MOLECULAR MARKER ANALYSIS FOR CASSAVA MOSAIC DISEASE RESISTANCE

By

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THESIS

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DECLARATION

I hearby declare that the thesis entitled **"Molecular marker analysis for cassava mosaic disease resistance"** is a bonafide record of research done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, fellowship or other similar title, of any other University or Society.

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LIST OF ABBREVIATIONS

%	Percentage
µg	Microgram
µl	Microlitre
µ*M*	Micromolar
A	Adenine
A_{260}	Absorbance at 260 nm wavelength
A_{280}	Absorbance at 280 nm wavelength
APS	Ammonium persulfate
bp	Base pair
C	Cytosine
cDNA	Complementary DNA
cm	Centimetre
CTAB	Cetyl trimethyl ammonium bromide
DNA	Deoxyribo nucleic acid
dNTPs	Deoxy nucleotide tri phosphates
EDTA	Ethylene diamine tetra acetic acid
F	Forward primer
G	Guanine
g	gram
g	standard acceleration due to gravity at the earth's surface
h	Hour
ha	Hectare
kbp	Kilo basepair
kDa	Kilodalton
kg	Kilogram
M	Molar
mg	milligram
Mg^{2+}	Magnesium ion

min	Minute
ml	Millilitre
m*M*	Millimolar
Mn^{2+}	Manganese ion
mRNA	Messenger ribonucleic acid
NaCl	Sodium chloride
ng	Nanogram
nm	Nanometre
°C	Degree Celsius
OD	Optical density
PCR	Polymerase chain reaction
PVP	Polyvinyl pyrrolidone
R	Reverse primer
RAPD	Random amplified polymorphic DNA
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolution per minute
RT	Reverse transcriptase
RT-PCR	Reverse transcription-polymerase chain reaction
s	Second
sp.	Species
spp.	Species (plural)
SSR	Simple sequence repeat
t	Tonne
T	Thymine
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

V	Volt
v/v	volume/volume
w/v	weight/volume

1. INTRODUCTION

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Cassava (*Manihot esculenta* Crantz), family Euphorbiaceae, is the fifth most important staple food crop of the world among the 102 countries where it is cultivated. Its tuberous roots contain starch up to 85% of their dry weight and provide food for over 500 million people, mostly in developing countries. Cassava is the third highest source of calories in the inter-tropical zone of the world (FAO, 2016) due to the high carbohydrate content in their storage roots (Ceballos *et al.*, 2004). Cassava is being grown in India for more than a century. In India, this crop is grown in an area of 2.44 lakhs ha, mostly in Kerala, Tamil Nadu, Andhra Pradesh, Karnataka, Maharashtra and a few North Eastern states with production of more than 60 lakhs tons. It is a famine reserve crop well suited to rain fed system of farming. It is nutrient efficient and possesses very high potential for food energy production. Cassava is an industrial crop used in the production of chips, starch, sago, ethanol and also used as an animal feed. Cassava is also known as the 'Orphan crop' that provides a basic daily source of dietary energy to the poorest population of the world.

Cassava is monoecious with 36 chromosomes and highly heterozygous due to its out crossing nature. It is vegetatively propagated mainly by means of stem cuttings. It is semi woody perennial plant and the only edible cultivated dicotyledonous species in the genus *Manihot* (Family: Euphorbiacae). This tropical crop possesses high photosynthetic ability coupled with capacity to yield under poor marginal soils and adverse weather conditions.

Cassava is affected by a number of diseases causing substantial losses in yields. Besides fungal, bacterial, and mycoplasmal infection, it is severely affected by few viral diseases. Of all the diseases reported, Cassava Mosaic Disease (CMD) is one of the most serious disease, which leads to heavy loss of the crop in India and Africa. In India, the yield loss reported due to cassava mosaic disease incidence is in the range

of 17-88% (Malathi *et al.,* 1985) depending on the varieties infected. Since the crop is propagated vegetatively, it is particularly prone to viral infection carried through the infected stem cuttings as planting material, which tends to build up in successive cycles of propagation.

CMD is caused by at least seven different Gemini viruses *viz* , African Cassava Mosaic Virus (ACMV) , the East African Cassava Mosaic Virus (EACMV), Ugandan Variant of East African Cassava Mosaic Virus (UgV), South African Cassava Mosaic Virus (SACMV), Madagascar Cassava Mosaic Virus (MCMV), Indian Cassava Mosaic Virus (ICMV) and Sri Lankan Cassava Mosaic Virus (SLCMV). CMD virus belonging to genus Begomo viruses of the family Gemini viruses, which are characterized by small, geminate particle containing circular single stranded DNA molecule (Briddon *et al.*, 1993). The genome has two compartments *viz*; DNA-A and DNA-B. The DNA–A encodes functions associated with virus replication and encapsulation and DNA-B encodes movement protein functions. Both the components are required for infectivity (Bock, 1982). The viruses are transmitted by the white fly *Bemisia tabaci*, and spread through infected stem cuttings, which is the usual method of cassava propagation (Fragette *et al;* 1998).

Since 1963, when intensive breeding work started in India, nearly 20 varieties were released from CTCRI, Kerala Agricultural University and Tamil Nadu Agricultural University. Most of the varieties are highly susceptible to CMD. Continuous vegetative propagation resulted in very high virus load and led to clonal deterioration of these varieties. A number of indigenous cultivars like Kalikalan, Ariyan and Burmah were found 100 per cent infected by the virus.

Breeding for resistance has been considered a feasible strategy for the control of CMD (Thresh *et al.*, 1994; Calvert and Thresh, 2002; Thottappilly *et al.*, 2003). Host plant resistance to CMD is the principal method of control, which was first identified in the third back cross derivatives of an inter specific cross of cassava and its wild

relative *M.glaziovii.* Recently a second source of CMD resistance was also identified in several Nigerian landraces, which is controlled by a single dominant gene. In cassava, several varieties remain healthy as symptom free carry the CMD virus. Molecular techniques will help to detect the presence / absence of virus in symptom free plants.The discovery of the host plant resistance, polygenic / monogenic dominant, is helpful in the development of resistant varieties using a combination of conventional and marker assisted breeding methods. The study of variability and diversity present in diverse cassava land races and pre-breeding lines maintained at the National repository of CTCRI, is essential to design the marker assisted breeding program of cassava for developing CMD resistant varieties suitable for cultivation in Kerala.

Molecular markers are powerful tools for marker-assisted selection (MAS) in plant breeding (Collard and Mackill 2008). MAS is more efficient, effective, reliable and cost-effective than conventional selection for many traits in crop plants. DNA markers are robust and abundant and are being used at large scale for germplasm characterisation in cassava (Peroni *et al.*, 2007). Of the different DNA markers, microsatellites have emerged as the marker of choice for plant genetic resources applications owing to their co dominant nature, high levels of polymorphism, abundant and uniform distribution throughout the genome, simplicity of detection through PCR and reproducibility.

The present project aims at screening the CMD symptom free lines to detect the presence/ absence of cassava mosaic virus using molecular techniques. Study on DNA polymorphism will help to understand the genetic diversity present among the cassava genotypes using SSR markers and to identify divergent lines for future breeding programs. Also the study aims at identification and validation of molecular markers linked to CMD resistance that can be used for marker assisted breeding programmes in future.

2 REVIEW OF LITERATURE

2.1 Introduction

Cassava (*Manihot esculenta* Crantz) is a species native to tropical America (Olsen and Schaal 2001). The genus *Manihot* comprises 98 species that spread throughout the Neotropics (Rogers and Appan, 1973). It has been cultivated in tropical America for more than 5,000 years. Cassava was first cultivated by native Latin Americans and then brought into Africa and Asia by the Portuguese traders in the 16th century (Jennings, 1976; Jones, 1959).

Cassava is the most important starchy root crop grown in the tropics and is mainly cultivated in Southern peninsular India. Introduced during seventeenth century by Portuguese, the crop played a significant role to overcome food shortage among the low income group of people in Kerala. Its underground tuber is rich in starch and mainly consumed after cooking. Processed products like chips, sago and vermicelli made of tapioca are also popular in the country. It is also widely used for the production of industrial alcohol, starch and glucose. It forms an important ingredient in poultry and cattle-feeds.

According to FAO (2016), Africa constitutes about 53% of the world production of cassava (230 million tons). The productivity of cassava in 2016 was estimated to be 12.40 tons ha^{-1} (FAO, 2016). According to the Kerala agricultural statistics 2013-2014, the production of cassava in Kerala is 24,79,070 tonnes with a productivity of 36.68 t ha^{-1}. Cassava is one of the most adopted crops in Kerala which is cultivated in about 14 districts in Kerala.

2.2 Botanical Description

Cassava is a tropical perennial shrub that can grow to a height of 3m. It has erect smooth stems radiating from the roots. The stems contain nodes at intervals that give rise to new plants. Leaves are large lobed, borne on a long, slender stalk joining a leaf. The color of leaves appear dark green but in some varieties yellow or purple pigmentation may occur. Male and female flowers are found on the same plant. In

some varieties of cassava cyanide producing sugar derivative occurs in varying amounts. There are many wild relatives of cassava. Based on morphological, ecological, and geographical evidence,*M.carthaginensis, M.aesculifolia, M. grahami, M. flabellifolia*, and *M. saxicola* were considered as the most closely related species to cultivated cassava.

Cassava is a very adaptable plant which can tolerate a wide range of soil conditions. It grows well in soils of low fertility status and produces satisfactory yield. Cassava grows best on sandy loam while poorly drained heavy and rocky soil is unsuitable for it. Maximum production of cassava has been reported from well drained, medium to heavy, fertile soils with a pH value of about 5.5 to 7.0. In high textured soil the tubers can be harvested easily. Cassava is sensitive to acidic soils and the yield is reduced in these soils. Cassava is known to be drought tolerant crop. However, growth and yield of cassava is best in warm, humid tropical conditions. Generally, well distributed rains (ranging from 100 to 200 cm) during the growing months are considered ideal. Wide spread adaptations of cassava in different soil and environmental conditions is caused primarily by the physiological traits possessed. El-Sharkawy, (2007) reported some of the characteristics like high photosynthetic capacity, possession of a tight stomatal control over leaf gas exchange, ability to extract water from deep soils, ability to rapid multiplication through cuttings etc contributing to high productivity and wider adaptability of cassava. Additionally, Jarvis *et al.*, (2012) pointed out that, cassava is a crop with high flexibility in adjusting to future climatic changes and therefore has a potential to become a crop of choice when other food crops are challenged.

2.3 Importance of cassava

Cassava has a reputation as a poor person's crop, *i.e.* a crop of last resort (Hillocks *et al.*, 2001a). Generally cassava is used as food, industrial raw material and substitute in animal feeds. About 90% of cassava root production is utilized as food and it is an important source of carbohydrates. In Kerala cassava is produced mainly by small

scale farmers on marginal and sub-marginal lands. The bulk of cassava grown in India is utilized as food in the form of fresh roots and processed products such as flour and fermented meal preparations. Furthermore, cassava leaves are consumed as vegetable and are the source of proteins and minerals (Lancaster and Brooks, 1983). Cassava leaves contain an average of 21% protein, which is high among non-leguminous plants (Ravindran, 1993). Industrial use of cassava involves production of commodities as ethanol, binding agent, paper, textiles, flavoring agent and starch.

2.4 Pests and diseases

Cassava production in Asia and Africa is particularly exposed to numerous biotic stresses. Common constrains include pests and diseases, poor agronomic practices, high cyanide levels, lack of clean planting materials, low yielding varieties, and long maturity periods (Thresh *et al.* 1994). Pests and diseases are the most economically important constraints to the cassava production. Pests infesting cassava include mealy bugs (*Phenacoccus manihot*), green spider mite (*Mononychellu stanajoa*) , cassava green mite (*Mononychellu stanajoa*), cassava hornworm (*Erinnyisello*), scales, thrips and whitely (*Bemisia tabaci) (*Montero, 2003*).*

Diseases affecting cassava include cassava bacterial blight, cassava virus diseases, cassava anthracnose disease, cassava bud necrosis and root rots (Calvert and Thresh, 2002). Economic importance of cassava diseases depends on the extent of damage a disease causes to the productive part of cassava. In Asia and Africa virus diseases of cassava are the most important production constraints (Taylor and Fauquet,1997; Thresh *et al.* 1994; Thresh *et al.* 1997). Cassava is reported to be vulnerable to at least 20 different viral diseases among which Cassava Mosaic Disease (CMD) and Cassava Brown Streak Disease (CBSD) are the most devastating diseases (Patil and Fauquet, 2009). Sources of CMD and CBSD in cassava are believed to be viruses already present in the indigenous African flora (Legg and Thresh, 2003). Factors influencing perpetuation of the virus diseases in cassava plant include: abundance of efficient insect vectors for transmission, planting of susceptible varieties and continuous use of unclean planting materials normally selected from the

previous seasons.

With the evident success on biological control of cassava mealy bug and cassava green mite, CMD and CBSD remained the challenge. More information on the causative pathogens and efficient diagnostic tools are the prerequisite for the formulation of sustainable management approaches. Thus, CMD and CBSD are now one of research priority of many root and tuber crop programs in Asian and Africa countries (Legg and Thresh, 2003).

2.4.1 Cassava Mosaic begomo viruses

Cassava mosaic disease (CMD) is caused by cassava mosaic begomo viruses (CMBs), and was first described from Tanzania towards the end of the 19th century. It constitutes one of the most widespread and devastating diseases of cassava in Africa (Thresh *et al.*, 1998). Early studies suggested that CMD is caused by a virus; however for many years viral etiology of CMD remained unclear until 1938 when another study by Storey and colleagues from Aman research station in north eastern Tanzania confirmed that the disease is caused by cassava mosaic gemini viruses (CMGs) (family; *Geminiviridae*: genus; Begomovirus) (Storey, 1936).

The virus is systemically transmitted in a persistent manner by whitefly (*Bemisia tabaci* Gennadius) (Homoptera: *Aleyrodidae*) (Dubern, 1994). CMGs greatly reduce the growth and yield of cassava particularly local unimproved varieties (Thresh *et al.*, 1997). CMGs spread easily from one field to another through planting of infected stem cuttings from the previous crop (Fauquet *et al.*, 1988). Incidence, spread, severity and the extent of yield loss depend on the variety susceptibility and stage of plant growth at which infection occurs. Recently, it was established that the severity of CMD is influenced by synergistic effects of co-infection of viruses and its associated DNA satellites (Ndunguru *et al.*, 2008). Losses are attributed to damage on leaves and stems, which interfere with the way in which the plant makes food for storage in the roots. The damaged photosynthesis areas reduce the growth of the plants, number of storage roots and the ability of the storage roots to enlarge and mature. Loss of planting material also occurs in infected cassava, where stem cuttings

are unhealthy and unsuitable for planting.

Viruses of the family *Gemini viridae* comprise a single stranded DNA genome that is encapsulated in characteristic twinned (so called geminate) particles (Bull *et al.*, 2006) The genome consist of two parts namely DNA-A and DNA-B components (Stanley *et al.*, 2005; Bull *et al.*, 2006). DNA-A component replicates autonomously (Rogers and Appan, 1973; Klinkenberg and Stanley, 1990) and comprises of six specific protein encoding open reading frames (ORFs), *AV1* & *AV2* on the virion-sense strand, and *AC1-AC4* on the complementary sense strand. *AC1* encodes for replication associated protein (Rep) required for initiation of viral DNA replication, *AC2* gene encodes for transcriptional activator protein (TrAP) that control gene expression, *AC3* encodes for replication enhancer protein (REn) while RNA silencing suppressor protein is coded by *AC4* gene. *AV1* on virion-sense strand codes CP responsible for virus transmission from plant to plant by whitefly (*Bemisia tabaci)* and *AV2* for pre coat protein (Patil and Fauquet, 2009). Replication of DNA-B depends on DNA-A. DNA B has two ORFs one each on the virion and on complementary strand; *BV1* is a shuttle protein encoder (NSP) while *BC1* is responsible for movement protein (MP) encoding. The virus move within and between cells of host plants by a co-operative action of the two genes (Hanley-Bowdoin *et al.*, 2004). Virus infection and subsequent symptom development in host plant requires presence of both virus components (DNA-A and DNA-B) (Stanley and Gay, 1983).

Taxonomic guidelines developed recently (Fauquet *et al.*, 2008) provided a frame work for defining species and strains. This approach sets sequence demarcation between members of different species to be 89% of begomo viruses DNA-A component nucleotide sequences.

Using this criterion seven distinct but similar virus species of CMD in Africa and 2 in Indian sub-continent have been described. These are; African cassava mosaic virus (ACMV), East African cassava mosaic Kenya virus (EACMKV), East African cassava mosaic virus (EACMV), East African cassava mosaic Zanzibar virus

(EACMZV), East African cassava mosaic Cameroon virus (EACMCV), East African cassava mosaic Malawi virus (EACMMV) and South African cassava mosaic virus (SACMV). Indian subcontinent has Indian cassava mosaic virus (ICMV) and Sri Lankan cassava mosaic virus (SLCMV). Each of these species can induce CMD on cassava plants both in single and in co-infections resulting in severe disease (Harrison *et al.*1995; Thresh *et al.*, 1998; Berry and Rey, 2001).

2.4.2 Geographical distribution and severity

Among the diseases and pests of cassava, cassava mosaic disease (CMD) is the serious factor limiting the productivity of cassava, which can lead to yield reduction of 70-80% (Fauquet and Fargette, 1990). Twenty different viruses have been reported from cassava (Thresh *et al.*, 1994). The disease was reported first in the South Indian state of Tamil Nadu during 1966.

Indian Cassava Mosaic Disease (ICMD) is a serious constraint to cassava production in India. This disease is reported to be widespread in South India mainly in Kerala, Tamil Nadu and Andhra Pradesh (Narasimhan and Arjunan, 1976). Yield losses up to 88% in highly susceptible cultivar 'Kalikalan' and 17 to 36 per cent in improved varieties released by CTCRI were reported (Malathi *et al.*, 1985). The symptoms of CMD are identical in Africa and India. Two distinct begomo viruses, *viz.* Indian cassava mosaic virus (Hong *et al.*, 1993) and Sri Lankan cassava mosaic virus (Saunders *et al.*, 2002) cause CMD in Asia.

Complete nucleotide sequencing of two cloned ICMV DNAs, one from the Southern state of Kerala (Hong *et al.*, 1993) and another from the central state of Maharashtra (Saunders et al.,2002) showed that they were highly similar to each other, indicating them to be isolates of the same virus.

In a PCR-RFLP study to analyze the genetic diversity of gemini virus associated with CMD, it was found that both ICMV and SLCMV were present in mosaic-affected cassava; ICMV was geographically restricted to certain regions, whereas SLCMV was widespread (Patil *et al.*, 2007).

In the germplasm maintained at CTCRI most of the accessions are found to be susceptible to CMD and white fly infestation. However a few of them were found to be phenotypically symptom free. Cassava leaves infected with *Indian cassava mosaic virus* exhibit bright yellow patches separated by normal green tissue. The disease significantly reduces stem girth and yield. Tuber splitting is also observed. The primary spread of the disease is through infected planting materials and secondary spread is by an insect vector, white fly (*Bemisia tabaci* Genn.), in the field in a persistent manner. All these viruses are transmitted by the whitefly, *Bemisia tabaci* to an extent of 5 percent only. The use of disease-free cuttings and elimination of the chance of infection through insect vectors are the main principles of management of this disease.

A survey was conducted on the incidence of cassava mosaic disease (CMD) in Kerala. Cassava mosaic disease occurred throughout the state at low to high incidences (44.5-96.75%) in the 35 regions surveyed. The disease incidence was higher in Thiruvananthapuram and Kollam districts and lower incidence was noticed in Wayanad district. Maximum white fly population was also observed in Thiruvananthapuram district of Kerala followed by Kollam. Survey results also indicated that Sri Lankan Cassava Mosaic Virus (SLCMV) is wide spread in Kerala (Anitha *et al.*,2011).

2.4.3. CMD Resistance

The studies conducted by Hahn *et al.*, (1980) indicated the possibility of several genes responsible for resistance. The disease is best kept under control by the deployment of resistant varieties (Thresh *et al.*, 1997).

Fauquet and Fargette (1990) have identified six different components of resistance to CMD, such as field resistance (percentage of infected plants), vector resistance (number of adult whiteflies per plant), inoculation resistance, virus resistance, symptom severity, and virus diffusion resistance (development of symptoms over time).

The search for host plant resistance to CMD started in Africa in 1920s, but the most rewarding programmes began in Tanzania during the late 1930s (Jennings, 1994). Resistance to CMD was first obtained from a cross between cassava and its relative *Manihot glaziovii* Muller von Argau (Nichols, 1947). After three backcrosses into cassava to obtain suitable storage roots, the clone 58308 was selected, and for decades this clone and its derivatives have been extensively used as the main source of resistance in breeding for resistance to the disease. This has resulted in the selection of several improved cultivated cassava genotypes of the Tropical *Manihot* Selection (TMS) series, with resistance to CMD (Hahn *et al.*, 1980).

The IITA clones and seed stocks have also been widely distributed for use as parents, or for further evaluation in national breeding programmes and clones such as TMS30337, TMS30395, TMS30001 (MNga-1) and TMS30572 were notable.

Meanwhile some African landraces, the Tropical Manihot Esculenta series (TME), collected in West Africa have also been identified as resistant to CMD (Mignouna and Dixon, 1997) and served as alternative sources of resistance to increase the genetic base of resistance to the disease. The genetics of currently deployed CMD resistance in the TMS lines derived from *M. glaziovii* is polygenic, and this involves recessive genes that are additively inherited, with a heritability of over 60% (Hahn *et al.* 1989; Jennings, 1994; Mignouna,1997; Mba and Dixon, 1997).Quantitative genetic analysis has also shown allelic differences and complementarity of genes for resistance to CMD in the African germplasm (Lokko *et al.*, 2005).

Evaluations at IITA identified an excellent source of resistance to CMD in some Nigerian landraces (Dixon 2004), namely TME3, TME7, TME5, TME8, TME14 and TME28. This resistance is effective against all known strains of the virus, including the virulent Ugandan variant (UgV) (Akano *et al.*, 2002). CIAT, in collaboration with IITA in Ibadan, Nigeria, and with support from the Rockefeller Foundation, developed several molecular markers for this source of CMD resistance,

revealed to be controlled by a single dominant gene designated as *CMD2* (Akano *et al.*, 2002).

From IITA, CTCRI received few resistant clones through tissue culture and were evaluated for their yield and CMD resistance in India. The clone TMS30001 (MNga-1), having better resistance to CMD remained with inconspicuous symptoms. After, ten years of field trial , MNga-1 was found to be highly resistant and high yielding. So it was released as CMD resistant variety, Sree Padmanabha, for cultivation in Tamil Nadu. It was also used for developing more resistant clones through intervarietal hybridisation (Unnikrishnan *et al.*, 2004). In interspecific breeding programme, hybrids of *M. caerulescens* exhibited high level of resistance and were used as donor parents for transferring resistance to elite Indian cultivars (Sheela *et al.*, 2002, 2004, Unnikrishnan *et al.*, 2002, Pillai *et al.*,2001). Among the crosses, one interspecific hybrid cassava with *M. caerulescens* (CMC-1) showed very high resistance to CMD while other field tolerant interspecific hybrids showed varying degrees of incidence during the same periods.

2.5 Molecular markers

A molecular marker is a DNA sequence in the genome which can be located and identified. As a result of genetic alterations (mutations, insertions, deletions), the base composition at a particular location of the genome may be different in different plants. These differences, collectively called as polymorphisms can be mapped and identified. Molecular markers provide true representations of the genetic makeup at the DNA level. They are consistent and not affected by environmental factors. Molecular markers can be detected much before development of plants occur. A large number of markers can be generated as per the needs.

Molecular markers may be broadly classified into three categories in the chronological order of their development. The first Generation of markers were the hybridization based markers. The second generation of markers were the PCR based markers, as their assay was carried out through amplification using either arbitrary or sequence specific primers. The third generation markers are the most recent ones,

called as SNPs. Their detection requires sequence information. With the advancement in the field of DNA sequencing, SNPs have become very popular in the last few years.

Molecular markers have several advantages over the phenotype based markers that were previously available to plant breeders. They offer great scope for improving the efficiency of plant breeding by carrying out selection indirectly on the trait of interest and are used to construct the linkage maps, locate the genetic loci on specific chromosomes, characterize the germplasm for its genetic diversity and finally to exercise Marker Assisted Selection (MAS).

Microsatellites or SSR are tandem repeats of 1 – 6 nucleotides. For example, $(A)_n$, $(AT)_n$, $(ATG)_n$, $(GATT)_n$, $(CTACG)_n$, $(TACGAC)_n$, and so on. They are abundant in genomes of all organisms. The sequence of unique flanking regions of SSR can be used to design primers and carry out PCR to amplify SSR containing sequences. The polymorphism can be detected by agarose gel electrophoresis if differences are large enough (agarose gels can detect differences greater than 10 base pair), or polyacrylamide gel electrophoresis or capillary electrophoresis (sensitive enough to detect differences as low as 1 to 2 bases).

Marker systems such as Isozymes, RFLPS, RAPDs, SSRs and ESTs have been used to develop a cassava framework map consisting of two geographic divergent parents (Fregene *et al.*, 1997, Carvalho *et al.*,2001, Moyib *et a*l.,2007, Raghu *et al.*, 2007).

Akano *et al.* (2002) used BSA of landraces to identify an SSR marker linked to a dominant CMD-resistance gene. To date, two CMD-resistance genes *CMD1* and *CMD2* have been placed on the map (Akano *et a*l., 2002; Fregene *et al.*, 2001). The detection of more molecular markers associated with resistance to CMD in cassava will enhance the development of improved cassava varieties with high and durable CMD resistance.

Using single marker analysis (SMA), four CMD resistance markers were detected *viz*. SSRY28, SSRY235, SSRY44 and NS136. SSRY28 and SSRY235 were

located on linkage group G and SSRY44 and NS136 on linkage group P of cassava genetic map developed by Fregene *et al.* (1997).

The predominant source of resistance to the cassava mosaic disease (CMD) is known to be polygenic requiring evaluation in multiple environments to characterize resistant genotypes, which makes the detection of genes for resistance using segregation analysis inefficient. A study was conducted to determine the extent of genetic diversity among African cassava accessions resistant to the cassava mosaic virus disease (CMD), using simple sequence repeat (SSR) markers. In this study, molecular markers associated with resistance to CMD in a resistant landrace were identified, using F1 progenies derived from a cross between the CMD resistant landrace TME7 and the susceptible line TMS30555, as a first step in marker assisted breeding for CMD resistance. Bulk segregant analysis (BSA) on the parents, resistant and susceptible DNA pools, using simple sequence repeat (SSR) and amplified fragment length polymorphism (AFLP) markers revealed that an SSR marker, SSRY28-180, donated by the resistant parent was linked with resistance to CMD. Marker-trait association detected by regression analysis showed that the marker, accounted for 57.41% of total phenotypic variation for resistance. The analysis further showed that another SSR marker, SSRY106-207 and an AFLP marker, E-ACC/M-CTC-225, accounted for 35.59% and 22.5% of the total phenotypic variation for resistance, respectively (Lokko *et al.*, 2005).

To generate a genetic linkage map of cassava, 58 F_1 progenies from a cross between Rayong 90 (female) and Rayong 5 (male) were examined in amplification fragment length polymorphism (AFLP) and simple sequence repeat (SSR) analyses. A total of 469 polymorphic markers consisting of 378 AFLPs generated from 76 primer combinations and 91 SSRs were identified. The genetic linkage map generated in this study will be useful for genetic studies in cassava particularly for the identification of genetic markers linked to traits of interest (Kunkeaw *et al.* 2010).

The molecular markers RME1, SSRY28 and/or NS158, which link with the CMD resistance loci *CMD2* in cassava, were found in three cultivars (11Q, T7 and N13) with moderate resistance to CMD (Huiping Bi *et al.* 2010).

2.6 Molecular diagnostic techniques

The development of PCR based and Enzyme Linked Immuno Sorbent Assay (ELISA) diagnostic techniques enabled the separation of virus species and strains and the detection of mixed infections. Auxiliary buds and bark samples of resistant and susceptible (control) cassava genotypes either naturally infected under field conditions or experimentally inoculated by grafting were indexed for virus. Virus detection was carried out using ELISA and PCR to determine the distribution of the virus within the plant and elucidate the genotypes response to virus movement. Bud and bark samples were positive for virus on the susceptible genotype TME 117 than resistant genotypes TMS 3001 and TMS 91/02319. Detectable virus concentration was significantly lower in the buds of resistant genotypes (Ogbe *et al.*, 2002).

A multiplex PCR was developed for simultaneous detection of African cassava mosaic virus (ACMV) and East African cassava mosaic Cameroon virus (EACMCV) in cassava affected with cassava mosaic disease (CMD). One set of three primers consisting of an upstream primer common for both viruses and two downstream virus-specific primers were designed for simultaneous amplification of 368 base pair (bp) and 650 bp DNA fragments specific to the replicase gene of ACMV and EACMCV, respectively. Similarly, a second set of three primers were designed for simultaneous amplification of 540 bp and 655 bp fragments specific to the coat protein gene of EACMCV and ACMV, respectively. Primers that can amplify a 171 bp fragment of the large subunit of ribulose bisphosphate carboxylase oxygenase L were included as an internal control in these assays to determine the reliability of multiplex PCR. A simplified, cost-effective and rapid sample preparation method was adapted in place of the conventional plant DNA extraction procedure for multiplex PCR detection of ACMV and EACMCV. The method was validated using CMD-infected cassava samples obtained from farmers' fields in

Nigeria. The multiplex PCR is useful for reliable assessment of the prevalence of CMBs in epidemiological studies and for crop improvement and phytosanitary programs in African countries. (Alabi *et al.*, 2008)

2.7 Principal Component Analysis (PCA)

The binary data generated from the 36 cassava cultivars with 36 SSR primers were subjected to principal component analysis (PCA) by Sree Lekha *et al.,* 2011. The first three principal components contributed to 28.16 %, 16.76 % and 8.11 %, respectively of the total variation present in the data. PCA helped to identify primers which contributed much to the variation present in the population.

Kawuki *et al.* (2009) assessed variability in 848 landraces and 553 cultivars of cassava from seven different countries by using principal component analysis (PCA). The first two PCs accounted for 56 % and 10 % of the genetic variation in landraces from the seven countries.

Principal component analysis using 10 quantitative traits indicated that the first three principal components (PC1, PC2, and PC3) contributed 55.68 % of the total variation observed among the 150 cassava genotypes. PC1 contributed 22.68 % of the total variation and was associated with root yield, number of roots per plant, harvest index and number of leaves. The second PC contributed 20.36 % of the total variation and was associated with the height at branching, plant height, number of leaf lobes and length of petiole. The third PC axis with a contribution of 12.64 % was associated with plant height, angle at branching, and root dry matter content (Adjebeng *et al.,* 2016).

Babu Rao *et al.* (2016) assessed variability in 18 cassava accessions by using principal component analysis and observed that, four components with eigen value more than one explained 84.17 % of the cumulative variation among traits. Principal component one (PC1), with eigen value of 4.48, contributed 31.99 % of the total variability, PC2, with eigen value of 3.31, revealed 23.63 % of total variability, PC3 had eigen value of 2.60 and contributed with 18.58 % to the total observed variability, while PC4, with eigen value of 1.39, accounted for 23.63 % of total

variability observed among the 18 cassava genotypes. PC1 was more related to petiole length, number of leaves per plant, total leaf area, plant height, stem diameter, number of tubers per plant, tuber dry matter content, HCN content and tuber yield per hectare.

In a study by Djirabaye *et al.,* 2016, the first six axes of the principal component analysis explained 83.27 % of the total variation (Djirabaye *et al.,* 2016). The length and median diameter of the roots, diameter of the stem, weight of fresh roots and Harvest Index were correlated to axe 1 and contributed to 29.56 % of the total variation.

Sixteen cassava genotypes were evaluated for variability by principal component analysis (PCA) based on five agronomic traits by Patrick *et al.,* 2016. It revealed that the first three principal components (PCs) accounted for 83.5 % of the total variation. The PC1 accounted for 44.0 % total variation with an Eigen value of 2.20. The major contributors for the first PC were total fresh biomass, root size and fresh root yield. PC2 and PC3 accounted for 20.5 % and 18.9 % of variability respectively. The major factors for PC2 were total fresh biomass, harvest index, leaf retention and root size. For PC3 the major factors were harvest index, leaf retention and root size.

3. MATERIALS AND METHODS

The study entitled "Molecular marker analysis for cassava mosaic disease resistance" was carried out at the Division of Crop Improvement, ICAR- Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram during 2013-2014. In this chapter, detailed information of the experimental material and procedures used in the study are elaborated.

3.1 Source of plant material

In the study of molecular marker analysis for cassava mosaic resistance, the molecular characterization using SSR markers were carried out among the following cassava genotypes (Table1). The plant materials used for the study comprised of 60 accessions of cassava collected from germplasm /pre breeding lines maintained in the field gene bank at ICAR-Central Tuber Crops Research Institute, Thiruvananthapuram. Genotypes used for the study comprised of resistant, susceptible and recovery type genotypes with varying levels of resistance to cassava mosaic disease.

3.2 Molecular marker analysis

3.2.1 Source of primers:

The primers were selected from literatures based on linkage with CMD resistance. Primers were collected from SSR primer collection in the Division of Crop Improvement, ICAR- CTCRI, Sreekariyam. List of SSR primers used for molecular characterization is given in Table 2.

3.2.2 Glass wares and materials in Molecular Biology Lab

Eppendorf tubes, pestle and mortar, micropipette tips for 1 ml, 200 µl and 10 µl, PCR tubes were autoclaved and used. Eppendorf tube stand, PCR tube holders,

micropipettes, ice bags, polythene covers, labels, wipes, bottles, spatula are other materials used for molecular work.

Table 1. Accessions of wild cassava used for study

Sl.no	Name of sample	Genotype	Source
1	Sree Padmanababha	Released variety	CTCRI
2	MN1	Pre breeding line	CTCRI
3	MNGA8	Pre breeding line	CTCRI
4	MN2	Pre breeding line	CTCRI
5	CR43-11	Pre breeding line	CTCRI
6	1135-D	Pre breeding line	CTCRI
7	11S18	Pre breeding line	CTCRI
8	11S17	Pre breeding line	CTCRI
9	IMS1-2	Pre breeding line	CTCRI
10	IMS1-9	Pre breeding line	CTCRI
11	I-13-S5-20	Pre breeding line	CTCRI
12	I-13-S5-17	Pre breeding line	CTCRI
13	I-99/14/3-S2-1	Pre breeding line	CTCRI
14	I-99/14/3-S2-2	Pre breeding line	CTCRI

15	IES1-1	Pre breeding line	CTCRI
16	IES1-3	Pre breeding line	CTCRI
17	8W5	Pre breeding line	CTCRI
18	9S127	Pre breeding line	CTCRI
19	CI273	Landrace	CTCRI
20	CO-1	Released variety	CTCRI
21	CR43-2	Pre breeding line	CTCRI
22	SreeReksha	Released variety	CTCRI
23	H226	Released variety	CTCRI
24	CR20A-2	Pre breeding line	CTCRI
25	CR43-1	Pre breeding line	CTCRI
26	CE185	Exotic accession	CTCRI
27	CE775	Exotic accession	CTCRI
28	CR54A3	Pre breeding line	CTCRI
29	7IVC4	Pre breeding line	CTCRI
30	9S132	Pre breeding line	CTCRI
31	CR21-10	Pre breeding line	CTCRI
32	CR43-7	Pre breeding line	CTCRI

33	CR52A41	Pre breeding line	CTCRI
34	Sree Visakham	Released variety	CTCRI
35	9S172	Pre breeding line	CTCRI
36	99/14/S17	Pre breeding line	CTCRI
37	M4	Popular variety	CTCRI
38	Sree Swarna	Pre breeding line	CTCRI
39	CR35-8	Pre breeding line	CTCRI
40	CR54A-19	Pre breeding line	CTCRI
41	Sree Apoorva	Landrace	CTCRI
42	CI-896	Landrace	CTCRI
43	IPS1-1	Pre breeding line	CTCRI
44	Sree Harsha	Released variety	CTCRI
45	9S165	Pre breeding line	CTCRI
46	H165	Released variety	CTCRI
47	CMR104	Pre breeding line	CTCRI
48	TCR56	Landrace	CTCRI
49	CMR129	Pre breeding line	CTCRI
50	TCR63	Pre breeding line	CTCRI

51	CR20A4	Pre breeding line	CTCRI
52	S1284	Pre breeding line	CTCRI
53	CR63-3	Pre breeding line	CTCRI
54	CR59-8R	Pre breeding line	CTCRI
55	Sree Athulya	Released variety	CTCRI
56	PDP1	Pre breeding line	CTCRI
57	Vellayani Hraswa	Released variety	CTCRI
58	Sree Vijaya	Released variety	CTCRI
59	CR57-6	Pre breeding line	CTCRI
60	9S98	Pre breeding line	CTCRI

3.2.3 Instruments

The equipments *viz.* ice machine(Icematic),electronic precision balance(Afcoset), water bath(MEMMERT), vortexer, table top centrifuge(Labnet), nanodrop spectrophotometer, pH meter, electrophoresis apparatus(SCIE PLAS), autoclave(SANYO), microwave oven (IFB), hot air oven, autoclave, PCR machine, alpha imager, UV transilluminator, deep freezer(-20^{0}C,-80^{0}C), refrigerator and distilled water unit were used for the study.

3.3 DNA extraction

From the field, leaf samples of CMD resistant, CMD susceptible and recovery cassava varieties were collected. From the collected leaf samples, DNA was isolated

by manual method (C-TAB method). The quality of isolated DNA was checked using agarose gel electrophoresis. The presence and absence of virus was confirmed by Multiplex PCR using ICMV and SLCMV specific primers.

The extraction of genomic DNA from plant material requires cell lysis inactivation of cellular nucleases and preparation of desired genomic DNA from cellular debris. The cetyl trimethyl ammonium sulfate is suitable for the elimination of polysaccharides polyphenolic compounds and helps in the recovery of pure DNA of good quality. Plant cells can be lysed with ionic detergent CTAB, and it forms an insoluble complex with nucleic acids in a low salt environment. Under these conditions, polysaccharides; phenolic compounds and other contaminants remain in the supernatant and can be washed away. The DNA complex is solubilized by raising the salt concentration and precipitated with ethanol isopropanol.

3.3.1. Sample collection

Fresh tender young leaves of cassava accessions were collected from germplasm of CTCRI, Thiruvananthapuram. Leaves were collected in polythene bags and transferred to lab in ice box.

3.3.2. Manual method

DNA was extracted from fresh and tender young leaves using modified CTAB (Cetyl Trimethyl ammonium Bromide) method.

From the samples collected 200 mg of leaf samples were weighed and grinded in frozen liquid nitrogen with 1ml CTAB buffer. The grinded samples were taken in microfuge tubes and it was incubated at 65℃ for 30 minutes. Then it was centrifuged at 12000 rpm for 10 minutes at 4° C. The supernatant was collected and equal volume of chloroform: isoamyl alcohol (24:1) was added and mixed by inversion. Then again centrifuged at 12000 rpm for 10 minutes at 4℃ and supernatant was taken. To the supernatant 5µl RNase was added and mixed by inversion. Then it was incubated at

65℃ for 10minutes.To that equal volume of chloroform: isoamyl alcohol was added and mixed by inversion. After that it was centrifuged at 10,000 rpm for 10 minutes. Supernatant was collected. To the supernatant equal volume of chloroform: isoamyl alcohol was added and was mixed by inversion. It was centrifuged at 10,000 rpm for 10 minutes. Supernatant was collected. To the supernatant added double volume ice cold ethanol. Then centrifuged at 10,000 rpm for 10 minutes. Pellet was saved. Washed the pellet in 70% ethanol. Pellet was then air dried and dissolved in 1x TE buffer.

3.3.3. Checking the quality of extracted DNA

Agarose gel electrophoresis:

Agarose gel electrophoresis separates DNA fragments according to their size. An electric current is used to move the DNA molecules across an agarose gel, which is a polysaccharide matrix that functions as sieve to "catch" the molecules as they are transported by electric current. When DNA is on a field with an electric current, these negatively charged DNA molecules (due to the presence of phosphate molecules) migrate towards the positive end of the field. The agarose gel being in a cross linked matrix, impart a resistance to the movement of DNA molecules and the smaller ones navigate the mesh faster than the longer ones, and hence separation of the molecules based on size and conformation occurs. Ethidium bromide is an intercalating dye, which intercalate between bases that are stacked in the centre of DNA helix. The fluorescence under UV and the DNA molecules can be visualized. Unknown fragments are compared with the ladder fragments (size known) to determine the approximate size of the unknown DNA bands.

The edges of a clean, dry glass plate which was supplied with the electrophoresis apparatus was sealed with a tape to make a mould and placed on a perfect horizontally leveled plateform.1.5% agarose gel was used to check the quality and integrity of the extracted DNA. 1.5% agarose solution was prepared by 0.75g agarose in a conical flask. Added 50 ml of 1x TBE buffer and gently boiled the

solution in a microwave oven (IFB) with occasional mixing until all particles are completely dissolved. Allowed to cool to 45^0C. To that added 1.2µl of ethidium bromide. Prepared the gel mould and kept the comb in position. Then the cooled gel was poured and kept till agarose was solidified. Filled the horizontal electrophoresis chamber with 1x TBE buffer and removed the comb from the gel and placed it in the electrophoresis chamber. Loaded the PCR product (3µl) mixed with 2µl bromophenol blue dye into the wells. A 100bp DNA ladder (Genei) was used as standard. Then the gel was run at 80 V for 30 minutes. The run was stopped after the dye front reached $3/4^{th}$ of the gel. Then it was visualized under ultraviolet light using a gel documentation system. Horizontal gel electrophoresis unit was used to run the gel. Spectrophotometer was used to check the quality of the DNA.

3.4. Multiplex PCR for virus detection

The DNA isolated from the samples was subjected to PCR, for the detection of ICMV and SLCMV. PCR amplification was carried out using ICMV and SLCMV specific forward and reverse primers.

Primer sequence:

MULTI_IC_A_F: 5'- GCT TCT GGC ATT TGT AN-3'

MULTI_SL_A_F: 5'- TGT AAT TCT CAA AAG TTA CAG TCN-3'

MULTI_IC/SL_A_R: 5'- ATA TGG ACC ACA TCG TGT CN-3'

3.4.1PCR reaction mixture:

The amplification was performed in a total volume of 20µl, PCR mix containing master mix: 2µl, primer1: 0.5µl, primer2: 0.5µl, primer3: 0.5µl, taq DNA polymerase: 0.2µl, template DNA: 3µl , SDW: 13.1µl.The reaction mixture along with template DNA samples was amplified in PCR machine (Bio Rad).

Then the PCR products were eletrophoresed in 2% agarose at 75V in TBE(Tris Borate EDTA) buffer of pH 8. 100bp DNA ladder plus was used as standard. The gel was observed and photographed using UV Transilluminator and documented image on Alpha imager. The images finally scored to detect polymorphism.

Table3. Details of PCR reaction mixture for multiplex PCR

Ingredients	**Stock concentration**	**Required concentration**	**Required volume for one reaction (20µl)**
Buffer	10X	1X	2 µl
dNTP mix	25mM	0.25mM	0.2 µl
P_1	10µM	0.25mM	0.5 µl
P_2	10µM	0.25mM	0.5 µl
P_3	10µM	0.25mM	0.5 µl
Taq DNA polymerase	5U/ µl	1U	0.2 µl
Template DNA	10ng/ µl	30ng	3.0 µl
Sterile distilled water	For a final volume of 20 µl		13.1 µl

3.4.2 PCR programme:

Initial denaturation : $94^0$2 min

Denaturation : 94^0C for 1min

Annealing : 56^0C for 1min

Extension : 72^0C for 2 min

} 30 cycles

Final extension : 72^0C for 5 min

The whole program is of 30 cycles and the holding temperature is at 4^0C.

3.5SSR ANALYSIS

3.5.1 SSR primer screening

SSR primers were screened to detect polymorphism among the genotypes with respect to cassava mosaic disease resistance. The different primers were screened by using bulk DNA of resistant, susceptible and recovery type varieties. Based on preliminary screening, the primers selected for characterization of the genotypes were SSRY 28, SSRY 44, SSRY 45, SSRY105, SSRY 100, SSRY 234, SSRY MeE 19.

3.5.2 PCR Amplification

The reaction mixture (25µl) consists of 1x buffer (Banglore Genei).

Table4. Details of PCR reaction mixture for SSR marker analysis

Components	Stock concentration	Required concentration	Volume for one reaction (25µl)
Buffer -(without $MgCl_2$)	10X	1X	2.5 µl
$MgCl_2$	25 mM	1.5 mM	1.5 µl
dNTP mix	25 mM	0.4 mM	0.4 µl
Primer (F)	10 mM	0.4 mM	1.0 µl
Primer (R)	10 mM	0.4 mM	1.0 µl
Taq DNA polymerase	5U/ µl	1U	0.2 µl
Template DNA	30ng/ µl	30ng/ µl	3.0 µl
Sterile distilled water	For a final volume of 25 µl		15.4 µl

Table 2.List of SSR primers used for molecular characterization

Sl No.	Primer name	Sequence(forward)	Sequence(reverse)	Product Size
1	SSRY 28	5'-TTGACATGAGTGATATTTTCTTGAG- 3'	5'GCTGCGTGCAAAACTAAA AT -3'	180
2	SSRY 44	5'GGTTCAAGCATTCACCTTGC-3'	5'GACTATTTGTGATGAAGGCTTGC-3'	194
3	SSRY 45	5'-TGAAACTGTTTGCAAATTACG A-3'	5'TCCAGTTCACATGTAGTTGCT-3'	228
4	SSRY105	5'-GGAAACTGCTTGCACAAAGA -3'	5'CAGCAAGACCATCACCAGTTT-3'	225
5	SSRY100	5'ATCCTTGCCTGACATTTTGC-3'	5'TTCGCAGAGTCCAATTGTTG- 3'	210
6	SSRY 234	5'TTGCCAGAACCCTAGGAGTAA-3	TGTCCCTAGGAAGGTTGCTG	196
7	SSRY MeE19	5'- TTCTCGTCG GCTCCTTTC TA-3'	5'-CCCCACTTGATCTGCCTT TA- 3'	208

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3.6 PCR conditions

PCR was carried out in Thermal cycler (Biorad). The program for SSR primers are as follows:

Initial denaturation : 95^0 for 3 min

Denaturation : 95^0 for 40 sec

Annealing : 56^0 C for 40 sec } 30 cycles

Extension : 72^0 C for 1 min

Final extension : 72^0C for 5 min

The amplified products were resolved in a 2% agarose gel using 100bp ladder).

3.6.1 Real time quantitative estimation to study the presence of virus in field tolerant plants

The real time PCR assay (q- PCR) was done as per the method described by Deepthi (2017), which as follows:

3.6.2 Primers used for qPCR

For absolute quantification of DNA A was done using the following primers in qPCR.

Sy- SLCMV A_F (5' TTCATCCATCCATATCTT 3')

Sy- SLCMV A_R (5' CCATATAGGTAAGGTCAT 3')

All primers were tested using DNA extracted from cassava leaves affected with SLCMV and total DNA from non- infected plants as controls.

3.6.3 PCR set up

Working solutions of primers, DNA samples from plants and plasmid dilutions were stored at -20^{0}C and was not thawed more than twice prior to use. Master mixes and water kept in small aliquots at 4^{0}C. To avoid DNA contamination and carry over, DNA extraction, preparation of master mixes and set up of PCR was done in separate rooms. All buffers and solutions for qPCR were pipetted using sterile filter tips and composed on ice to avoid errors from evaporation during pipetting. For each sample, triplicate qPCR reactions were composed in 96-well microplate formats and microplates were sealed immediately after loading. Samples, standards, non- template controls (NTC) and negative water controls were run on each plate to minimize variations between plates.

Table 5.Standard real time PCR mix (25µl)

Maxima SYBR Green q PCR Master Mix(2X)	12.5 µl
Forward primer	0.3 µl
Reverse primer	0.3 µl
Template DNA	>100ng
Water, nuclease free	To 25 µl
Total volume	25 µl

3.6.4. Real time PCR assay for sensitive detection and quantification of SLCMV DNA in cassava using SYBR green.

A Sybr Green qPCR assay for SLCMV was established and conducted during the growing season to quantify the viral DNA. Absolute quantification of DNA A and DNA B of SLCMV was done using primer Sy- SLCMV A_ F, Sy- SLCMV A_ R. For each DNA sample, three technical replicate reactions were prepared. A qPCR master mix was prepared by adding 12.5 µl of Maxima SYBR Green qPCR Master

mix (2X), 0.3 µM forward and reverse primer each, ≤100ng of template DNA and nuclease free sterile water to a volume of 25µl. The programme consists of 40 cycles, each consisting of 95^{0}C for 15 seconds (denaturation) 53^{0}C for 20 seconds (annealing) and 72^{0}C for 20 seconds (extension). All real time PCR assays were performed in an Eppendorf realplex Mastercycler (Eppendorf, Germany).

To determine copy numbers of DNA- A molecules the qPCR was calibrated.

3.7PAGE: Sequencing Gel Apparatus and its Reagents

The amplified SSR products were run on vertical PAGE gel. At first the large plate and small plate were washed and wiped with distilled water followed by wiping with absolute ethanol. Then laboline (repellent) was uniformly spread in large plate whereas bind silane was used in small plate. The spacer and comb was kept on it (coated side towards spacer). Assembled the unit with side clamp, bottom is assembled and the unit is locked. The prepared 6 % acrylamide gel (appendix) added with 10 % ammonium per sulphate and TEMED (1: 10 ratio) is injected using a syringe, uniformly in between the plates without air bubbles. After solidification, the pre- running was done for 15 minutes. The PCR products with loading dye were denatured (94^{0}C for 5 minutes) using thermal cycler and 4 µl of the denatured PCR products were loaded in gel. The gel was run at 100 W constantly for 20 to 45 minutes duration depending upon the size of PCR products loaded in the gel. After running, the small plate was separated from the assembly and silver staining was carried out.

3.7.1Silver staining

The staining procedure has different steps. It started with the fixing of the gel in 10% acetic acid for 30 minutes and staining with 0.1% $AgNO_3$for 10 seconds, developing (ice cold 3% Na_2CO_3, 3 ml formaldehyde and 400 µl sodium thiosulphate) until the ladder properly run, followed by 1 min fixing and washing for

30 minutes. The scoring was done after the gel get dried. The gel plate was also scanned to store the image for future use.

Sequencing gel apparatus consists of two plates (Large and Small), Clamps, Spacers, Comb (two type: (Transparent) and pseudo comb (Red color)), Buffer tank and power pack.

- ❖ Gel plate clearing:

Small and Large plate: Initially wiped with distilled water and then with alcohol.

- ❖ Application of Bind silane:

For small plates, on the gel forming surface.

Preparation: 99.5% ethanol: 496.5 ml

0.5% acetic acid: 2.5 ml

Bind silane: 1ml

- ❖ Application of repellent

For large plate only. Laboline can be used.

- ❖ 40% Acrylamide (100 ml)

Acrylamide: 38 g

Bis acrylamide: 2 g. Made up to 100ml.

- ❖ Gel solution preparation (6%)

Urea : 42 g

10x TBE : 10 ml

Double distilled water: 15 ml

To dissolve warm for 30 sec and filtered it to 15 ml 40% ml acrylamide. Made up the volume to 100 ml by adding distilled water. Then to that at the time of gel filling added freshly prepared 600µl APS (10%) and 60µl TEMED.

- PAGE dye preparation (50ml)
  Formamide: 49 ml
  Xylene cyanol: 500 mg
  Bromophenol blue: 50 mg
  0.5 M EDTA: 1ml
- Running buffer
  2L of 1X TBE buffer
- Fixer (10%)
  Acetic acid: 200 ml
  Distilled water: 1800 ml
- Developer
  Sodium carbonate: 60g
  Sodium thiosulphate: 400µl (at the time of use)
  Formaldehyde (37%): 3ml (at the time of use)
  Distilled water: 2000L
- Silver stain
  Silver citrate: 2g
  Formaldehyde: 3 ml
  Distilled water: 2000L
- Washing solution
  2% Sodium hydroxide
- Steps in Silver staining
  1. Kept the plates 20 minutes in fixer.
  2. Washed in double distilled water for 5 min.

3. Treated with silver stain for 20 min.
4. Washed in distilled water.
5. Treated with developer till the marker bands clear.
6. Treated with stopper for 5 min.

3.8Morphological analysis

The plant and tuber morphology was identified using descriptors of cassava. In the study13 descriptors were used for characterization.

3.8.1List of qualitative characters used for morphological analysis

1. Stem colour : Recorded the colour on the middle third of the stem was recorded during active vegetative growth.
2. Petiole colour : Recorded the most frequent occurrence from the leaves taken from mid height position during active vegetative growth
3. Colour of unopened leaf: The predominant colour of the unopened leaf was recorded at three months after planting.
4. Leaf colour: Observed a leaf from the middle of the plant and recorded the most frequent occurrence. No intermediates were allowed.

3.8.2 List of quantitative characters used in morphological analysis

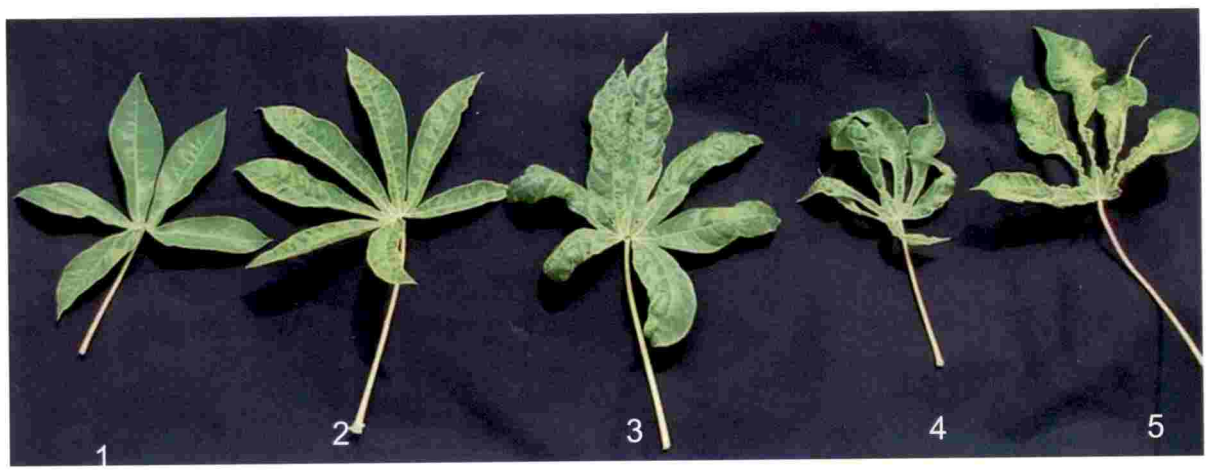
1. Leaf retention : No of leaves retained nine months after planting was recorded
2. Harvest index : It is calculated using the following formula

   Harvest Index (HI) = Tuber Yield (kg)/Foliage weight (kg)

3. Stem weight : Total weight of the stem was recorded and expressed in kg
4. Leaf weight : Total weight of the leaves was recorded and expressed in kg
5. Weight of tubers: Total weight of the tubers/plant was recorded and expressed in kg
6. Total biomass : Total weight of the biomass(stem=leaves=tubers) was calculated and expressed in kg
7. Number of tubers: Total number of tuber per plant was recorded.

8. Stem girth: The girth of was measured with a tape at mid portion of the stem and expressed in centimeters.
9. Cassava mosaic disease
10. Plant height: It was measured in centimeters from the base of the stem to tip of the growing point with the measuring scale and expressed in centimeters.
11. CMD disease scoring (1-5 scale)

Different grades of CMD infection were scores from 1 to 5 scales (Hahn 1980)



- Grade 1 – No symptom
- Grade 2 - Mild chlorosis, mild distortions at bases of most leaves
- Grade 3 - Pronounced mosaic pattern on most leaves, narrowing and distortion of the lower one-third of the leaflets
- Grade 4 - Severe mosaic distortion of two thirds of most leaves and general reduction of leaf size and stunting of shoots

  Grade 5 - Very severe mosaic symptoms on all leaves, distortion, twisting, mis-shapen and severe leaf reduction of most leaves accompanied by severe stunting of plants.(Photo Courtsey: Dr.T.Makesh Kumar)

3.9Data analysis

50

3.9.1 Scoring of bands in the gels

The bands were scored visually as 1 and 0 based on the presence and absence of bands respectively. The data scoring was done for all 7 primers across 60 cassava accessions. The scored data was analyzed in NTSYpc programme.

3.9.2 Statistical analysis of molecular data

Clear and reproducible bands were only selected for scoring. 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. The scored data was analyzed using NTSYpc (version 2.21 f) software (Rohlf,2009). A binary matrix of presence/ absence of alleles was obtained used in clustering analysis by Unweighted Pair Group Method with Arithmetic Average (UPGMA). The SAS statistical package was used for hierarchal cluster analysis based on Euclidean distance. Dendrogram grouping the 60 accessions based on SSR marker data was constructed based on complete linkage method using Jaccard's distance. The similarity was calculated for qualitative data with SM (Simple matching) coefficient and used for cluster analysis. Principal component analysis (PCA) was done using the PROC PRINCOMP procedures of SAS (1999).

4. RESULT

The results of study entitled "Molecular marker analysis for cassava mosaic disease resistance" carried out at the Division of Crop Improvement, ICAR- Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram during 2013-2014 are presented in this chapter.

4.1 Molecular characterization of CMD resistant clones

The isolation of DNA from fresh leaf samples was carried out using the CTAB method and the DNA isolation kit (QUIAGEN). The genomic DNA was isolated from sixty cassava varieties and the quality was analyzed by Agarose gel Electrophoresis (1.5% agarose) and spectrophotometer (Table6) and pure DNA was obtained by RNase treatment.

4.1.1Multiplex DNA for virus detection

Cassava samples collected from CTCRI were diagnosed for the presence of Indian Cassava Mosaic Virus (ICMV) and Sri Lankan Cassava Mosaic Virus (SLCMV) through multiplex PCR analysis. It was done using primers specific for ICMV and SLCMV. Multiplex PCR was useful in differentiating Sri Lankan Cassava Mosaic Virus (SLCMV) which produced 600bp fragment and Indian Cassava Mosaic Virus (ICMV) that produced 900bp fragment after multiplex PCR. 11S18-0T, I-99/14/3-S2-2, I-13-S5-20, IES1-3, 9S127, C0-1, CE775, 9S132, Sree Visakham, 99/14/S17, M4, TR5-3, CI-896, Sree Harsha, 9S165, H165, TCR56, TCR63, CR63-3, Sree Athulya, Vellayani Hrazwa, 9S98 were found to be infected with virus.

Multiplex PCR helped to differentiate resistant and susceptible varieties even in plants that do not show any cassava mosaic disease symptoms.

SL .no:	Name	DNA conc (ng/µl)	SL no:	Name	DNA conc (ng/µl)
1	Sree Padmanababha	1566	31	CR21-10	578
2	MN1	2876	32	CR43-7	1298
3	MNGA8	490	33	CR52A41	2674
4	MN2	967	34	Sree Visakham	3875
5	CR43-11	1467	35	9S172	1243
6	1135-D	2789	36	99/14/S17	2476
7	11S18	2346	37	M4	1465
8	11S17	789	38	Sree Swarna	1357
9	IMS1-2	568	39	CR35-8	980
10	IMS1-9	789	40	CR54A-19	2570
11	I-13-S5-20	2400	41	Sree Apoorva	976
12	I-13-S5-17	1398	42	CI-896	1788
13	I-99/14/3-S2-1	908	43	IPS1-1	3456
14	I-99/14/3-S2-2	1788	44	Sree Harsha	986
15	IES1-1	1805	45	9S165	2784
16	IES1-3	865	46	H165	3085
17	8W5	390	47	CMR104	457
18	9S127	2576	48	TCR56	1309
19	CI273	3298	49	CMR129	3005
20	CO-1	1689	50	TCR63	1567
21	CR43-2	2100	51	CR20A4	567
22	SreeReksha	2091	52	S1284	876
23	H226	1634	53	CR63-3	1209
24	CR20A-2	1200	54	CR59-8R	1873
25	CR43-11	568	55	Sree Athulya	657
26	CE185	876	56	PDP1	1277
27	CE775	900	57	Vellayani Hraswa	1765
28	CR54A3	1356	58	Sree Vijaya	786
29	7IVC4	587	59	CR57-6	986
30	9S132	689	60	9S98	1678

4.2 Screening of cassava accessions for CMD resistance

Visual scoring was done in a 0-5 range scale. Score of 0 indicates that the variety is highly resistant and the score of 5 indicates the variety is highly susceptible. From the 60 accessions evaluated, 39 were resistant and 21 were found to be susceptible.

4.3 Primer Screening for Identification of SSR Markers Linked to Cassava Mosaic Disease Resistance

The SSR primers selected based on review of literature were screened by Bulk Segregant Analysis. Primers screened include SSRY 28, SSRY 44, SSRY 45, SSRY 100, SSRY 105, MeESSRY19 and SSRY 234. Out of the 7 primers screened SSRY28, SSRY 44 showed association with cassava mosaic disease resistance.

The marker SSRY 28 resulted in 2 bands having 180bp and 200bp. All are found to be polymorphic. The percentage of polymorphism for this marker was 100%.

The marker SSRY 44 amplified 3 alleles (230bp, 250bp and 280bp). Polymorphic bands were obtained and the percentage of polymorphism was 100%.

The marker SSRY 45 amplified three alleles (200bp, 250bp and 280bp) and all the three alleles were found to be polymorphic.

The percentage polymorphism of the different SSR primers is depicted in Fig.1 to Fig7.

From the markers studied it was found that SSRY28 amplified the expected product size of 180 bp in eighteen highly resistant genotypes. However the allele was amplified in susceptible varieties like Vellayani Hrazwa too. Hence it was not found to be highly linked to CMD resistance. SSRYMe19 amplified the allele in eighty seven percent of the resistant genotypes. However it was amplified in 95 percent of

the susceptible genotypes too. None of the alleles of the markers studied could distinguish completely between CMD resistant and CMD susceptible genotypes. Among the markers tested SSRY44 amplified the allele 250bp in 20 highly resistant genotypes and was amplified only in five susceptible genotypes. Hence SSR Y 44 (250 bp) can be used in marker assisted selection for CMD resistance in cassava after further validation.

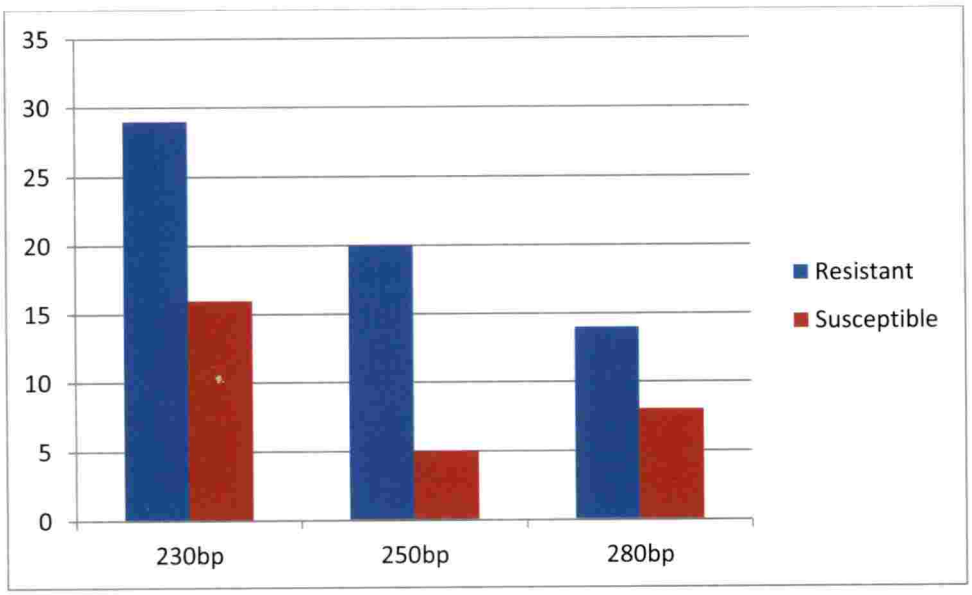


Fig.1. Percentage distribution of resistant genotypes among different alleles of SSRY 44

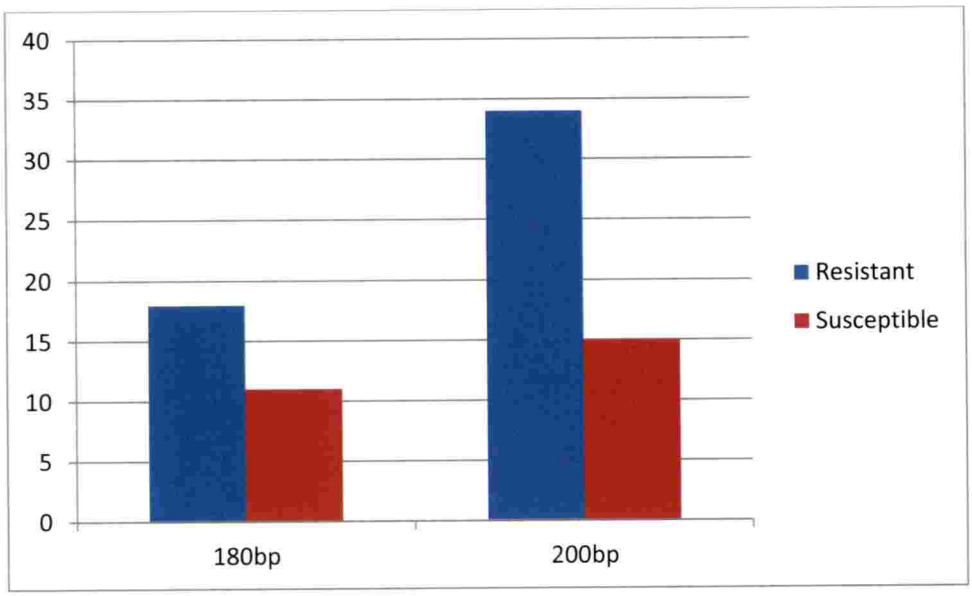


Fig.2. Percentage distribution of resistant genotypes among different alleles of SSRY 28

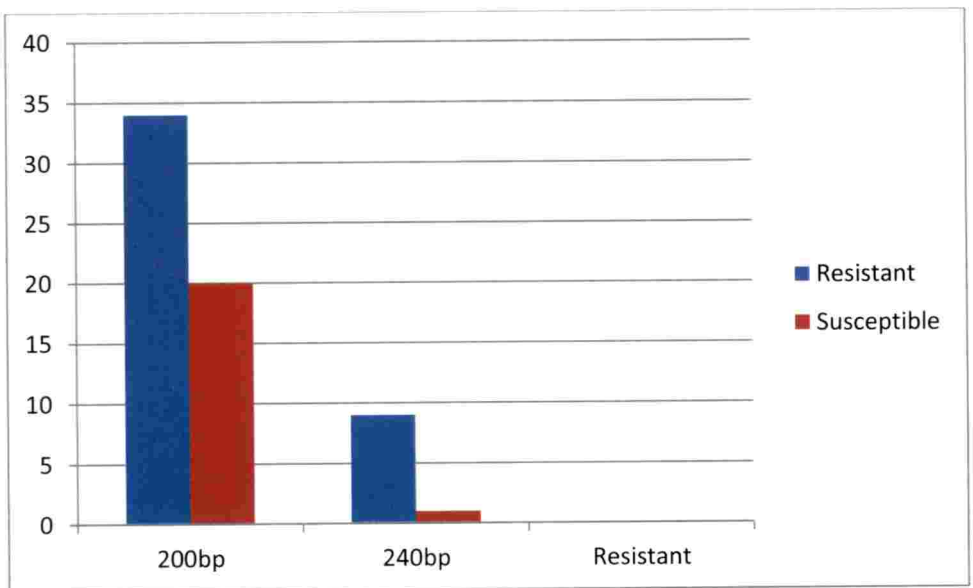


Fig.3. Percentage distribution of resistant genotypes among different alleles of SSRY 45

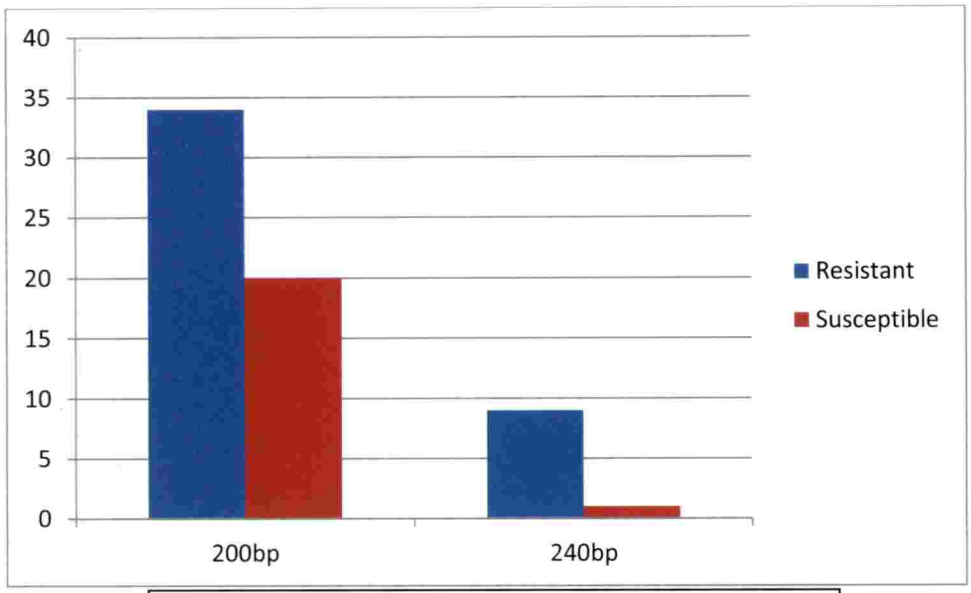


Fig.4. Percentage distribution of resistant genotypes among different alleles of SSRYMeE19

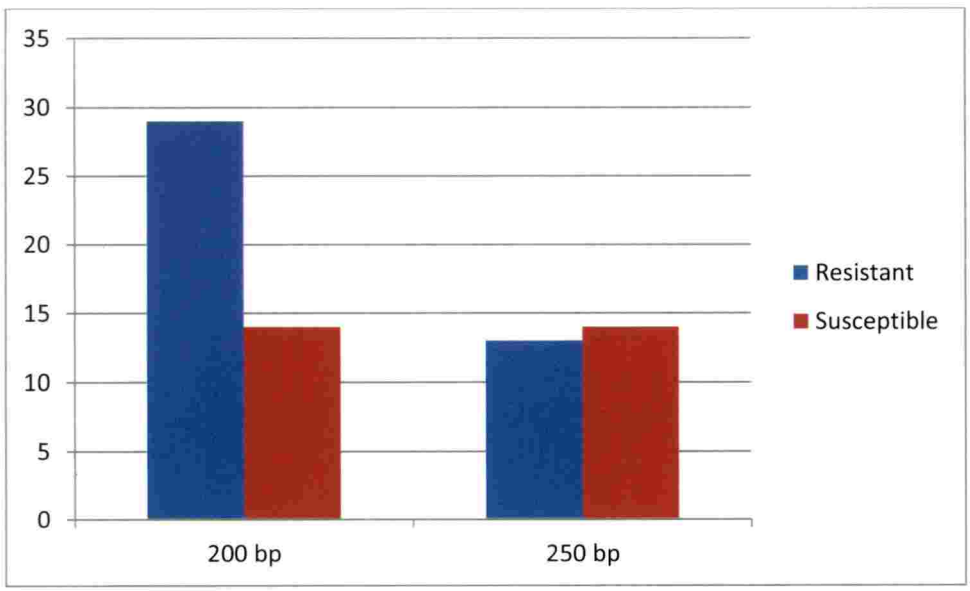


Fig.5 Percentage distribution of resistant genotypes among different alleles of SSRY105

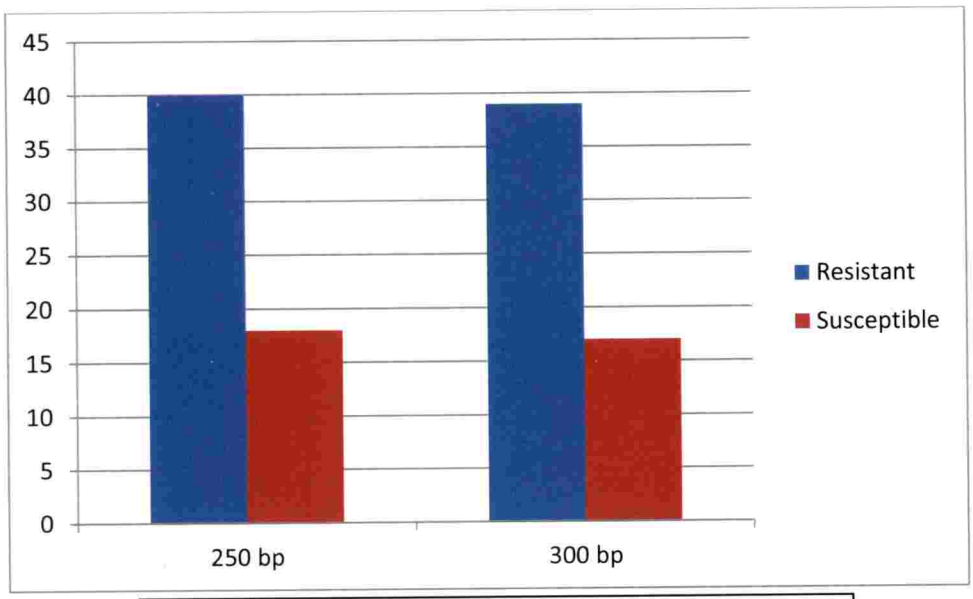


Fig.6. Percentage distribution of resistant genotypes among different alleles of SSRY234

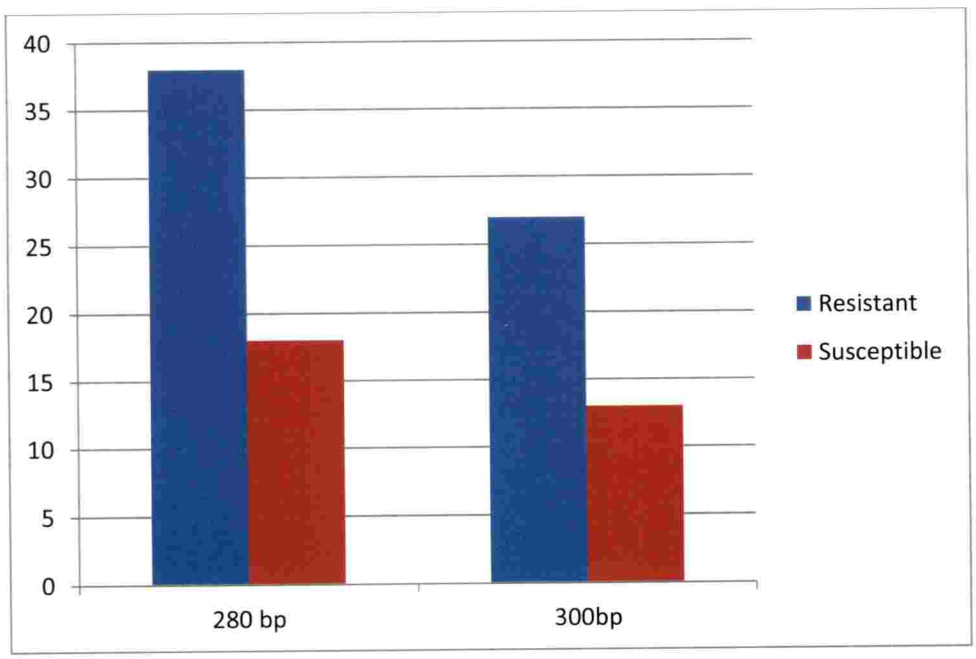


Fig.7 Percentage distribution of resistant genotypes among different alleles of SSRY100

Marker	Total No. of alleles	No. of polymorphic alleles	% of polymorphism	Major Allele Frequency	He	PIC	Gene Diversity
SSRY-28	2	2	100	0.6750	0.4747	0.3567	0.3886
SSRY-44	3	3	100	0.6556	0.6297	0.5586	0.4419
SSRY-45	3	3	100	0.7667	0.6096	0.5218	0.3578
SSRMe9	2	2	100	0.8667	0.2637	0.2289	0.2289
SSRY105	2	2	100	0.7000	0.4973	0.3737	0.4111
SSRY234	2	2	100	0.9500	0.4998	0.3749	0.0944
SSRY100	2	2	100	0.8417	0.4880	0.3690	0.2353

Table 7. Simple sequence repeat fingerprinting results with number of alleles per locus, major allele frequency, Expected heterozygosity, PIC values and Gene diversity index.

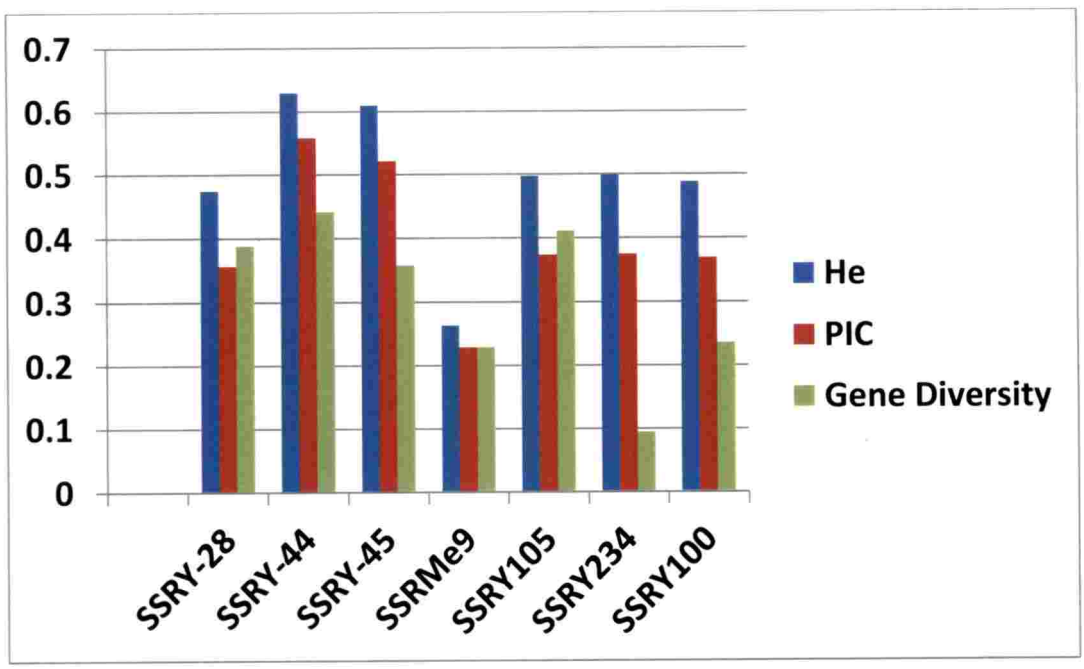
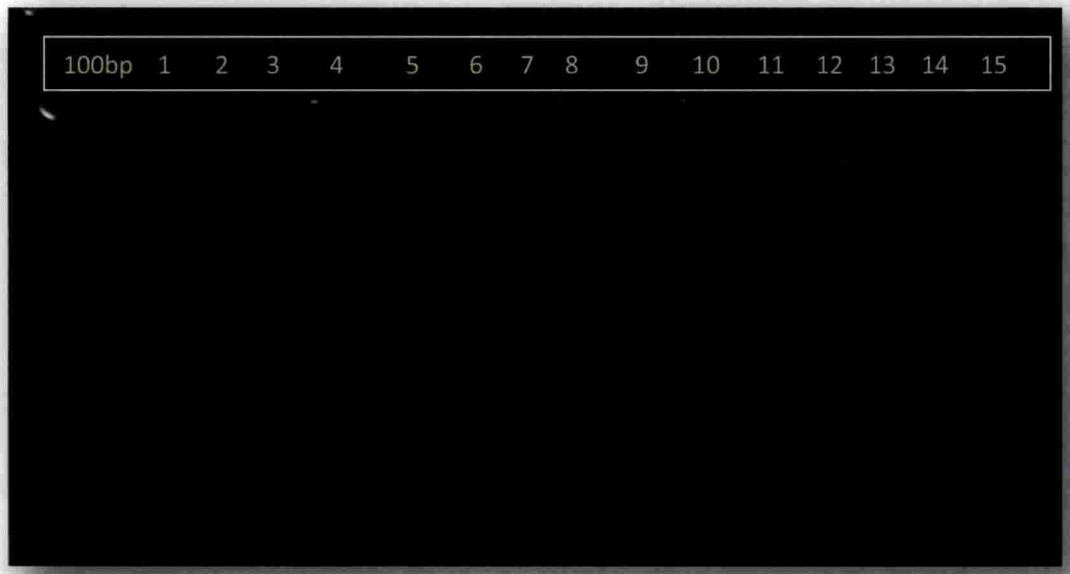


Fig. 8. Simple sequence repeat fingerprinting results with number of alleles per locus, major allele frequency, Expected heterozygosity, PICvalues and Gene diversity index

SL No:	Accessions	Accession Code
1	Sree Padmanabha	V_1
2	MN1	V_2
3	MN2	V_3
4	MNGA8	V_4
5	CR43-11	V_5
6	1135-D	V_6
7	11S18	V_7
8	11S17	V_8
9	IMS1-2	V_9
10	IMS1-9	V_{10}
11	I-13-S5-20	V_{11}
12	I-13-S5-17	V_{12}
13	I-99/14/3-S2-1	V_{13}
14	I-99/14/3-S2-2	V_{14}
15	IES1-1	V_{15}
16	IES1-3	V_{16}
17	8W5	V_{17}
18	9S127	V_{18}
19	CI273	V_{19}
20	CO-1	V_{20}
21	CR43-2	V_{21}

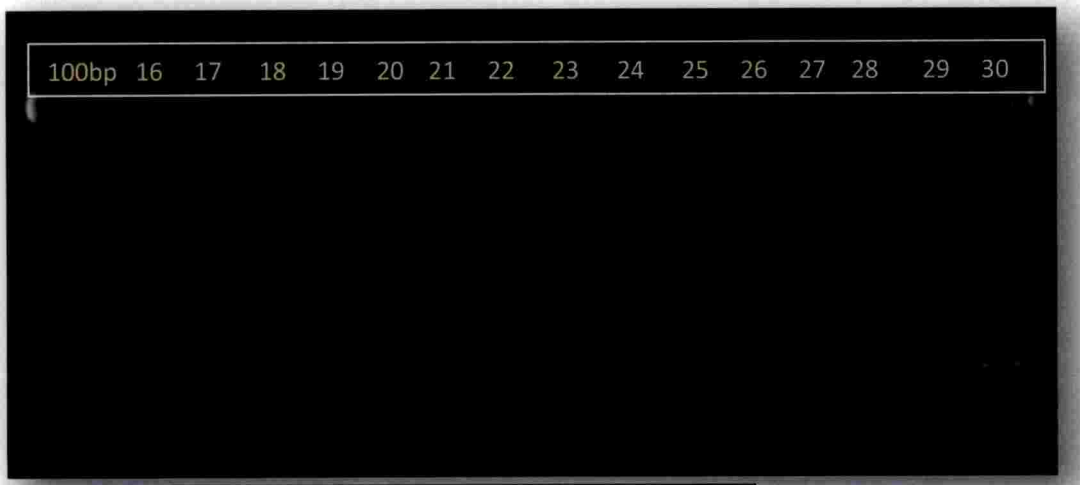
22	Sree Reksha	V_{22}
23	H226	V_{23}
24	CR20A-2	V_{24}
25	CR43-11	V_{25}
26	CE185	V_{26}
27	CE775	V_{27}
28	CR54A3	V_{28}
29	7IVC4	V_{29}
30	9S132	V_{30}
31	CR21-10	V_{31}
32	CR43-7	V_{32}
33	CR52A41	V_{33}
34	Sree Visakham	V_{34}
35	9S172	V_{35}
36	99/14/S17	V_{36}
37	M4	V_{37}
38	Sree Swarna	V_{38}
39	CR35-8	V_{39}
40	CR54A-19	V_{40}
41	Sree Apoorva	V_{41}
42	CI-896	V_{42}
43	IPS1-1	V_{43}
44	Sree Harsha	V_{44}
45	9S165	V_{45}

46	H165	V_{46}
47	CMR104	V_{47}
48	TCR56	V_{48}
49	CMR129	V_{49}
50	TCR63	V_{50}
51	CR20A4	V_{51}
52	S1284	V_{52}
53	CR63-3	V_{53}
54	CR59-8R	V_{54}
55	Sree Athulya	V_{55}
56	PDP1	V_{56}
57	Vellayani Hraswa	V_{57}
58	Sree Vijaya	V_{58}
59	CR57-6	V_{59}
60	9S98	V_{60}



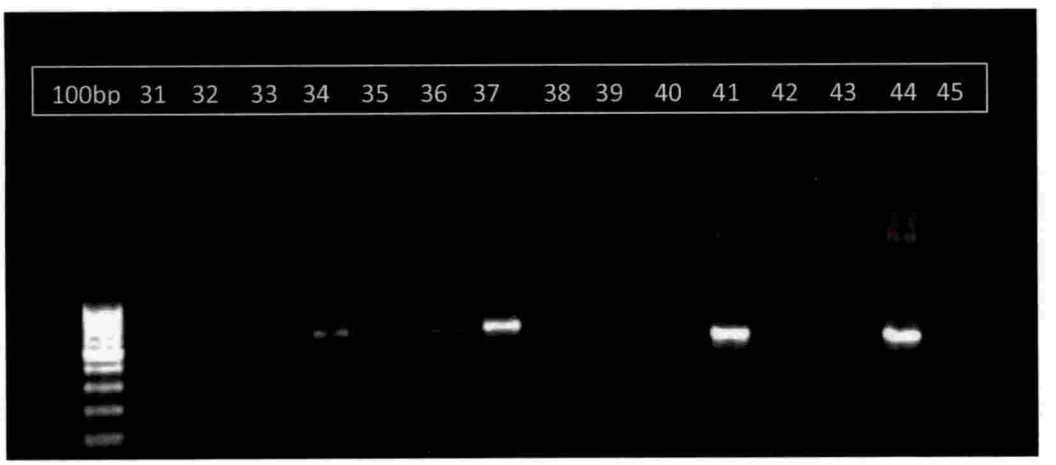
Gel image of multiplex PCR

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
100bp DNA ladder	V_1	V_2	V_3	V_4	V_5	V_6	V_7	V_8	V_9	V_{10}	V_{11}	V_{12}	V_{13}	V_{14}	V_{15}



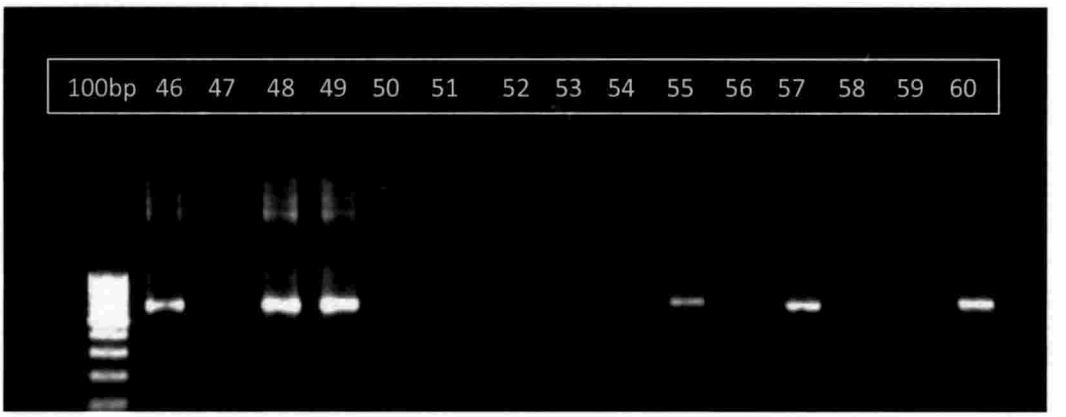
Gel image of multiplex PCR

DNA ladder	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
100bp	V_{16}	V_{17}	V_{18}	V_{19}	V_{20}	V_{21}	V_{22}	V_{23}	V_{24}	V_{25}	V_{26}	V_{27}	V_{28}	V_{29}	V_{30}



Gel image of multiplex PCR

DNA ladder	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45
100bp	V31	V_{32}	V_{33}	V_{34}	V_{35}	V_{36}	V_{37}	V_{38}	V_{39}	V_{40}	V_{41}	V_{42}	V_{43}	V_{44}	V_{45}



Gel image of multiplex PCR

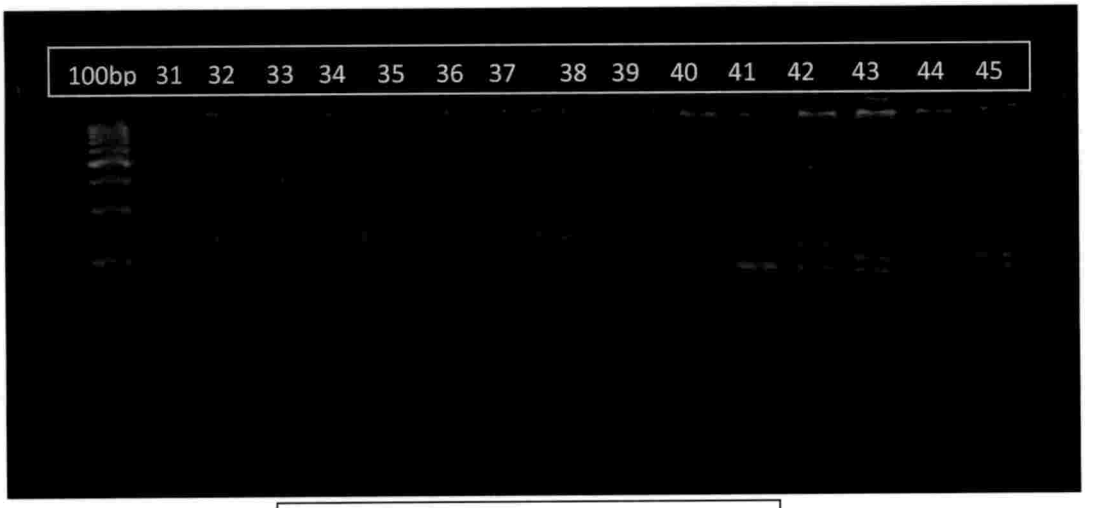
DNA ladder 100bp	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
	V_{46}	V_{47}	V_{48}	V_{49}	V_{50}	V_{51}	V_{52}	V_{53}	V_{54}	V_{55}	V_{56}	V_{57}	V_{58}	V_{59}	V_{60}

2 % agarose gel image of primer SSRY 45

DNA ladder	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
100bp	V_1	V_2	V_3	V_4	V_5	V_6	V_7	V_8	V_9	V_{10}	V_{11}	V_{12}	V_{13}	V_{14}	V_{15}

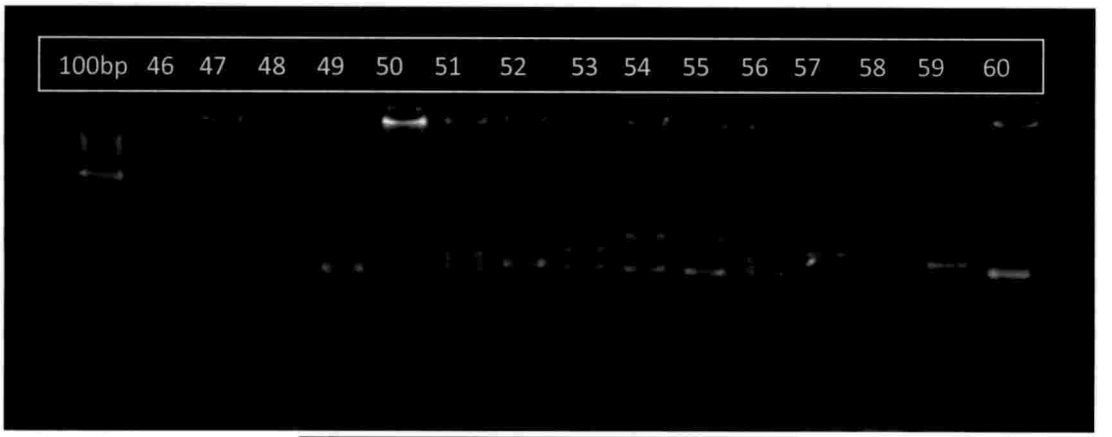
2 % agarose gel image of primer SSRY 45

DNA ladder	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
100bp	V_{16}	V_{17}	V_{18}	V_{19}	V_{20}	V_{21}	V_{22}	V_{23}	V_{24}	V_{25}	V_{26}	V_{27}	V_{28}	V_{29}	V_{30}



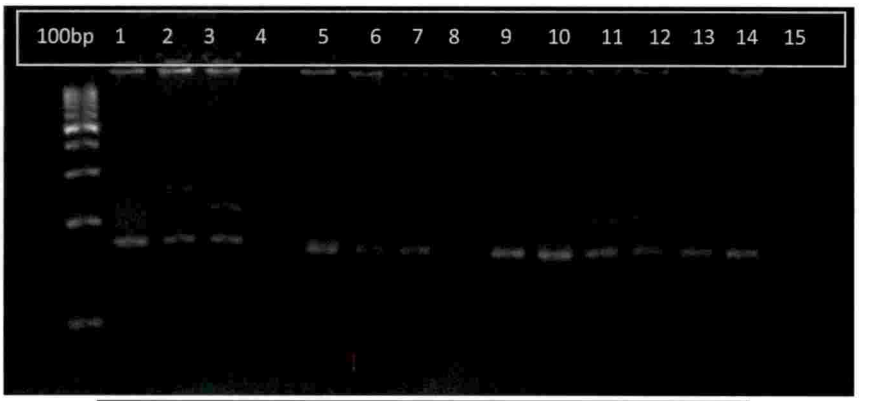
2 % agarose gel image of primer SSRY 45

DNA ladder	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45
100bp	V31	V_{32}	V_{33}	V_{34}	V_{35}	V_{36}	V_{37}	V_{38}	V_{39}	V_{40}	V_{41}	V_{42}	V_{43}	V_{44}	V_{45}



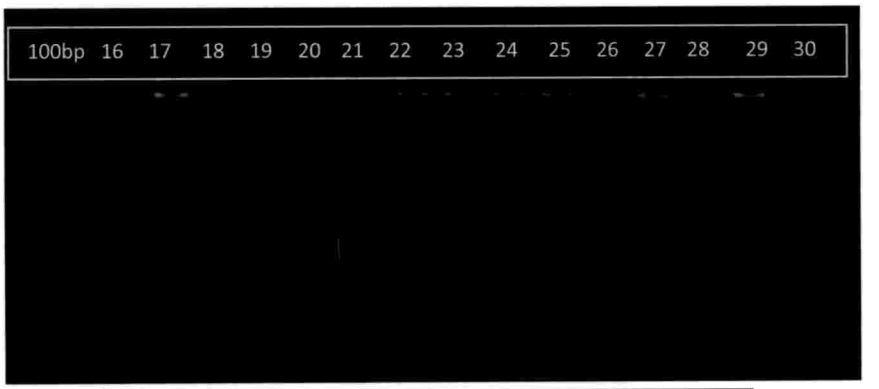
2 % agarose gel image of primer SSRY 45

DNA ladder 100bp	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
	V_{46}	V_{47}	V_{48}	V_{49}	V_{50}	V_{51}	V_{52}	V_{53}	V_{54}	V_{55}	V_{56}	V_{57}	V_{58}	V_{59}	V_{60}



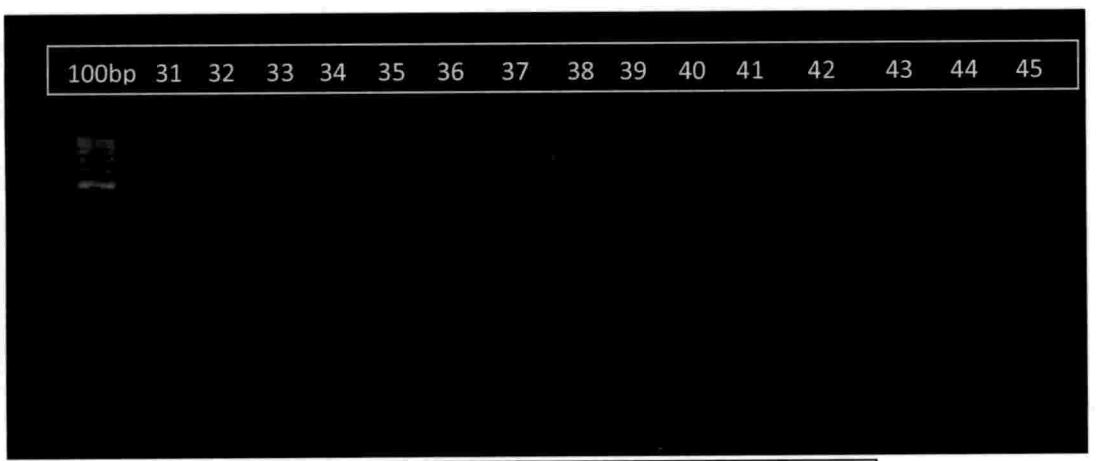
2% agarose gel image of primer SSRY100

DNA ladder	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
100bp	V_1	V_2	V_3	V_4	V_5	V_6	V_7	V_8	V_9	V_{10}	V_{11}	V_{12}	V_{13}	V_{14}	V_{15}



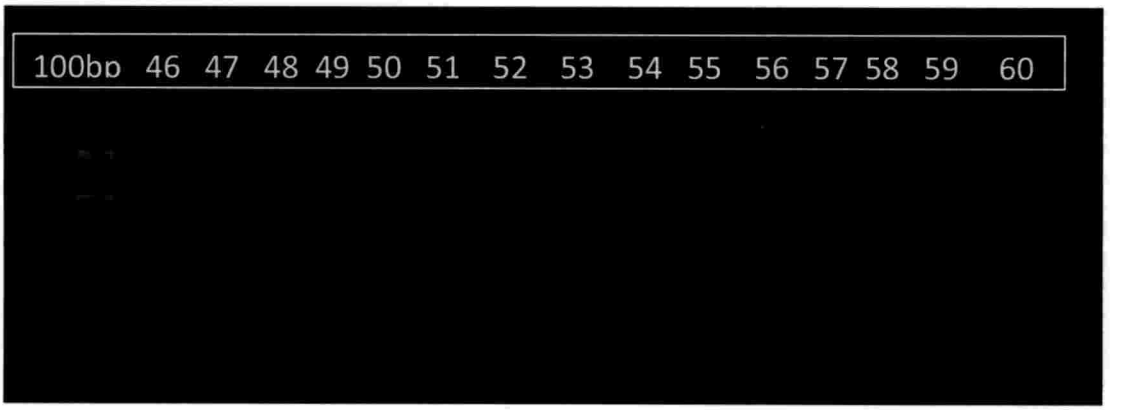
2% agarose gel image of primer SSRY100

DNA ladder	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
100bp	V_{16}	V_{17}	V_{18}	V_{19}	V_{20}	V_{21}	V_{22}	V_{23}	V_{24}	V_{25}	V_{26}	V_{27}	V_{28}	V_{29}	V_{30}



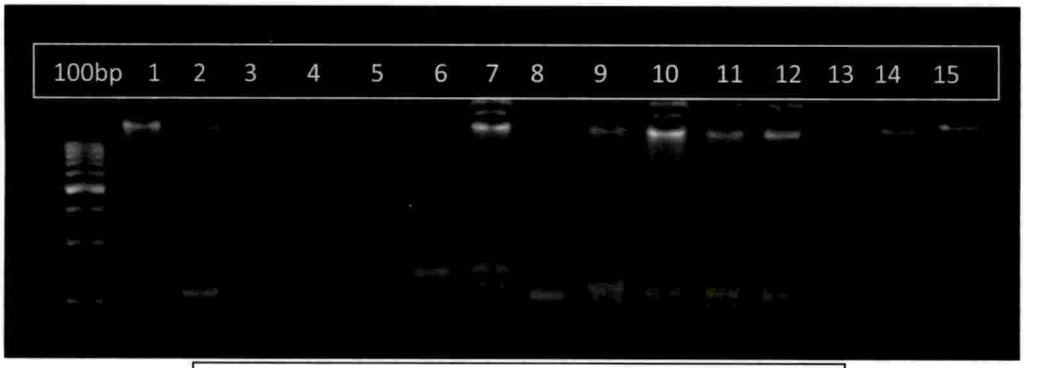
2% agarose gel image of primer SSRY100

DNA ladder	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45
100bp	V31	V_{32}	V_{33}	V_{34}	V_{35}	V_{36}	V_{37}	V_{38}	V_{39}	V_{40}	V_{41}	V_{42}	V_{43}	V_{44}	V_{45}



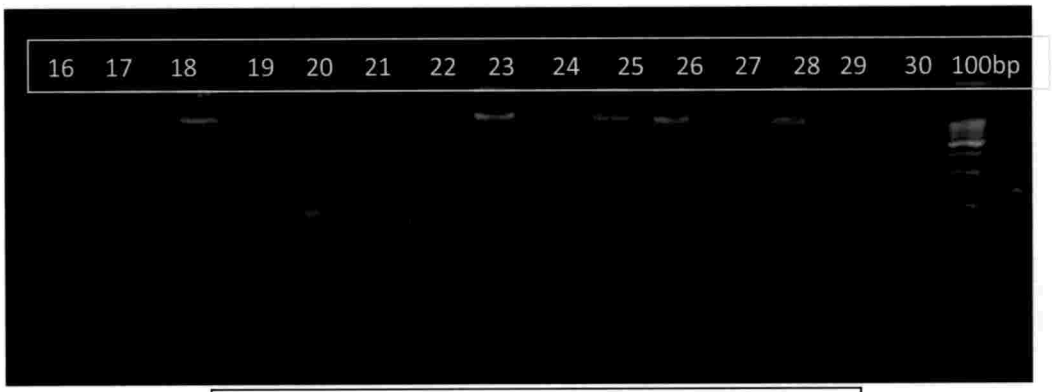
2% agarose gel image of primer SSRY100

DNA ladder 100bp	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
	V_{46}	V_{47}	V_{48}	V_{49}	V_{50}	V_{51}	V_{52}	V_{53}	V_{54}	V_{55}	V_{56}	V_{57}	V_{58}	V_{59}	V_{60}



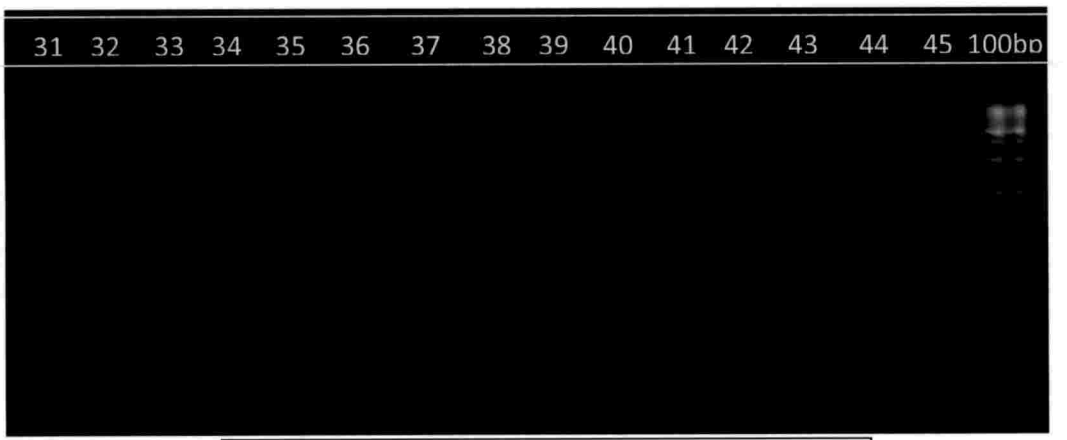
2% agarose gel image of primer MeESSRY19

DNA ladder 100bp	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
	V_1	V_2	V_3	V_4	V_5	V_6	V_7	V_8	V_9	V_{10}	V_{11}	V_{12}	V_{13}	V_{14}	V_{15}



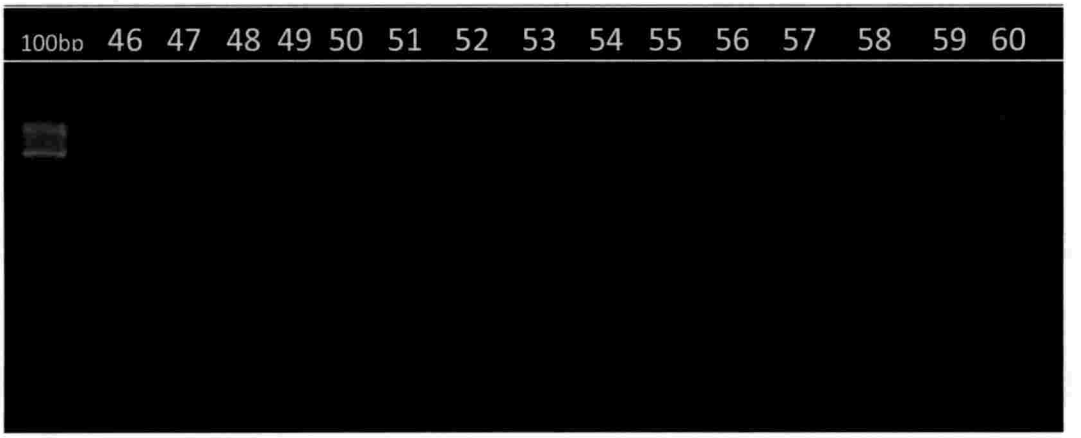
2% agarose gel image of primer MeESSRY19

DNA ladder	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
100bp	V_{16}	V_{17}	V_{18}	V_{19}	V_{20}	V_{21}	V_{22}	V_{23}	V_{24}	V_{25}	V_{26}	V_{27}	V_{28}	V_{29}	V_{30}



2% agarose gel image of primer MeESSRY19

DNA ladder	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45
100bp	V31	V_{32}	V_{33}	V_{34}	V_{35}	V_{36}	V_{37}	V_{38}	V_{39}	V_{40}	V_{41}	V_{42}	V_{43}	V_{44}	V_{45}



2% agarose gel image of primer MeESSRY19

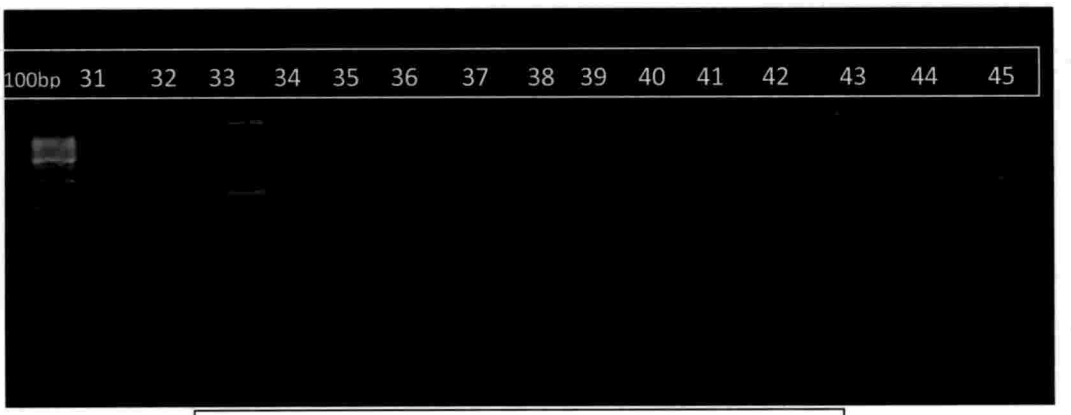
DNA ladder 100bp	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
	V_{46}	V_{47}	V_{48}	V_{49}	V_{50}	V_{51}	V_{52}	V_{53}	V_{54}	V_{55}	V_{56}	V_{57}	V_{58}	V_{59}	V_{60}

2% agarose gel image of primer SSRY 234

DNA ladder	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
100bp	V_1	V_2	V_3	V_4	V_5	V_6	V_7	V_8	V_9	V_{10}	V_{11}	V_{12}	V_{13}	V_{14}	V_{15}

2% agarose gel image of primer SSRY 234

DNA ladder	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
100bp	V_{16}	V_{17}	V_{18}	V_{19}	V_{20}	V_{21}	V_{22}	V_{23}	V_{24}	V_{25}	V_{26}	V_{27}	V_{28}	V_{29}	V_{30}

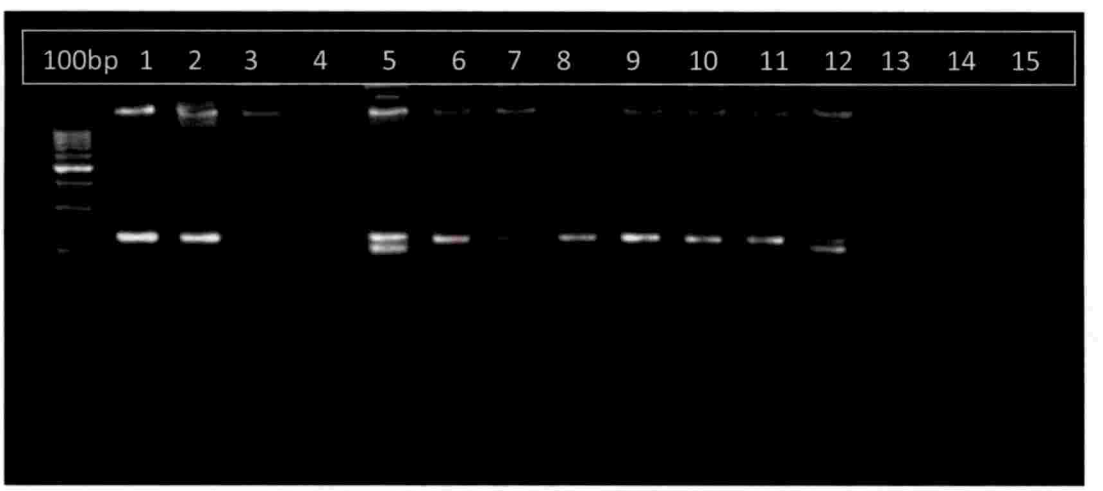


2% agarose gel image of primer SSRY 234

DNA ladder	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45
100bp	V31	V_{32}	V_{33}	V_{34}	V_{35}	V_{36}	V_{37}	V_{38}	V_{39}	V_{40}	V_{41}	V_{42}	V_{43}	V_{44}	V_{45}

2% agarose gel image of primer SSRY 234

DNA ladder 100bp	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
	V_{46}	V_{47}	V_{48}	V_{49}	V_{50}	V_{51}	V_{52}	V_{53}	V_{54}	V_{55}	V_{56}	V_{57}	V_{58}	V_{59}	V_{60}

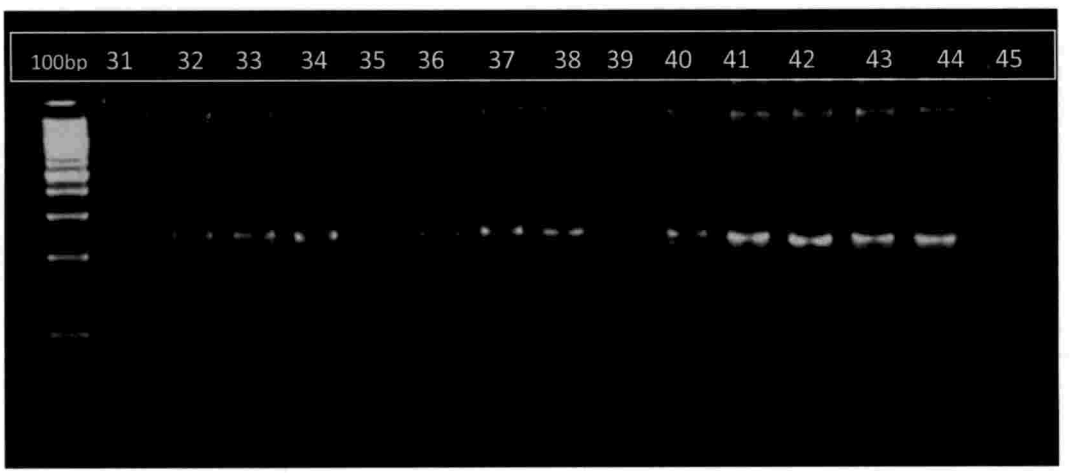


2% agarose gel image of primer SSRY 105

DNA ladder	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
100bp	V_1	V_2	V_3	V_4	V_5	V_6	V_7	V_8	V_9	V_{10}	V_{11}	V_{12}	V_{13}	V_{14}	V_{15}

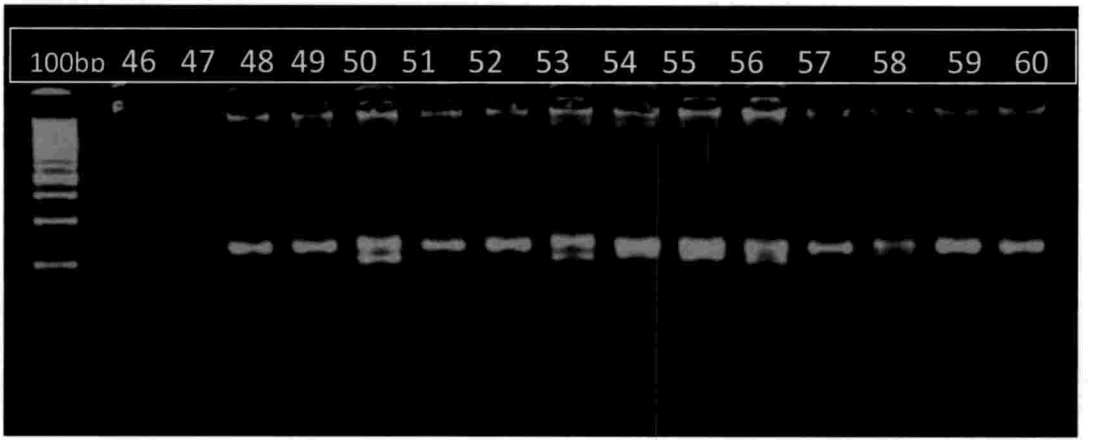
2% agarose gel image of primer SSRY 105

DNA ladder	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
100bp	V_{16}	V_{17}	V_{18}	V_{19}	V_{20}	V_{21}	V_{22}	V_{23}	V_{24}	V_{25}	V_{26}	V_{27}	V_{28}	V_{29}	V_{30}



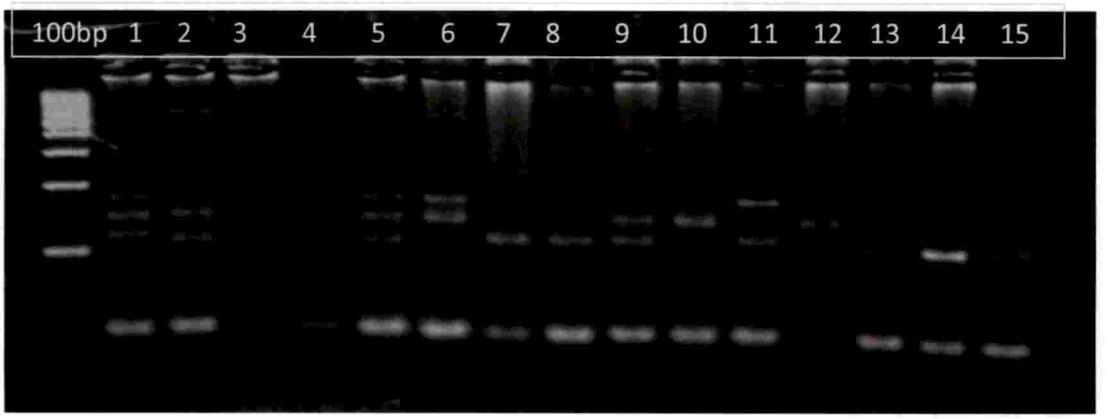
2% agarose gel image of primer SSRY 105

DNA ladder	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45
100bp	V31	V_{32}	V_{33}	V_{34}	V_{35}	V_{36}	V_{37}	V_{38}	V_{39}	V_{40}	V_{41}	V_{42}	V_{43}	V_{44}	V_{45}



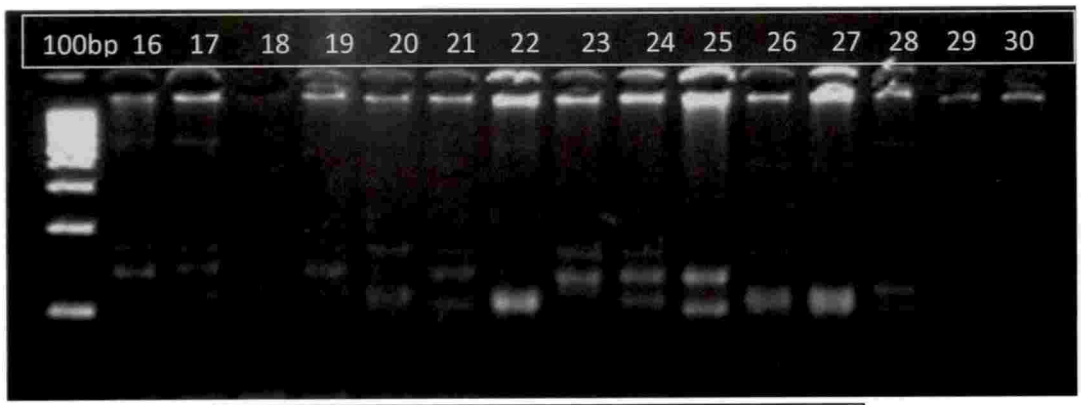
2% agarose gel image of primer SSRY 105

DNA ladder 100bp	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
	V_{46}	V_{47}	V_{48}	V_{49}	V_{50}	V_{51}	V_{52}	V_{53}	V_{54}	V_{55}	V_{56}	V_{57}	V_{58}	V_{59}	V_{60}



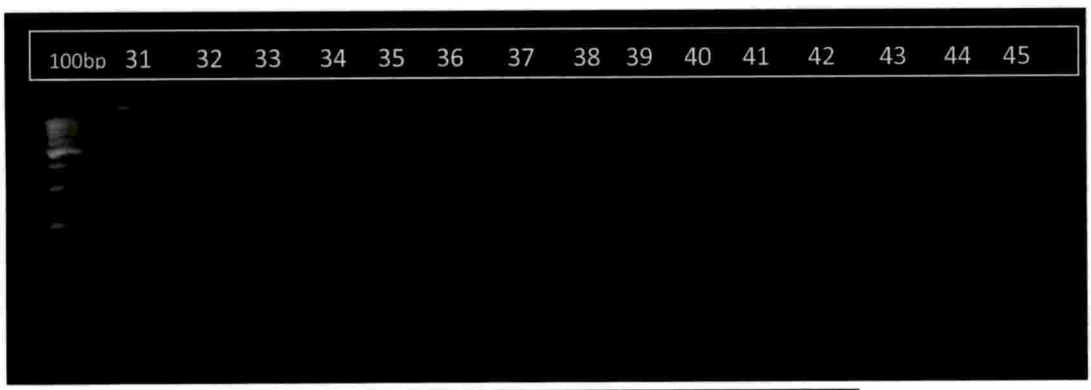
2% agarose gel image of primer SSRY 44

DNA ladder	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
100bp	V_1	V_2	V_3	V_4	V_5	V_6	V_7	V_8	V_9	V_{10}	V_{11}	V_{12}	V_{13}	V_{14}	V_{15}



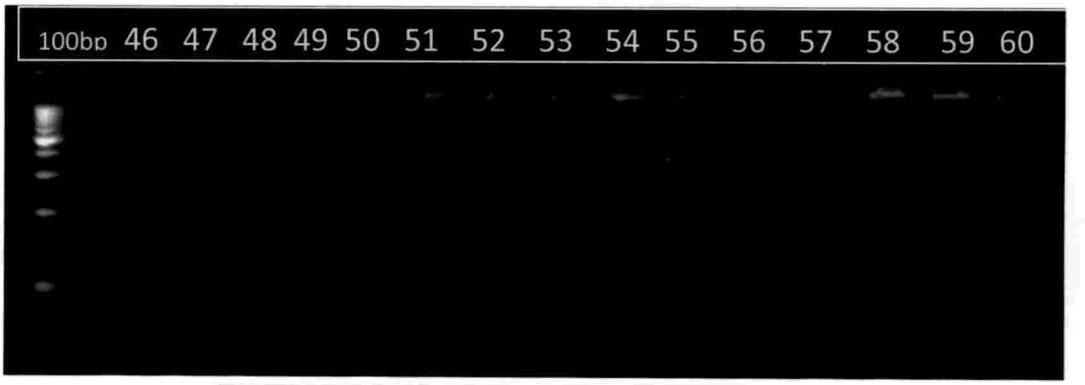
2% agarose gel image of primer SSRY 44

DNA ladder	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
100bp	V_{16}	V_{17}	V_{18}	V_{19}	V_{20}	V_{21}	V_{22}	V_{23}	V_{24}	V_{25}	V_{26}	V_{27}	V_{28}	V_{29}	V_{30}



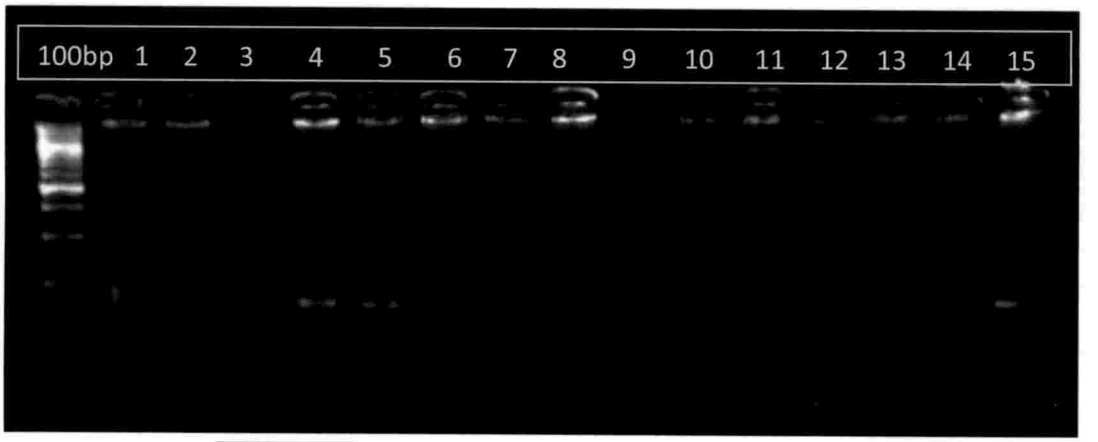
2% agarose gel image of primer SSRY 44

DNA ladder	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45
100bp	V31	V_{32}	V_{33}	V_{34}	V_{35}	V_{36}	V_{37}	V_{38}	V_{39}	V_{40}	V_{41}	V_{42}	V_{43}	V_{44}	V_{45}



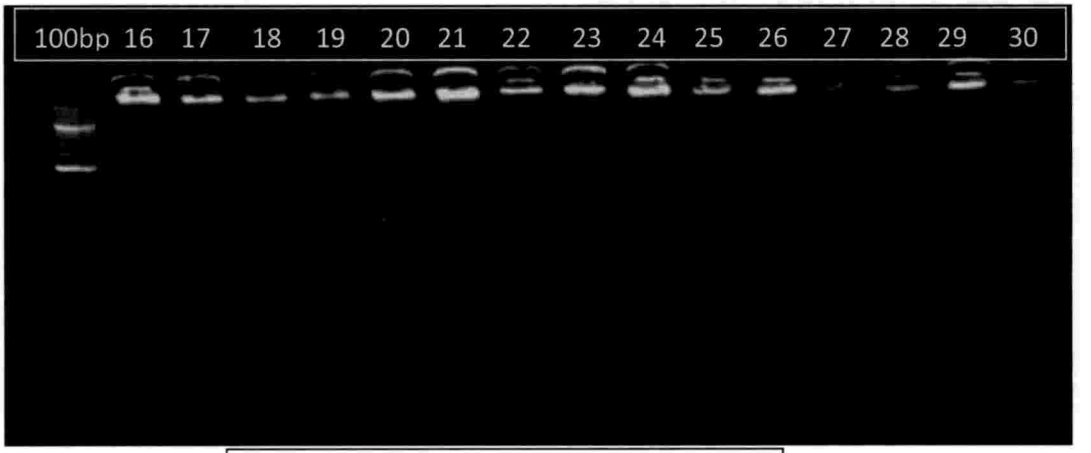
2% agarose gel image of primer SSRY 44

DNA ladder 100bp	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
	V_{46}	V_{47}	V_{48}	V_{49}	V_{50}	V_{51}	V_{52}	V_{53}	V_{54}	V_{55}	V_{56}	V_{57}	V_{58}	V_{59}	V_{60}



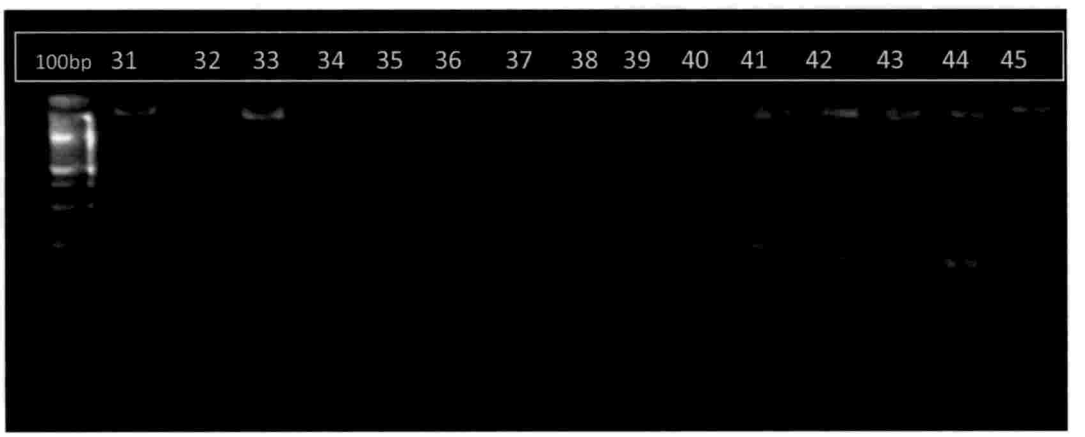
2% agarose gel image of primer SSRY 28

DNA ladder 100bp	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
	V_1	V_2	V_3	V_4	V_5	V_6	V_7	V_8	V_9	V_{10}	V_{11}	V_{12}	V_{13}	V_{14}	V_{15}



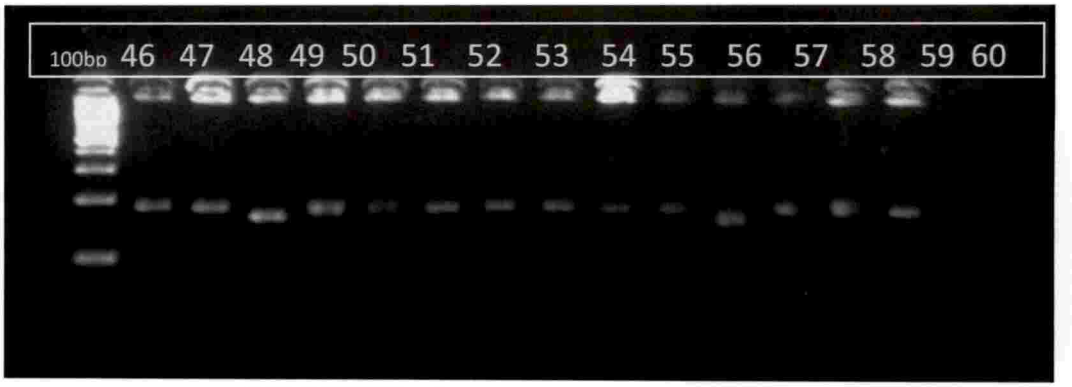
2% agarose gel image of primer SSRY 28

DNA ladder	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
100bp	V_{16}	V_{17}	V_{18}	V_{19}	V_{20}	V_{21}	V_{22}	V_{23}	V_{24}	V_{25}	V_{26}	V_{27}	V_{28}	V_{29}	V_{30}



2% agarose gel image of primer SSRY 28

DNA ladder	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45
100bp	V31	V_{32}	V_{33}	V_{34}	V_{35}	V_{36}	V_{37}	V_{38}	V_{39}	V_{40}	V_{41}	V_{42}	V_{43}	V_{44}	V_{45}



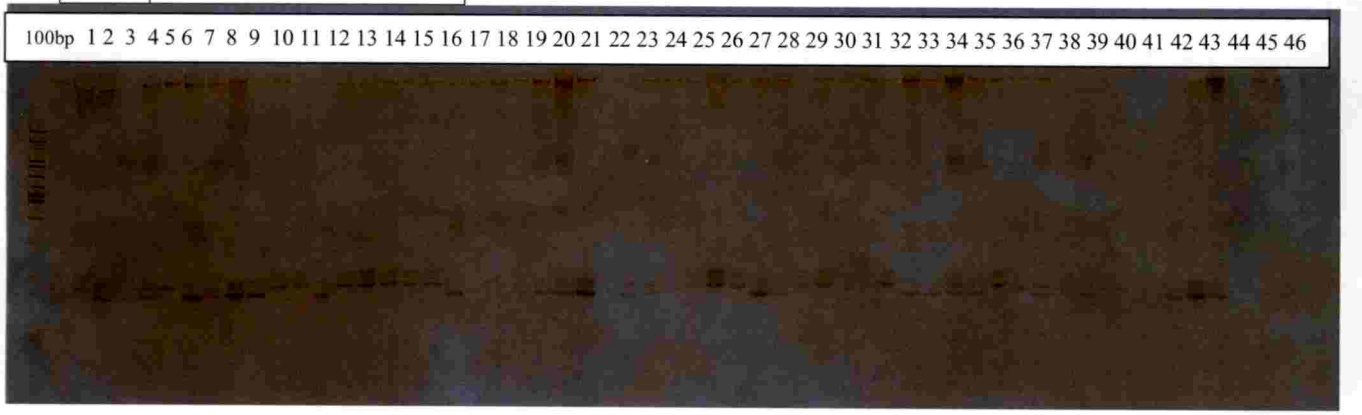
2% agarose gel image of primer SSRY 28

DNA ladder 100bp	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
	V_{46}	V_{47}	V_{48}	V_{49}	V_{50}	V_{51}	V_{52}	V_{53}	V_{54}	V_{55}	V_{56}	V_{57}	V_{58}	V_{59}	V_{60}

Sl No:	Accessions
1	100bp DNA ladder
2	Sree Padmanabha
3	MN1
4	MN2
5	MnGA8
6	CR43-11
7	IMS1-2-no spot
8	IMS1-9
9	I-99/14/3-S2-1
10	I-99/14/3-S2-2
11	IES1-1
12	9S127
13	CI273
14	CR43-2
15	CR24-4

16	H226
17	CR20A-2
18	CR63-3
19	CE185
20	CE775
21	CR54A3
22	7IVC4
23	9S132
24	CR21-10
25	CR43-7
26	CR52A41
27	CR35-8
28	9S172
29	CR20A4
30	CR59-8R
31	CR57-6
32	9S98
33	CMR104
34	99/14/3 S2-2
35	11S18-0T
36	CO-1
37	H226
38	9S172
39	8W5
40	M4
41	TR5-3
42	CI896
43	H165
44	TCR56
45	TCR63
46	Sree Athulya
47	Vellayani Hrazwa
48	Sree vijaya

49	I-13-S5-20
50	I-13-S5-17
51	1ES1-3
52	CE775
53	9S165
54	S1284
55	11S35dwarf
56	11S17-0T
57	CMR129
58	CE185
59	Sree Harsha
60	Sree Swarna



Simple sequence repeat fingerprinting results with number of alleles per locus, major allele frequency, Expected heterozygosity, PICvalues and Gene diversity index is given table 7 and depicted in Fig 8

The major allele frequency ranged from 0.6556 (SSR Y 44) to 0.95 (SSRY234). The expected heterozygosity ranged from 0.2637 (SSRMe9) to 0.6297 (SSRY44). SSRY 45 also recorded high expected heterozygosity (0.6096).

The maximum PIC value was recorded by the marker SSRY44 (0.5586) followed by SSRY45 (0.5218). SSRMe9 recorded the lowest PIC value (0.2289) among the markers tested.

The gene diversity index was found to be the lowest for the primer SSRY234 (0.0944). The maximum gene diversity was recorded for the marker SSRY44 (0.4419) followed by SSRY105 (0.4111), SSR Y 28 (0.3886) and SSR Y45 (0.3578).

4.5 Morphological characterization

The morphological traits of 60 genotypes were recorded and presented in Table 7-8.

4.5.1 Leaf retention

No. of leaves ranged from 50 (CMR 12) to 1800 (Vellayani Hraswa). The genotypes viz. 8W5, CR 43-7, CR 21-10 and CI-273, 9S 172 recorded very high leaf retention ability (>1000 leaves/plant).

4.5.2 Leaf weight

The leaf weight/plant ranged from 0.04kg (CE 185) to 1.45 kg(Vellayani Hraswa). CR21-10 and 9S172 also recorded high leaf weight of 1.25kg and 1.45 kg per plant respectively.

	Sample(vareity)	Leaf retenion	HI	Leaf weight	stem weight	Wt. of tubers	Total biomass	No: of tub	Stem girth	CMD
1	Sree Padmanabha	400	0.565	0.2	2.5	3.5	6.2	4	8.5	1
2	MN-1	600	0.774	0.25	1.5	6	7.75	2	7	1
3	MN-2	600	0.776	0.3	1	4.5	5.8	9	7.3	1
4	MNGA8	450	0.734	0.18	1.2	3.8	5.18	7	9	1
5	CR43-11	500	0.66	0.22	2	4.3	6.52	10	10	1
6	11S 35	185	0.604	0.05	1	1.6	2.65	3	4.5	1
7	11S18	200	0.68	0.075	1.1	2.5	3.675	8	6.8	1
8	11S17	380	0.67	0.08	1.4	3	4.48	9	7.8	3
9	IMS1-2	375	0.769	0.12	1.5	5.4	7.02	14	10.5	1

10	IMS1-9	485	0.797	0.22	1	4.8	6.02	12	11	1
11	I-13-S5-20	650	0.712	0.24	1.3	3.8	5.34	8	9.5	3
12	I-13-S5-17	950	0.741	0.35	1.4	5	6.75	11	12.5	1
13	I-99/14/3-S2-1	875	0.674	0.42	1.8	4.6	6.82	15	9.5	1
14	I-99/14/3-S2-2	945	0.65	0.45	1.6	3.8	5.85	10	8.8	3
15	IES1-1	925	0.536	0.375	1.7	2.4	4.475	8	7	1
16	IES1-3	860	0.587	0.48	1.7	3.1	5.28	9	7.9	3
17	8W5	1200	0.495	0.56	2.5	3	6.06	15	8.5	1
18	9S127	700	0.587	0.32	2.5	4	6.82	12	9	1
19	CI-273	1500	0.633	0.72	4.5	9	14.22	19	11.5	1

20	CO-1	400	0.661	0.125	0.9	2	3.025	4	5.5	3
21	CR43-2	700	0.617	0.345	3	5.4	8.745	11	10.5	1
22	Sree Reksha	800	0.625	0.4	2	4	6.4	7	8.5	1
23	H226	200	0.64	0.05	2.2	4	6.25	12	10	3
24	CR20A-2	1000	0.699	0.62	3	8.4	12.02	15	8	1
25	CR43-1	500	0.619	0.65	2	4.3	6.95	10	10	3
26	CE185	140	0.697	0.04	3	7	10.04	19	9.5	1
27	CE775	600	0.426	0.2	2.5	2	4.7	11	7	1
28	CR54A3	700	0.624	0.26	3	5.4	8.66	11	10.8	1
29	7IVC4	700	0.542	0.32	4.5	5.7	10.52	15	12	1

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30	9S132	200	0.579	0.045	2.5	3.5	6.045	13	8.5	1
31	CR21-10	1400	0.556	1.02	4.5	6.9	12.42	20	13.5	1
32	CR43-7	1200	0.586	0.95	3.5	6.3	10.75	9	10	1
33	CR52A41	1000	0.7	0.78	2	6.5	9.28	13	12.5	1
34	Sree Visakham	500	0.564	0.32	2	3	5.32	7	9.5	3
35	9S172	1500	0.534	1.25	1.8	3.5	6.55	12	8	1
36	99/14/S17	890	0.692	0.46	1.1	3.5	5.06	14	12	3
37	M4	300	0.485	0.075	1.2	1.2	2.475	3	8	3
38	Sree Swarna	1000	0.439	0.63	5	4.4	10.03	13	10	1

39	CR35-8	800	0.676	0.3	2.1	5	7.4	11	8	1
40	CR54A-19	1000	0.731	0.59	1.8	6.5	8.89	15	7	1
41	Sree Apoorva	300	0.294	0.2	1	0.5	1.7	5	14.5	1
42	CI-896	600	0.423	0.25	1.8	1.5	3.55	3	9	3
43	IPS1-1	380	0.643	0.12	1.1	2.2	3.42	7	8.5	1
44	Sree Harsha	400	0.654	0.14	0.6	1.4	2.14	5	13.1	3
45	9S165	425	0.842	0.24	1.35	8.5	10.09	16	8.6	1
46	H165	200	0.637	0.14	1	2	3.14	5	8	3
47	CMR104	100	0.741	0.05	1	3	4.05	6	9	1
48	TCR56	200	0.237	0.11	1.5	0.5	2.11	1	5	3
49	CMR12	50	0.3	0.2	0.5	0.3	1	4	5	1

50	TCR63	200	0.682	0.4	1	3	4.4	7	5	3
51	CR20A4	680	0.703	0.28	1.2	3.5	4.98	5	8.5	1
52	S1284	800	0.631	0.6	3.5	7	11.1	10	9	1
53	CR63-3	465	0.764	0.23	1.1	4.3	5.63	8	7.4	1
54	CR59-8R	800	0.761	0.455	3	11	14.455	8	11	1
55	Sree Athulya	500	0.633	0.3	1.9	3.8	6	8	10	3
56	PDP-1	500	0.599	0.34	1	2	3.34	5	8	3
57	Vellayani Hraswa	1800	0.465	1.45	2	3	6.45	6	7	3
58	Sree Vijaya	300	0.582	0.15	1	1.6	2.75	3	6	3
59	CR57-6	1000	0.337	0.45	2.5	1.5	4.45	5	10	1
60	9S98	1000	0.677	0.62	2	5.5	8.12	19	7	3

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	Sample(vareity)	Stem colour	Petiole colour	Col of unoponed leaf	Leaf colour
1	Sree Padmanabha	Grey	Green	Green	Green
2	MN-1	Whitish grey	Green	L.green	Dark green
3	MN-2	Whitish grey	Green	Green	Green
4	MNGA8	Whitish grey	Green	Green	Green
5	CR43-11	Whitish grey	Red	Green	Green
6	11S 35	Brown	Red	Brownish green	Green
7	11S18	Brown	Red	Green	Green
8	11S17	Brown	Red	Green	Green
9	IMS1-2	Whitish grey	Green	Green	Green
10	IMS1-9	Whitish grey	Green	Green	Green
11	I-13-S5-20	Brown	Red	Light green	Green
12	I-13-S5-17	Brown	Green	Brownish green	Green
13	I-99/14/3-S2-1	Brown	Red	Purple	Green

14	I-99/14/3-S2-2	Brown	Red	Brownish green	Green
15	IES1-1	Brown	Green	Brownish green	Green
16	IES1-3	Grey	Green	Brownish green	Green
17	8W5	Brown	G.red on tp	Light green	Dark green
18	9S127	Red	Red	Green	Green
19	CI-273	Brown	Red	G.with L.purple	Green
20	CO-1	Brown	Red with green	Purple green	Green
21	CR43-2	Brown	G with L.brown	Light green	Dark green
22	Sree Reksha	Whitish grey	Red	Green	Green
23	H226	Grey	Purple	L.green	Green
24	CR20A-2	Grey	Purple	L.green	Green(narrow)
25	CR43-1	Ash	Purple	Green	Green
26	CE185	Grey	Green	Light green	Green
27	CE775	Grey	Red	Purple	Green

28	CR54A3	Brown	G.L red	Green	Green
29	7IVC4	Brown	Red	Green	Green
30	9S132	Brown	Red green	Green	Dark green
31	CR21-10	R.brown.G	Green	Green	Green
32	CR43-7	Red	Red	Green	Light green
33	CR52A41	Grey	L.green	L.green	Green
34	Sree Visakham	Brown	G.L red on top	Purple	Green
35	9S172	Whitish grey	Red	Light green	Green
36	99/14/S17	Brown	Red	Green	Green
37	M4	Grey	Red	L.green	Green
38	Sree Swarna	Red	Dark red	Purple red	Green
39	CR35-8	Brown	Red	L.green	Green
40	CR54A-19	Brown	Red	Green	Green
41	Sree Apoorva	Grey	Reddish yellow	L.purple	Green

42	CI-896	Red	Red	Brownish green	Green
43	IPS1-1	Grey	Yellow with purple	Dark purple	Green
44	Sree Harsha	Grey	Pinkish red	L.green	Green
45	9S165	Grey	L.green	L.green	Green
46	H165	Grey	L.green	L.green	Green
47	CMR104	Grey	Yellow green	L.green	Dark green
48	TCR56	Red green	Red brown	L.green	Green
49	CMR12	Black	Green with red vein	L.purple	Green
50	TCR63	Brown	Red	L.green	Green
51	CR20A4	Brown	Red	Green	Green
52	S1284	Grey	Red	L.green	Green
53	CR63-3	Brown	Red	Purplish green	Green
54	CR59-8R	Red	Red	L.green	Green
55	Sree Athulya	Grey	Dark Brown	Purple	Green

56	PDP-1	Brown	Green	L.green	Dark green
57	Vellayani Hraswa	Red	L.green	Green	Green
58	Sree Vijaya	Grey	Red green	Purple	Green
59	CR57-6	Brown	Green	L.green	Dark green
60	9S98	Grey	Reddish green	Purple	Green

4.5.3 Stem weight

The stem weight was highest in Sree Swarna (5kg/plant) followed by CI273 and 7IVC4 (4. 5) and was lowest in CMR12 (0.5 kg/plant).

4.5.4 Weight of tubers/plant

The tuber weight ranges from 0.3 (CMR 12) to 11 (CR59-8R) kg per plant. All the high yielding genotypes *viz.* MN-1, CR43-7, CR54A-19, CR52A41, CR21-10, CE185, S1284, CR20A-2, 9S165, CI273 and CR59-8R that recorded very high weight of tubers per plant(>6 kg/plant) were found to be CMD resistant too.

4.5.5 Total biomass

The total biomass ranged from 1(CMR 12) to 14.46 (CR59-8R). Very high biomass was recorded by the CMD resistant genotypes CI-273(14.22kg/plant), CR21-10 (12.42 kg/plant), CR20A-2 (12.02 kg/plant) and S1284 (11.1 kg/plant).

4.5.6. Harvest Index

The maximum value for this trait among the lines was recorded in 9S165 (0.84) followed by IMS1-9 (0.80), MN-2 (0.78), CR63-3 (0.76 and CR59-8 (0.76). The lowest harvest index value was recorded in TCR56 (0.24) followed by the triploid variety Sree Apoorva (0.29.)

4.5.6 Number of Tubers

Number of tubers per plant ranged from 1 (TCR56) to 20 (CR21-10). CI273, 9S98 and CE185 also recorded higher number of tubers per plant (19).

4.5.7 Stem Girth

The stem girth ranged from 4.5 cm (11S35) to 14.5cm (Sree Apoorva). TCR56, CMR12 and TCR63 also recorded low value (5cm) for stem girth while CR21-10 (13.5 cm), Sree Harsha (13.1 cm), CR52A41(12.5cm) and I-13S5-17(12.5cm) recorded high values for stem girth.

4.5.8. Cassava mosaic disease score

The CMD score ranged from 1-3. Forty genotypes were found to be CMD symptom free with a score of 1.

4.6 Molecular Data Analysis:

4.6.1 Cluster analysis for diversity study

Cluster analysis was done based on Euclidian distance of 60 genotypes of the SSR markers studied. The similarity coefficient matrix was calculated and dendrogram was constructed using UPGMA cluster analysis. Dendrogram showed the partition of genotypes into 3 main clusters (Fig.9). At 0.4% similarity it was subdivided into 3 major clusters. First cluster belongs to only one accession of genotype MN2. Second cluster have only one accession 11S17. Third cluster was divided to many sub clusters. Considerable variability was observed. From the dendrogram it was found that H226 showed similarity with Sree Apoorva.CI273 showed similarity with CR43-11and CE185. CR54A3 and CR54A19 were genetically similar. 11S17, CMR12, MNGA8 and 7IVC4 showed similarity. At 0.52% similarity fourth sub cluster is developed. Considerable variability was observed among the 60 genotypes.

Based on morphological data seven clusters were formed (Fig.10). Six clusters were again divided into two sub clusters and seventh cluster formed three sub

clusters. Vellayani Hrazwa was found to be highly divergent and formed separate cluster with early bulking genotypes like CR21-10(V31), 9S172, (V35), CR43-7 (V32) and 8W5 (V17). The cluster IIa consisted of four CMD resistant varieties (V54, V52, V39 and V22). In cluster IIIa& IIIb, both resistant and susceptible varieties were grouped together Resistant varieties were clustered together in Clusters Ivan (V49,V47, V26) and Vibe (V45,V53 , V4) .

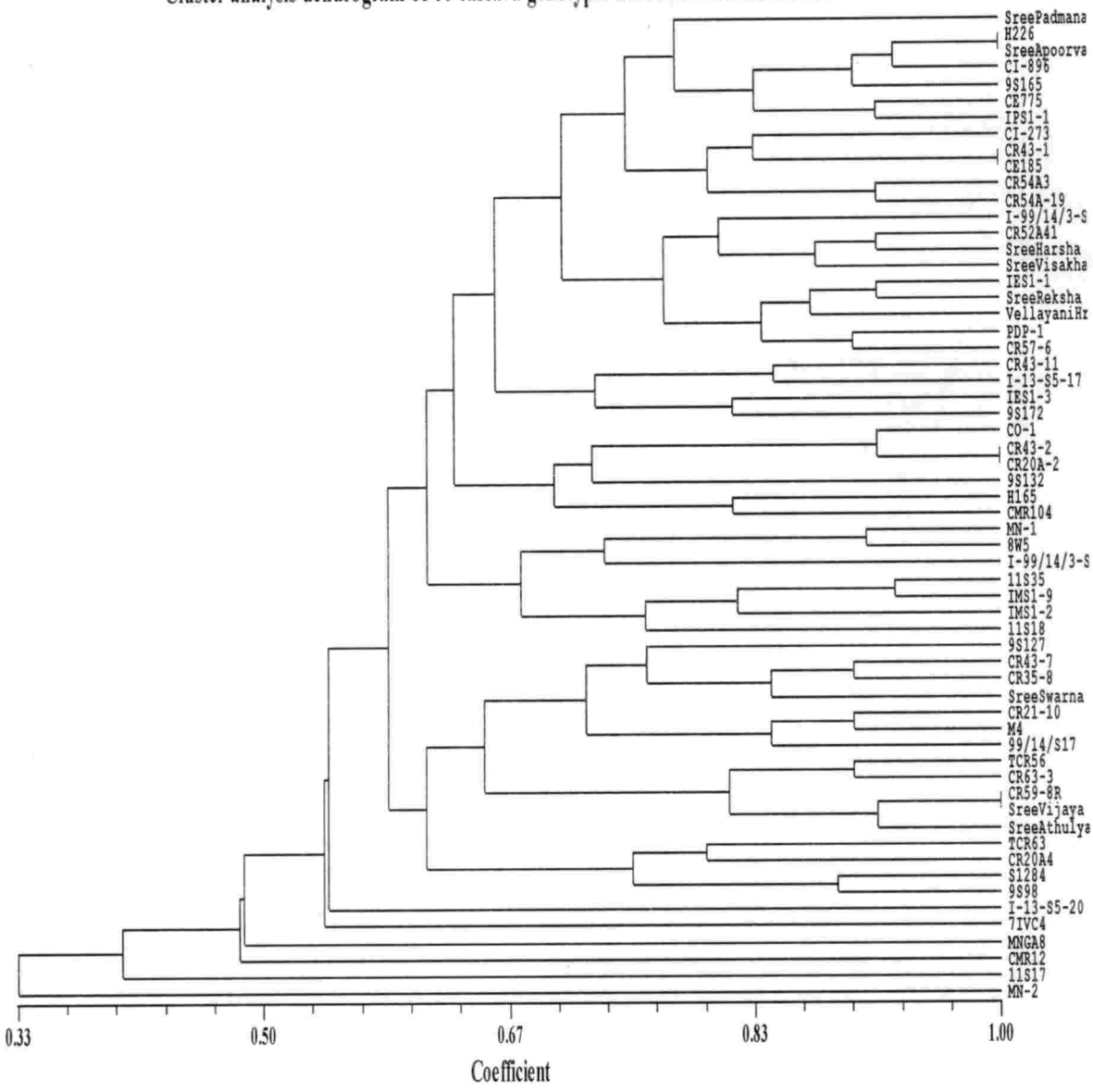

Fig.9 Dendrogram depicting the clustering of 60 cassava genotypes based on SSR marker data

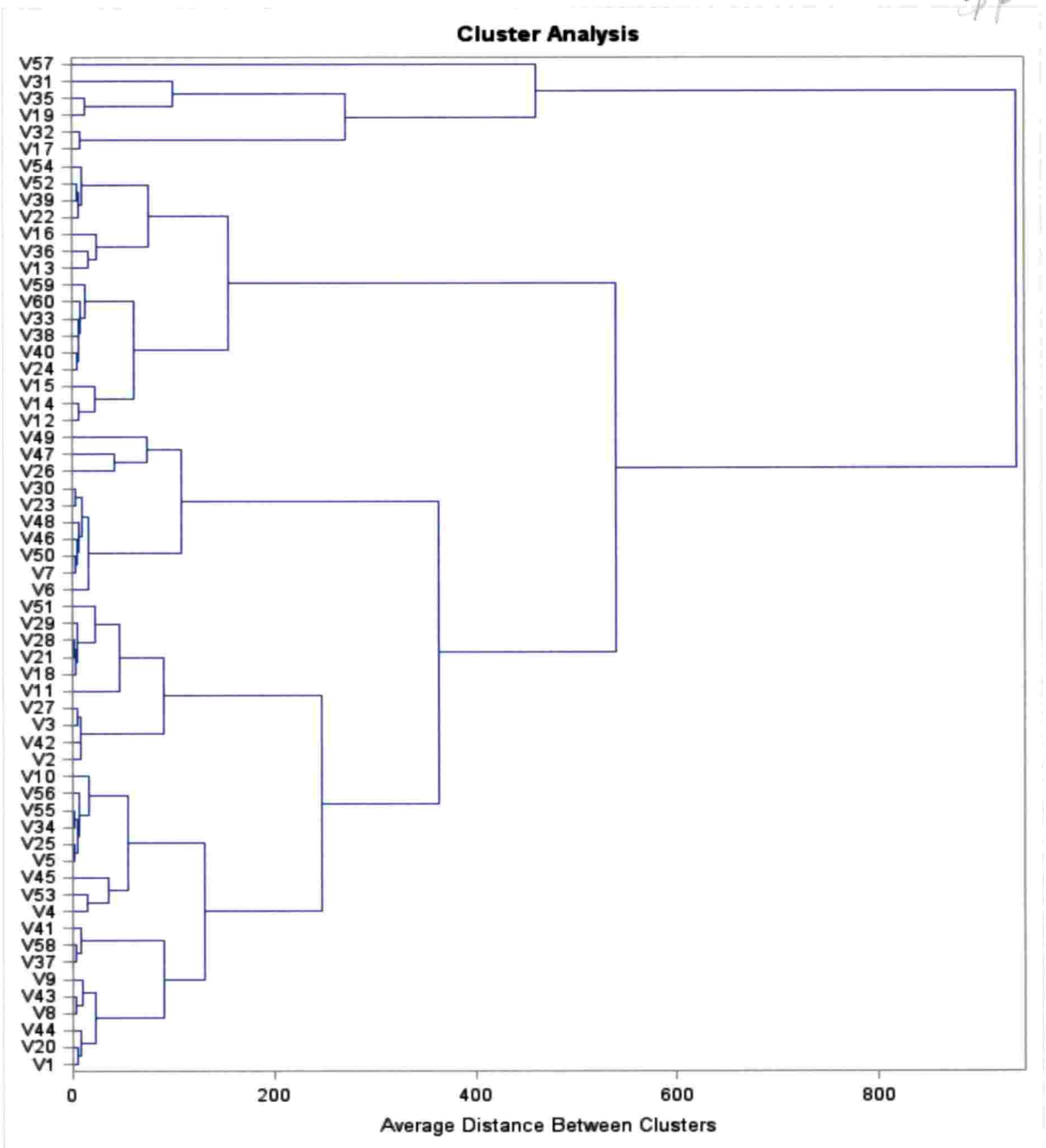

Fig.10 Dendrogram depicting the clustering of 60 cassava genotypes based on morphological data

4.6.2 Principal component analysis (PCA)

The patterns of variation and the relative importance of each morphological trait in explaining the observed variability was assessed through principal component analysis (PCA). Thirteen morphological traits were considered for PCA analysis. The PCA was performed using Eigen vector and 2-D and 3-D plot were obtained. The correlation matrix of different traits is given in Table 9. Eigen values, proportion, cumulative variance and component scores of the 12 principal components for morphological traits in 60 cassava genotypes are given in Table 10. The result of PCA grouped the variables into 12 components among which the first four are significant (Eigen value > 1) and explained 68.9% of the total variability (Table 10).

The first principal component (PC-1) accounted 34.18% of the total variation and was correlated positively with total biomass (0.4521), tuber weight/plant (0.4099), stem weight (0.3769) and leaf retention (0.3429) while CMD (-0.2091)contributed negatively as expected. The second principal component (PC-2) accounted 16% of the total variability while PC3 contributed to 10.25% of the variability among 60 cassava genotypes studied. Plotting the first and second principal components from the matrix showed MN2 as an outlier. From the plot on first three principal components, three genotypes 11S18, CMR104 and CR57-6 were found to be outliers. The highly divergent accessions identified include Vellayani Hrazwa and MN2. Being a CMD resistant variety, MN2 can be used as a donor source for introgression of CMD resistant genes into popular cassava varieties in future.

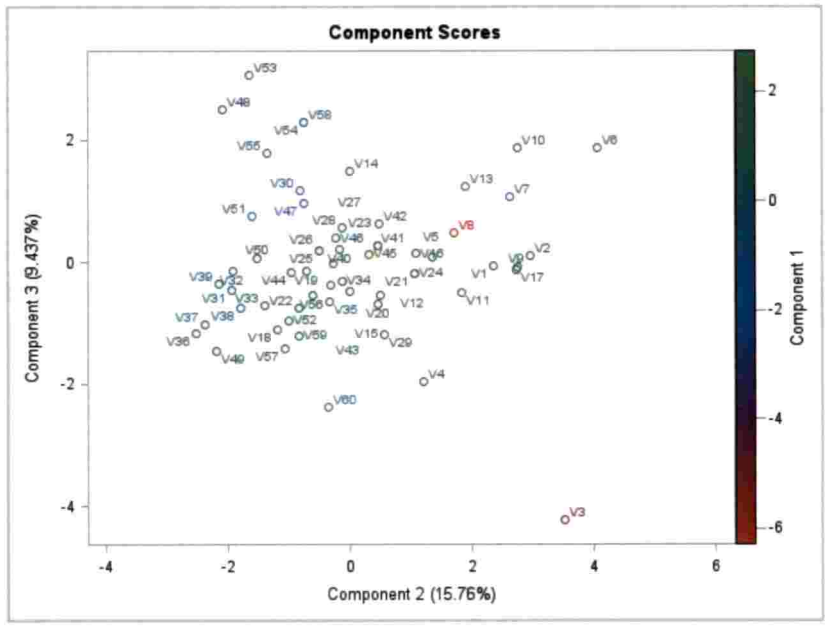

Fig.11 Projection of 60 cassava genotypes and morphological traits on PCI and PCII

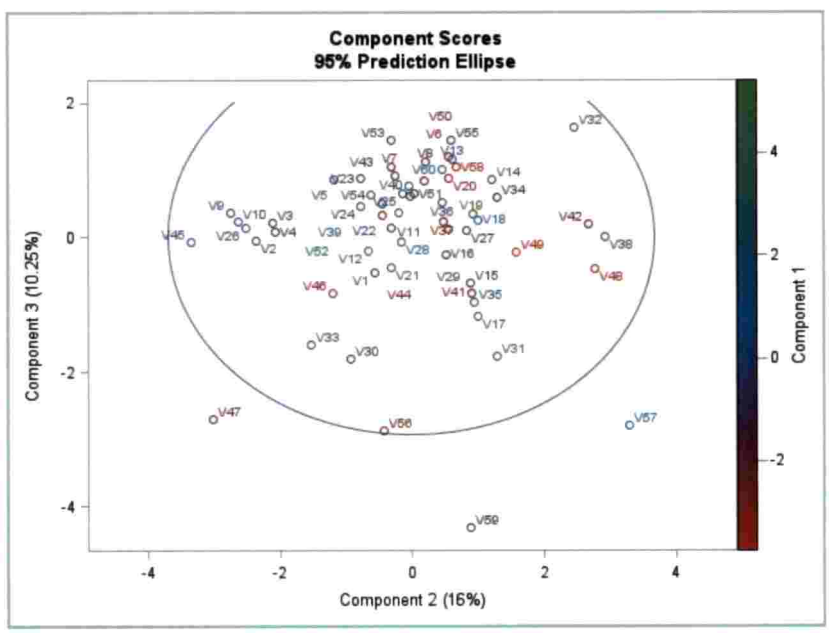

Fig.12 Projection of 60 cassava genotypes and morphological traits on PCI, PCII and PCIII

Table 10. Eigen values, proportion, cumulative variance and component scores of the first 12 principal components for morphological traits in 60 genotypes of cassava.

Table 10 Eigen values of the Correlation Matrix				
	Eigen value	Difference	Proportion	Cumulative
1	4.44395010	2.36390873	0.3418	0.3418
2	2.08004137	0.74707988	0.1600	0.5018
3	1.33296149	0.22120422	0.1025	0.6044
4	1.11175728	0.17294047	0.0855	0.6899
5	0.93881681	0.12838238	0.0722	0.7621
6	0.81043442	0.05553278	0.0623	0.8245
7	0.75490164	0.18237354	0.0581	0.8825
8	0.57252811	0.13471424	0.0440	0.9266
9	0.43781387	0.10257285	0.0337	0.9602
10	0.33524102	0.22674206	0.0258	0.9860
11	0.10849896	0.03544403	0.0083	0.9944
12	0.07305493	0.07305493	0.0056	1.0000

4.7 Real Time PCR

Among the 60 cassava genotypes studied, 39 were found to be resistant. A SYBR Green real time PCR assay for finding the virus load was established and conducted to quantify the viral DNA. Absolute quantification of DNA A and B of SLCMV has been carried out using by primers Sy- SLCMV A_F, Sy- SLCMV A_R. Three technical replicate reactions were prepared for each DNA sample. Results of qPCR

are given in Table 11. The resistant varieties were found to be free from viruses causing cassava mosaic disease.

Table 12. Results of qPCR viral load assay.

Standard	Values obtained	Positive (copies of amplification)	Accessions	VALUES	Negative control
10^{-1}	433000	414807	CR43-2	Nil	Nil
10^{-2}	43347	414807	CR20-A2	Nil	Nil
10^{-3}	4335	414807	CR35-8	Nil	Nil
10^{-4}	433	414807	Sree Padmanabha	Nil	Nil
10^{-5}	43.3	414807	CR43-7	Nil	Nil
10^{-6}	4.33	414807	CR24 - 4	Nil	Nil
10^{-7}	0.433	414807			Nil
10^{-8}	0.0433	414807			Nil

CR59-8R **CR20A2**

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CR24-4

5. DISCUSSION

Cassava (*Manihot esculenta* Crantz), family Euphorbiaceae, is the fifth most important staple food crop of the world. Its starchy tuberous roots provide food for over 500 million people, mostly in developing countries. In India, this crop is grown in an area of 2.44 lakhs ha, mostly in Kerala, Tamil Nadu, Andhra Pradesh, Karnataka, Maharashtra and a few North Eastern states with production of more than 60 lakhs tons. It is a famine reserve crop well suited to rain fed system of farming. Cassava is affected by a number of diseases causing substantial losses in yields. Of all the diseases reported, Cassava Mosaic Disease (CMD) is one of the most serious diseases, which leads to heavy loss of the crop in India and Africa. In India, the yield loss reported due to cassava mosaic disease incidence is in the range of 17-88% (Malathi *et al.,* 1985) depending on the varieties infected. Most of the popular cassava varieties grown in Kerala are highly susceptible to cassava mosaic disease caused by Indian Cassava Mosaic Virus (ICMV)/ Sri Lankan Cassava Mosaic Virus (SLCMV). Host plant resistance, polygenic / monogenic dominant, is helpful in the development of resistant varieties using a combination of conventional and marker assisted breeding methods. The study of variability and diversity present in diverse cassava land races and pre-breeding lines maintained at the National repository of CTCRI, is essential to design the marker assisted breeding program of cassava in Kerala.

The present investigation entitled **"Molecular marker analysis for cassava mosaic disease resistance**"was carried out at ICAR-Central Tuber Crops Research Institute, Sreekariyam Thiruvananthapuram to analyse the genetic variation among cassava mosaic disease resistant genotypes and to identify molecular markers linked to cassava mosaic disease resistance to facilitate the speed breeding of cassava mosaic disease resistant varieties suited to Kerala.

5.1 CMD screening

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Sixty cassava genotypes were screened for CMD incidence and visual scoring was done in a 0-5 range scale. Among the 60 accessions evaluated, 39 were resistant and 21 were found to be susceptible. Cassava samples collected from CTCRI were diagnosed for the presence of Indian Cassava Mosaic Virus (ICMV) and Sri Lankan Cassava Mosaic Virus (SLCMV) through multiplex PCR analysis. It was done using primers specific for ICMV and SLCMV. Multiplex PCR was useful in differentiating Sri Lankan Cassava Mosaic Virus (SLCMV) which produced 600bp fragment and Indian Cassava Mosaic Virus (ICMV) that produced 900bp fragment after multiplex PCR. A multiplex PCR was developed for simultaneous detection of different viral strains in cassava affected with cassava mosaic disease (CMD). Mostly results of multiplex PCR with ICMV/SLCMV specific primers was in agreement with field screening results.

5.2 Molecular marker analysis

The SSR primers selected based on review of literature were screened by bulk Segregant Analysis. Primers screened include SSRY 28, SSRY 44, SSRY 45, SSRY 100, SSRY 105, MeESSRY19 and SSRY 234. Out of the 7 primers screened SSRY28, SSRY 44 showed association with cassava mosaic disease resistance. From the markers studied it was found that SSRY28 amplified the expected product size of 180 bp in eighteen highly resistant genotypes. However the allele was amplified in susceptible varieties like Vellayani Hrazwa too. Hence it was not found to be highly linked to CMD resistance. According to Lokko *et al.,* 2005, the SSR marker, SSRY28-180, donated by the resistant parents was linked with resistance to CMD. Marker-trait association detected by regression analysis showed that the marker, accounted for 57.41% of total phenotypic variation for resistance. However in the present study, SSRY28 was not found to be tightly linked to CMD resistance.

SSRYMe19 amplified the allele in eighty seven percent of the resistant genotypes. However it was amplified in 95 percent of the susceptible genotypes too. None of the alleles of the markers studied could distinguish completely between CMD resistant and CMD susceptible genotypes. Among the markers tested SSRY44 amplified the allele 250bp in 20 highly resistant genotypes and was amplified only in five susceptible genotypes. Hence SSR Y 44 (250 bp) can be used in marker assisted selection for CMD resistance in cassava after further validation. Catia *et al.*,2015 reported that the *CMD2* gene was found in 5, 4 and 5 % of cassava accessions screened, with flanking markers NS169+RME1, NS158+RME1 and SSRY28+RME1. In his study, an individual analysis of the presence of the alleles that were associated with *CMD2* for the NS169, NS158, SSRY28 and RME1 markers showed that 19, 16, 23, and 27 % of the cassava accessions had alleles associated with CMD resistance. In the present investigation also, 48.3% of the population had the allele 180 bp amplified the marker SSR Y28. Eventhough tight linkage was not observed, SSR Y 28 and SSR Y44 were useful for marker assisted selection for CMD resistance in Cassava.

The major allele frequency ranged from 0.6556 (SSR Y 44) to 0.95 (SSRY234). The expected heterozygosity ranged from 0.2637 (SSRMe9) to 0.6297 (SSRY44). SSRY 45 also recorded high expected heterozygosity (0.6096). The high He value recorded for SSR 44 and SSR45 (>0.6) is in agreement with the reports of Tiago *et al.*,2015. High heterozygosity is due to the fact that cassava plants are able to perform natural crossed fertilization, open pollination and protogynous flowering (Fregene *et al.*, 2003).

The maximum PIC value was recorded by the marker SSRY44 (0.5586) followed by SSRY45 (0.5218). SSRMe9 recorded the lowest PIC value(0.2289) among the markers tested.

The gene diversity index was found to be the lowest for the primer SSRY234(0.0944) . The maximum gene diversity was recorded for the marker SSRY44 (0.4419) followed by SSRY105 (0.4111), SSR Y 28 (0.3886) and SSR Y45 (0.3578). The PIC and He values recorded for SSR Y28 in the present study was lesser than the earlier reports by Tiago *et al.*,2015.

5.3 Morphological characterization

Sixty cassava genotypes were characterized based on seven quantitative traits, five qualitative traits and CMD resistance. No. of leaves ranged from 50 (CMR 12) to 1800 (Vellayani Hraswa). The genotypes viz. 8W5, CR 43-7, CR 21-10 and CI-273, 9S 172 recorded very high leaf retention ability (>1000 leaves/plant). The leaf weight/plant ranged from 0.04kg (CE 185) to 1.45 kg(Vellayani Hraswa). The stem weight was highest in Sree Swarna (5kg/plant) followed by CI273 and 7IVC4 (4. 5) and was lowest in CMR12 (0.5 kg/plant).

The tuber weight ranges from 0.3 (CMR 12) to 11 (CR59-8R) kg per plant. All the high yielding genotypes *viz.* MN-1, CR43-7, CR54A-19, CR52A41, CR21-10, CE185, S1284, CR20A-2, 9S165, CI273 and CR59-8R that recorded very high weight of tubers per plant(>6 kg/plant) were found to be CMD resistant too.

The maximum value for harvest index among the cassava genotypes was recorded in 9S165 (0.84) followed by IMS1-9 (0.80), MN-2 (0.78), CR63-3 (0.76 and CR59-8 (0.76). The lowest harvest index value was recorded in TCR56 (0.24) followed by the triploid variety Sree Apoorva (0.29.). Since harvest index is one of the most important selection criteria in cassava, the genotypes with high harvest index will be useful for developing novel varieties in future.

Number of tubers per plant ranged from 1 (TCR56) to 20 (CR21-10). CI273, 9S98 and CE185 also recorded higher number of tubers per plant (19). The stem girth ranged from 4.5 cm (11S35) to 14.5cm (Sree Apoorva). TCR56, CMR12 and TCR63

also recorded low value (5cm) for stem girth while CR21-10 (13.5 cm), Sree Harsha (13.1 cm), CR52A41(12.5cm) and I-13S5-17(12.5cm) recorded high values for stem girth.

5.4 Cluster Analysis

Grouping of landraces based on their similarity is important in the formulation of speed breeding programmes. In the present investigation, cluster analysis was done based on Euclidian distance of 60 genotypes of the SSR markers studied. The similarity coefficient matrix was calculated and dendrogram was constructed using UPGMA cluster analysis. Dendrogram showed the partition of genotypes into 3 main clusters. At 0.4% similarity it was subdivided into 3 major clusters.

Based on morphological data seven clusters were formed. Six clusters were again divided into two sub clusters and seventh cluster formed three sub clusters. Vellayani Hrazwa was found to be highly divergent and formed separate cluster with early bulking genotypes like CR21-10(V31), 9S172, (V35), CR43-7 (V32) and 8W5 (V17). In agreement with the present finding, Ribeiro *et al.* (2011) reported similarity ranging between 0.16 and 0.96. The clustering pattern suggests the grouping of CMD resistant genotypes together in the same cluster.

5.5 Principal component analysis (PCA)

The PCA analysis provides information about associations of accessions, which are useful to formulate better strategies for breeding. The patterns of variation and the relative importance of each morphological trait in explaining the observed variability was assessed through principal component analysis (PCA). The first principal component (PC-1) accounted 34.18% of the total variation and was correlated positively with total biomass (0.4521), tuber weight/plant (0.4099), stem weight (0.3769) and leaf retention (0.3429) while CMD (-0.2091) contributed negatively as expected. The second principal component (PC-2) accounted 16% of

the total variability while PC3 contributed to 10.25% of the variability among 60 cassava genotypes studied. Plotting the first and second principal components from the matrix showed MN2 as an outlier. From the plot on first three principal components, three genotypes 11S18, CMR104 and CR57-6 were found to be outliers.

SYBR Green real time PCR assay for finding the virus load was established and conducted to quantify the viral DNA. Absolute quantification of DNA A and B of SLCMV has been carried out and the resistant varieties were found to be free from viruses causing cassava mosaic disease.

In the present investigation, 39 genotypes with complete resistant to cassava mosaic disease caused by ICMV/SLCMV were identified, Molecular marker analysis indicates the association of SSRY28 and SSRY44 with CMD resistance. However further validation is needed for using these markers for undertaking Marker assisted selection in cassava.

6. SUMMARY

The experiment entitled "Molecular marker analysis for cassava mosaic disease resistance" was carried out at Division of Crop Improvement, ICAR-CTCRI, Sreekariyam, Thiruvananthapuram during 2013-14. The main objective of the investigation was to analyse the genetic variation among cassava mosaic disease resistant genotypes and to identify molecular markers linked to cassava mosaic disease resistance incidence.

Sixty cassava genotypes were screened for CMD incidence and visual scoring was done in a 0-5 range scale. Among the 60 accessions evaluated, 39 were resistant and 21 were found to be susceptible. Cassava samples collected from CTCRI were diagnosed for the presence of Indian Cassava Mosaic Virus (ICMV) and Sri Lankan Cassava Mosaic Virus (SLCMV) through multiplex PCR analysis. It was done using primers specific for ICMV and SLCMV. Mostly results of multiplex PCR with ICMV/SLCMV specific primers was in agreement with field screening results.

For molecular analysis, the SSR primers were selected based on review of literature and were screened by Bulk Segregant Analysis. Primers screened include SSRY 28, SSRY 44, SSRY 45, SSRY 100, SSRY 105, MeESSRY19 and SSRY 234. From the markers studied, it was found that SSRY28 amplified the expected product size of 180 bp in eighteen highly resistant genotypes. However the allele was amplified in susceptible varieties like Vellayani Hrazwa too. SSRYMe19 amplified the allele in eighty seven percent of the resistant genotypes. However it was amplified in 95 percent of the susceptible genotypes too. None of the alleles of the markers studied could distinguish completely between CMD resistant and CMD susceptible genotypes. Among the markers tested, SSRY44 amplified the allele 250bp in 20 highly resistant genotypes and was amplified only in five susceptible genotypes. Hence SSR Y 44 (250 bp) can be used in marker assisted selection for CMD resistance in cassava after further validation.

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In the present investigation, 39 genotypes with complete resistant to cassava mosaic disease caused by ICMV/SLCMV were identified. These resistant lines can be further evaluated for developing a high yielding CMD resistant variety in future. Molecular marker analysis indicates the association of SSRY28 and SSRY44 with CMD resistance. These makers can be used for marker assisted selection to facilitate speed breeding of new cassava varieties.

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APPENDIX I

CTAB Extraction Buffer

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

APPENDIX II

TE buffer

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

APPENDIX III

TBE Buffer (5x) for 1 liter solution

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

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

APPENDIX IV

Chloroform : Isoamyl alcohol

Chloroform	24ml
Isoamyl alcohol	1ml

Mix 24 parts of chloroform with 1 part of isoamyl alcohol and store in a tightly capped bottle.

8. ABSTRACT

The experiment entitled "Molecular marker analysis for cassava mosaic disease resistance" was carried out at Division of Crop Improvement, ICAR-CTCRI, Sreekariyam, Thiruvananthapuram during 2013-14. The main objective of the investigation was to analyse the genetic variation among cassava mosaic disease resistant genotypes and to identify molecular markers linked to cassava mosaic disease resistance incidence.

Sixty cassava genotypes were screened for CMD incidence and visual scoring was done in a 0-5 range scale. Among the 60 accessions evaluated, 39 were resistant and 21 were found to be susceptible. Cassava samples collected from CTCRI were diagnosed for the presence of Indian Cassava Mosaic Virus (ICMV) and Sri Lankan Cassava Mosaic Virus (SLCMV) through multiplex PCR analysis and the results were in agreement with field screening results.

For molecular analysis, the SSR primers were selected based on review of literature and were screened by Bulk Segregant Analysis. Primers screened include SSRY 28, SSRY 44, SSRY 45, SSRY 100, SSRY 105, MeESSRY19 and SSRY 234. From the markers studied, it was found that SSRY28 amplified the expected product size of 180 bp in eighteen highly resistant genotypes. SSRYMe19 amplified the allele in eighty seven percent of the resistant genotypes.. None of the alleles of the markers studied could distinguish completely between CMD resistant and CMD susceptible genotypes. Among the markers tested, SSRY44 amplified the allele 250bp in 20 highly resistant genotypes and was amplified only in five susceptible genotypes. Hence SSR Y 44 (250 bp) can be used in marker assisted selection for CMD resistance in cassava after further validation.

The expected heterozygosity of the SSR markers ranged from 0.2637 (SSRMe9) to 0.6297 (SSRY44). The maximum PIC value was recorded by the marker SSRY44 (0.5586) followed by SSRY45 (0.5218). The gene diversity index

was found to be the lowest for the primer SSRY234 (0.0944). The maximum gene diversity was recorded for the marker SSRY44 (0.4419) followed by SSRY105 (0.4111), SSR Y 28 (0.3886) and SSR Y45 (0.3578).

Sixty cassava genotypes were characterized based on seven quantitative traits, five qualitative traits and CMD resistance. The genotypes *viz.* 8W5, CR 43-7, CR 21-10 and CI-273, 9S 172 recorded very high leaf retention ability (>1000 leaves/plant). The tuber weight ranged from 0.3 (CMR 12) to 11 (CR59-8R) kg per plant. All the high yielding genotypes *viz.* MN-1, CR43-7, CR54A-19, CR52A41, CR21-10, CE185, S1284, CR20A-2, 9S165, CI273 and CR59-8R that recorded very high weight of tubers per plant(>6 kg/plant) were found to be CMD resistant too. The maximum value for harvest index among the cassava genotypes was recorded in 9S165 (0.84) followed by IMS1-9 (0.80), MN-2 (0.78), CR63-3 (0.76 and CR59-8 (0.76).

In the present investigation, cluster analysis was done based on Euclidian distance of 60 genotypes of the SSR markers studied. Based on morphological data seven clusters were formed. Six clusters were again divided into two sub clusters and seventh cluster formed three sub clusters.

The PCA analysis provides information about associations of accessions, which are useful to formulate better strategies for breeding. The patterns of variation and the relative importance of each morphological trait in explaining the observed variability was assessed through principal component analysis (PCA). The first principal component (PC-1) accounted 34.18% of the total variation and was correlated positively with total biomass (0.4521), tuber weight/plant (0.4099), stem weight (0.3769) and leaf retention (0.3429) while CMD (-0.2091) contributed negatively as expected.

SYBR Green real time PCR assay for finding the virus load was conducted to quantify the viral DNA and the resistant varieties were found to be free from viruses causing cassava mosaic disease.

In the present investigation, 39 genotypes with complete resistant to cassava mosaic disease caused by ICMV/SLCMV were identified. These resistant lines can be further evaluated for developing a high yielding CMD resistant variety in future. Molecular marker analysis indicates the association of SSRY28 and SSRY44 with CMD resistance. These makers can be used for marker assisted selection to facilitate speed breeding of new cassava varieties.