. The fourth cluster has four genotypes and was again subdivided in to two sub clusters. The fifth cluster has three genotypes and also subdivided into two sub clusters 5a and 5b. The two genotypes included in 5b i.e. V19 and V24 were found to be similar . Sixth cluster has one genotype (V20). The seventh cluster is the largest one with 16 genotypes and consists of mostly dwarf genotypes and is divided into several sub clusters. The eighth cluster has one genotype (V15) and ninth cluster has two genotypes (V27 and V29), the clustering pattern indicated the grouping of the dwarf genotypes together.

Mantel's test was done to assess correlation between the markers used. The analysis established that there is high correlation existing between these data. The mantels test revealed significant correlation between the results of different type of markers in white yam. The correlation between different clusters was found to be high (>0.5). The cluster data from dendrogram was in agreement with data from cluster analysis after PCA suggesting that most of the tall and dwarf genotypes grouped separately. The present finding was in conformity with the results obtained by Ramirez *et al* (2014).

5.4 *In vitro* propagation studies

Sodium dichloroisocyanurate solution of 2 per cent for 15 minutes treatment found optimum for surface sterilization and got 70% recovery without any microbial contamination, when nodal explants collected from field were used as explants .Other concentrations tried lead to high microbial contamination with treatment time less than 15 minutes. Five different media were used for the study and identified MS medium without hormones enhanced sprouting and shoot growth. MS medium with 2mg kinetin enhanced rooting compared to other media. Major problem faced during

micropropagation is the delay of sprouting and contamination due to systemic microbes. The present finding was in conformity with the reports of Das *et al* (2013) in greater Yam. However initial establishment of the explants is very slow and took 82-115 days. Hence further research is needed for hastening the *in vitro* establishment in white yam.

In addition to identifying molecular markers linked to dwarfness, the present study could identify a high yielding (8 kg/plant), highly divergent white yam genotype, DR17 that could be used for the genetic improvement of white yam in future.

SUMMARY

6. SUMMARY

White Yam *(Dioscorea rotundata* Poir.) is an important tuber crop grown in Kerala and it is well known for its yield potential. It is one of the most important tuber crop grown globally and provides food security to the poor population especially in Africa. Use of stakes for trailing white yam led to high cost of cultivation limiting the spread of the crop. Recent studies revealed that the most critical problems facing the farmers cultivating yams includes, non availability of adequate staking materials and its high cost that accounts for 30 percent of the cost of cultivation. High labour cost for harvesting also necessitates the development of varieties with dwarf plant stature. A dwarf mutant, Sree Dhanya was released in 1993 as the first dwarf variety suitable for planting with closer spacing without any staking. Hence the present study aims at identifying molecular markers linked to genes affecting plant architecture (plant height/dwarfness) in white yam (*Dioscorea rotundata* Poir.), to assess variability and genetic diversity among white yam genotypes using molecular markers like RAPD, ISSR and SSR and also to standardize *in vitro* protocol for the rapid propagation of dwarf/bushy yam.

In the present study, DNA was isolated from 30 white yam (*Dioscorea rotundata* Poir.) genotypes having dwarf, semidwarf and tall plant type. 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 and RAPD primers gave better results. Molecular markers *viz.* ISSR, SSR and RAPD primers were selected based on previous reports in yams. For preliminary screening of ISSR, 19 ISSR primers were used of which, only 15 were selected based on their polymorphism and repeatability.

For preliminary screening of SSR, 14 SSR were used of which, only 10 were selected based on their polymorphism and repeatability. Nineteen RAPD primers were screened of which, only eight were selected based on their polymorphism and repeatability. Eleven RAPD primers were eliminated after preliminary screening due to poor amplification profile in tall genotypes.

Ten polymorphic ISSR markers were used for the molecular characterization of selected white yam genotypes. The total number of bands per ISSR primer ranged from 1 ($(ACC)_6Y$) to 10 (UBC 825). UBC 825 that recorded the highest number of polymorphic bands (10) followed by UBC 827 with UBC 864 with nine bands. In the case of primer UBC 810 all the eight bands were found to be polymorphic. The polymorphism of the ISSR primers studied ranged from 77.78% to 100%. UBC 809 recorded the lowest polymorphism (77.78%) among the primers followed by UBC 811 (85.71%). The Observed heterozygosity value (Hobs) of the ISSR primers ranged between 0 ($(ACC)_6Y$), UBC 864 to 0.88 (UBC 825 and UBC 864). In the present study, the ISSR primers produced an average of six polymorphic alleles with a mean Hobs and PIC values of 0.738 and 0.707 respectively. The higher PIC and Hobs values obtained in the present study for the ISSR markers indicated the high variability of the population studied. It also indicated the usefulness of the ISSR markers identified that can be used in elucidating genetic diversity of yams in future.

Among the ten SSR markers studied, the number of alleles per marker ranged from 2 to 5 while the number of polymorphic alleles ranged from 1 to 5. YM15 recorded the maximum number of polymorphic alleles (5) followed by Dab2D06 and YM26 with four alleles. The percentage of polymorphism ranged from 50 (Dab2C05, Dab2E07). The observed heterozygosity values ranged from 0.4339 (Dab2C05) to 0.775 (YM 15). The SSR makers *viz.* Dab2D06, YM15 and YM 26 recorded high

Hobs values (>0.7). The polymorphism information content ranged from 0.3398 (Dab2C05) to 0.7388 (Dab2D06). YM15 also recorded high PIC value of 0.7377. On average the SSR markers recorded Hobs value of 0.5715 and polymorphism information content of 0.4866.

Among the eight RAPD primers studied in white yam, OPW -16 was found be the best that produced ten polymorphic bands followed by OPG-02 and OPG-13 with six polymorphic bands. All the primers evaluated resulted in 100% polymorphism. The Hobs values ranged between 0.7377 (OPW1) to 0.8937 (OPW-16). OPG-13 and OPG-16 recorded high observed heterozygosity >0.8. The polymorphism information content ranged from 0.6997 (OPG-03) to 0.8838 (OPW-16). All the primers recorded high PIC value of >0.6.

The comparison of the different type of markers (ISSR, SSR, RAPD) studied showed that eventhough number of polymorphic bands are higher for ISSR and RAPD, the average polymorphism information content was not lesser for SSR as compared to RAPD and ISSR primers evaluated. The results implied that the all the different type of markers used were efficient in discriminating the genotypes studied.

The results indicated the association of three SSR markers **(Dab2C05, Dpr3F04, Dab2E07)** with dwarfness in white yam. One of the SSR marker Dab2C05 showed unique band of size 1.5Kbp for dwarf plant type. The ISSR primer **UBC 836** was also identified as better marker that showed specific band for dwarf genotypes.

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dissimilarity index. In the present study, the SSR markers gave unique results. Based on SSR markers, the white yam genotypes were grouped in to three clusters. The dwarf genotypes (12) clustered together in Cluster 3. The semi dwarf genotype SD15 was grouped together with other dwarf genotypes. More clusters were resulted in dendrogram based on RAPD markers. At 0.8 dissimilarity level, it formed nine clusters. The clustering pattern indicated the grouping of the dwarf genotypes together. Mantel's test was done to assess correlation between the markers used. The analysis established that there is high correlation existing between these data. The mantels test revealed significant correlation between the results of different type of markers in white yam. The correlation between different clusters was found to be high >0.5. The cluster data from dendrogram was in agreement with data from cluster analysis after PCA suggesting that most of the tall and dwarf genotypes grouped separately.

A preliminary study was also carried out to standardize the micropropagation protocol for the regeneration of plants from nodal explants taken from field. Sodium dichloroisocyanurate solution of 2 per cent for 15 minutes treatment found optimum for surface sterilization and got 70% recovery without any microbial contamination when nodal explants collected from field were used as explants .Other concentrations tried gave high microbial contamination with treatment time less than 15 minutes. Five different media were used for the study and identified MS medium without hormones enhanced sprouting and shoot growth. MS medium with 2mg kinetin enhanced rooting compared to other media. Major problem faced during micro propagation is the delay of sprouting and contamination due to systemic microbes. However initial establishment of the explants is very slow and took 82-115 days. Hence further research is needed for hastening the in vitro establishment in white yam.

In addition to identifying molecular markers linked to dwarfness, the present study could identify a high yielding (8 kg/plant), highly divergent white yam genotype, DR17 that could be used for the genetic improvement of white yam in future.

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APPENDIX I

CTAB Extraction Buffer

Tris- HCl (pH 8.0)	100 m*M*
EDTA	25 m*M*
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

TE buffer (10X)

Tris- HCl (pH 8.0)	10 m*M*
EDTA	1 m*M*

APPENDIX III

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.

APPENDIX 1V

40% Acrylamide solution

Acrylamide	38g
Bisacrylamide	2g

Dissolved 38g of acrylamide in 40 ml distilled water, stirred to dissolve and then added bisacrylamide, made up to 100ml.

APPENDIX V

Bind Silane

Ethanol	95%
Bind silane	1µl
0.5% acetic acid	2.5 ml

The solution is then made up to 100ml.

APPENDIX VI

Fixer (10%)

Acetic acid	200 ml
Distilled water	1800 ml

APPENDIX VII

Silver stain

2g of silver nitrate is dissolved in 2000ml distilled water with thorough mixing. Added 3 ml formaldehyde to this solution.

APPENDIX VIII

Developer

60g of sodium carbonate is dissolved in 2000ml distilled water. Stored the solution at 20^0C. Just before use, added 400µl chilled sodium thiosulphate (10 mg/ml) and 3 ml 37 % formaldehyde.

APPENDIX IX

Sodium hydroxide solution (2%)

40 g NaOH dissolved in 2000ml distilled water.

APPENDIX X

Page dye

Formamide	50ml
Xylene cyanol	50mg
Bromophenol blue	50mg
0.5M EDTA	1ml

APPENDIX XI

100bp marker for PAGE gel

100bp Ladder	5µl
Dye	40µl
Distilled water	55µl

APPENDIX XII

Master mix I

Dnase/Rnase free water	10µl	for one reaction
15mM EDTA	2µl	

APPENDIX XIII

Master mix II

3M Sodium acetate (pH 4.6)	2µl	for one reaction
Ethanol	50µl	

ABSTRACT

In Vitro propagation and identification of molecular markers linked to dwarfness in white yam (*Dioscorea rotundata* Poir.)

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Abstract of the thesis

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ABSTRACT

White Yam (*Dioscorea rotundata* Poir.) is a leading source of calories for over 300 million people in Africa, Asia, parts of South America, as well as the Caribbean and the South Pacific islands. It is an important tuber crop grown in Kerala owing to its very high yield potential and taste. Use of stakes for trailing white yam led to high cost of cultivation limiting the spread of the crop. Recent studies revealed that the most critical problems facing the farmers cultivating yams includes, non availability of adequate staking materials and its high cost that accounts for 30 percent of the cost of cultivation. Hence the present study was carried out with an objective to identify molecular markers linked to genes affecting plant architecture (plant height/dwarfness) in white yam (*Dioscorea rotundata* Poir.).The study also aims at assessing variability and genetic diversity among white yam genotypes using molecular markers like RAPD, ISSR and SSR and also to standardize *in vitro* protocol for the rapid propagation of dwarf/bushy white yam.

In present study, DNA was isolated from 30 white yam (*Dioscorea rotundata* Poir.) genotypes having dwarf, semidwarf and tall plant type. Based on preliminary screening, 15 ISSR, 10 SSR and 8 RAPD primers with high polymorphism were selected for the molecular characterization of white yam genotypes.

The total number of bands per ISSR primer ranged from 1 ($(ACC)_6Y$) to 10 (UBC 825). UBC 825 that recorded the highest number of polymorphic bands (10) followed by UBC 827 with UBC 864 with nine bands. In the present study, the ISSR primers produced an average of six polymorphic alleles with a mean Hobs and PIC values of 0.738 and 0.707 respectively. Among the ten SSR markers studied, YM15 recorded the maximum number of polymorphic alleles (5) followed by Dab2D06 and YM26 with four alleles. The percentage of polymorphism ranged from 50 (Dab2C05, Dab2E07). The polymorphism information content ranged from 0.3398 (Dab2C05) to

0.7388 (Dab2D06).YM15 also recorded high PIC value of 0.7377. On average the SSR markers recorded Hobs value of 0.5715 and polymorphism information content of 0.4866. Among the eight RAPD primers studied in white yam, OPW -16 was found be the best that produced ten polymorphic bands followed by OPG-02 and OPG-13 with six polymorphic bands. All the primers recorded high PIC value of >0.6.

The comparison of the different type of markers (ISSR, SSR, RAPD) studied, showed that the number of polymorphic bands are higher for ISSR and RAPD, the average polymorphism information content was lesser for SSR as compared to RAPD and ISSR primers evaluated. The results implied that the all the different type of markers used were efficient in discriminating the genotypes studied.

Cluster analysis done based on Euclidean distance of 30 accessions for ISSR, SSR and RAPD. Dendrogram showed the partition of most of the dwarf and tall genotypes in to two different clusters. Semi dwarf comes under the cluster of dwarf genotypes. The correlation between different clusters was found to be high (>0.5). The cluster data from dendrogram was in agreement with data from cluster analysis after PCA suggesting that most of the tall and dwarf genotypes grouped separately.

A preliminary study was also carried out to standardize the micropropagation protocol for the regeneration of plants from nodal explants taken from field. Sodium dichloroisocyanurate solution of 2 per cent for 15 minutes treatment found optimum for surface sterilization and got 76.6% recovery without any microbial contamination, when nodal cuttings collected from field were used as explants. MS medium with 2mg kinetin enhanced rooting compared to other media. Major problem faced during micropropagation is the delay of sprouting and contamination due to systemic microbes. However initial establishment of the explants is very slow and took 82-115 days. Hence further research is needed for hastening the *in vitro* establishment in white yam.

The results indicated the association of three SSR markers (**Dab2C05, Dpr3F04, Dab2E07**) with dwarfness in white yam. One of the SSR markers Dab2C05 showed unique band of size 1.5Kbp for dwarf genotype. The ISSR primer **UBC 836** was also identified as better marker that showed specific band for dwarf plant type.

In addition to identifying molecular markers linked to dwarfness, the present study helped to identify a high yielding (8 kg/plant), highly divergent white yam genotype, **DR17** that could be used for the genetic improvement of white yam in future.

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