CHARACTERIZATION OF SELECTED ACCESSIONS OF CASSAVA GERMPLASM USING MORPHOLOGICAL AND MOLECULAR MARKERS

By

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(2012-09-122)

THESIS

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DECLARATION

I hereby declare that the thesis entitled **"Characterization of selected accessions of cassava germplasm using morphological and molecular markers"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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LIST OF ABBREVATIONS

%	Percentage
A_{230}	Absorbance at 230 nm wavelength
A_{260}	Absorbance at 260 nm wavelength
A_{280}	Absorbance at 280 nm wavelength
AFLP	Amplified fragment length polymorphism
ANOVA	Analysis of Variance
bp	Base pair
cm	centimetre
CTAB	Cetyl Trimethyl Ammonium Bromide
DNA	Deoxyribonucleic acid
dNTPs	Deoxy nucleotide tri phosphates
E	East
EDTA	Ethylene diamine tetra acetic acid
EST	Expressed Sequence Tag
FAOSTAT	Food and Agriculture Organization Statistical Database
g	gram
ICAR-CTCRI	ICAR- Central Tuber Crops Research Institute
NBPGR	ICAR-National Bureau of Plant Genetic Resources
IPGRI	International Plant Genetic Resources Institute
ISSR	Inter simple sequence repeat
KAU	Kerala Agricultural University
kb	Kilo bases
kg	Kilogram
L	Litre
m	Meter
M	Molar
mg	milligram

$MgCl_2$	Magnesium Chloride
min	Minute
ml	Millilitre
mm	Millimeter
mM	Millimolar
N	North
NaCl	Sodium chloride
NCBI	National Centre for Biotechnology Information
ng	Nanogram
nm	Nanometer
NPGRL	National Plant Genetic Resources Laboratory
°C	Degree Celsius
OD	Optical density
PC	Principal Component
PCA	Principal Component Analysis
PCR	Polymerase chain reaction
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	revolutions per minute
RT	Reverse transcriptase
RT-PCR	Reverse transcription-polymerase chain reaction
s	second
SAS	Statistical sequence length polymorphism
SM	Simple matching
sp.	Species
spp.	Species (plural)

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SSLP	Simple sequence length polymorphism
SSR	Simple sequence repeat
Taq	*Thermusaquaticus*
TBE	Tris-borate EDTA buffer
TE	Tris-EDTA buffer
T_m	Melting temperature
Tris HCl	Tris (Hydroxy methyl) aminomethane hydrochloride
U	Enzyme units
UV	Ultra violet
V	Volt
v/v	volume/volume
W	Watt
w/v	weight/volume
µg	Microgram
µl	Microlitre
µM	Micromolar

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INTRODUCTION

INTRODUCTION

Root and tuber crops are plants yielding starchy roots, tubers, corms and stems that are used mainly as food for human, feed for animals and also for manufacturing starch, alcohol and fermented beverages including beer. They provide a substantial part of the world's food supply and on a global basis approximately 45% of root and tuber crops production is consumed as food, with the remainder used as animal feed or for industrial processing for products.

The consumption of root and tuber crops as food in developed countries is considerably smaller than it is in developing countries, but their use as animal feed is relatively higher. Roots and tuber crops are second in importance to cereals as a global source of carbohydrate. They also provide some minerals and vitamins, although a proportion of minerals and vitamins may be lost during processing. Many tropical tuber crops are used in the preparation of stimulants, tonics, carminatives and expectorants. India holds a rich genetic diversity of tropical root and tubers crops *viz.*, cassava, sweet potato, aroids, yams and several minor tuber crops.

Cassava is the most widely cultivated tuber crop in tropics and it is cultivated mainly for starchy roots. It is the most important food staple in tropics, where it is the fourth most important source of energy and carbohydrate. Cassava is grown for various end uses such as human food, animal feed and as industrial raw materials.

The centre of origin of cassava was first reported to be Central America, including Colombia, Venezuela, Guatemala and Southern Mexico, due to the large number of varieties present there (Rogers, 1963). The exact area of origin of cassava as a crop plant is unknown, although several theories have been put forth. Deborah (1998) suggested that cassava was first cultivated in north-eastern Brazil as he based this theory on the abundance of wild *Manihot* species in that region. South America is known as the native place of cassava, but nowadays cassava is extensively cultivated as an annual crop in many tropical and sub-tropical regions

of the world including Africa, India and Indonesia. Among these countries Africa is its largest centre of production.

Cassava, botanically known as *Manihot esculenta* Crantz is a perennial shrub belonging to the family *Euphorbiaceae* of class Dicotyledons. The *Manihot* genus is reported to have about 100 species among which *Manihot esculenta* is the commercially cultivated one. Cassava is a monoecious species with chromosome number in all species as 2n=36. In spite of this high chromosome number, *Manihot* species behave meiotically as diploid. Cassava is highly heterozygous in nature because of its outcrossing property. Increased genetic variablity in cassava is noted because of its increased species variation on wild cassava and also with the traditional farming practises. Cassava cultivars are lacking many economically important characters such as resistance to insects, diseases, drought and have low protein content (Nassar and Dorea, 1982; Nassar and Grattapaglia, 1986). This can be attributed to the mode of evolution of the species and modifications of the allogamy system of the plant (Nassar and O'hare, 1985).

Genetic diversity serves as a way for populations to adapt to changing environments. With more variation, it is more likely that some individuals in a population will possess variations of alleles that are suited for the environment. Those individuals are more likely to survive to produce offspring bearing that allele. The population will continue for more generations because of the success of these individuals. Genetic variability in cassava can be assessed using morphological and molecular markers.

The National and international research institutes maintain a very good number of germplasm collections of cassava. ICAR-CTCRI is the only institute in India dealing with the tropical tuber crops research in India. The genetic variability present in these germplasm collections of cassava is evaluated by using the agro-morphological descriptors which are generated by the International Plant Genetic Resources Institute (IPGRI) and the Indian Council of Agricultural Research-National Bureau of Plant Genetic Resources (ICAR-NBPGR). Morphological descriptors include both the above and below ground characters

such as the plant, stem, leaf, flower, fruit and tuber characters. The characters like plant height, stem diameter, internode length, leaf length and width, petiole length and width, number of tubers, tuber yield per plant, tuber length and weight etc. are collectively called as quantitative characters whereas the plant vigour, growth habit, stem colour, leaf scar, leaf colour, petiole colour, flowering, fruit colour, tuber outer skin, cortex and flesh colour etc. belongs to the qualitative characters. In the morphological characterization, it is difficult to make precise descriptors because the genotype phenotypically varies with the environmental factors. So in addition, along with morphological characterization, molecular characterization based mainly on DNA markers has been used to analyse/describe the germplasm genetic diversity (Fregene *et al.*, 1997).

Molecular characterization includes a number of molecular methods such as Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP) and most recently the Single Sequence Repeat (SSR - also known as microsatellites) markers have been used to study the genetic diversity in cassava (Fregene *et al.*, 2003). SSR molecular markers are a very effective tool for assessing the genetic diversity since they can be easily adapted for the classification and identification of many organisms and are particularly useful in studying the variation in allelic frequency of unlike loci.

This research work endeavours to combine both morphological and molecular data for considerable understanding of the distribution and range of genetic dissimilarity present within the selected cassava accessions collected from different states of South India.

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REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1 Cassava

2.1.1 Origin and History of cassava

Cassava (*Manihot esculenta* Crantz) is a shrub which belongs to the family Euphorbiaceae and is well known for its starchy roots. It is ranked the sixth most important source of calories in the diet of humans as reported by the food and agricultural organisation in 1999. Cassava is the major root crop in the tropical areas. During 1970s this crop was unknown in developed countries, but the situation was quite opposite in developing countries like Africa, West Indies, South America and Asia where cassava was one of the most important energy source of dietary carbohydrate and protein for millions (Allem, 1994).

Kingdom	:	Plantae
Order	:	Malpighiales
Family	:	Euphorbiaceae
Tribe	:	Manihoteae
Genus	:	*Manihot*
Species	:	*esculenta*

Belonging to the dicotyledon family *Manihot* is known to have about 200 species and among these the most important species is *M. esculenta* Crantz which is commonly known by the names Cassava, Manioc, Tapioca, Mandioca, Yuca etc. *Manihot* is the native of tropical Brazil, from where it spread to different parts of South America and to North America, then finally to sub-tropical humid regions like Asia and Africa. Production of cassava in India is mainly concentrated to 13 States *viz*., Andhra Pradesh, Assam, Karnataka, Kerala, Meghalaya, Nagaland, Tamil Nadu, Rajasthan, Arunachal Pradesh, Mizoram, Pondicherry, Tripura and Andaman and Nicobar Islands. Cassava is being cultivated as a subsistence crop by farmers in these places (Haysom *et al*., 1994).

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2.1.2 Cassava propagation

Cassava can be easily propagated by stem cuttings and by sexual seeds. Out of these two practices, stem cutting propagation is the most common. When done in favourable climatic conditions shoots and roots arise within a week. This crop can also be propagated from true seeds which will take longer time for establishment. Slow propagation is the main drawback of plants propagated from true seeds (Allem, 1994).

2.1.3 Morphology of cassava

Roots are the storage units in cassava plants which may vary in shape and size. External pigmentation of cassava roots may range from white to dark brown and the cortex colour from white to purple. The root shape may be conical, oval, cylindrical or irregular (Wheatley and Chuzel, 1993). Cassava plants have 3-14 storage roots and the length of it ranges from 15 to 100 cm and weight of one root up to 3 kilograms. Apart from the physiology of cassava roots anatomically the roots of cassava are not tuberous but true roots (Hillocks and Jenmings, 2003).

The stem of cassava is woody and cylindrical with alternating nodes and internodes. It has a correlation between cassava roots based on the pigmentation that is, higher roots have lighter stems and vice versa. Cassava stem have sympodial branching, they divide di, tri and tetrachotomously and produces secondary branches which in turn produces other successive branches which will turn to reproductive branches after flowering. Stem colour varies from silver, grey to dark brown. The branching habit of the plant ranges from erect, semi-erect to spreading types. Growth habit of stem can be straight or zigzag (Nartey, 1988).

Leaves are simple, alternate, laminate and have a phyllotaxy of 2/5. Petiole length ranges from 5 to 30 cm and reach up to 40 cm. Cassava leaves are palmately lobed and lobe number may vary from 3 to 9 consisting of 3-11 linear, obovate, pandurate lobes. The pigmentation of petioles also varies from different shades of green to red. Apical leaf colour may be light green or purple. Mature

leaves are glabrous and are surrounded by two stipules (Fukuda and Guevara, 1998).

Flowers of cassava are monoecious and heterozygous in nature which produces both male (pistillate) and female (staminate) flowers on different branches. Female flowers will be few in number as compared to male flowers. Female flowers are present on the lower part of the inflorescence whereas male flowers are numerous and can be seen on upper part of the inflorescence, which in turn found at the insertion point of reproductive branches. Both the flowers open at the same time facilitating the cross pollination by insects (Nartey, 1988).

Pedicel of male flower is thin, straight and very short and the basal disk divides to 10 lobes. Pollen colour ranges from yellow to orange and the size ranges from 122 to 148 micrometer. Pollen grains of cassava flowers are found to be larger as compared to other flowering plants. Ovary is tricarpellary with six ridges and ovule has three locules each (Ghosh *et al.*, 1988).

Fruit of cassava is a trilocular capsule. It is ovoid and globular, having 1 to 1.5 cm in diameter and has six straight aristae. Each of the locule contains a single caruncculate seed. Maturation of fruit occurs 75 to 90 days after pollination. Seeds of cassava are ovoid, ellipsoidal and approximately 100 mm long, 6mm wide and 4 mm thick. Weight varies from 95 to 136 mg per seed. It usually takes 16 days for germination soon after collection. The seed colour ranges from dark brown to grey (Rogers, 1973).

2.1.4 Economic importance

Cassava products being an item of export to the developed industrial countries have tremendous uses and play an important role in nutrition of people in the developing countries. It can also be used as food and animal feed. The current status and development on *Manihot esculenta* is based on the usefulness of cassava plant and their products. Cassava, the perennial shrub belonging to the dicotyledon family has a height of 1-4 metres. Two different plant types are

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known, one is erect (with or without branching on top) and the other is spreading type. The botanical traits of cassava are highly variable in nature.

Utilisation of cassava products will enhance the rural industrial development and increase the income of producers, processors and traders thereby promoting the nutrition and livelihood of more than five hundred million people, producers and traders all over the world. The utilisation of *M. esculenta* as one of the important food crop faces two major problems. One is the moderate content of protein in cassava and the consumption might result in nitrogen imbalance which leads to kwashiorkor, a protein deficiency syndrome. Second is the release of large quantities of hydrogen cyanide from the cassava food materials which will result in chronic cyanide intoxication, goitre, ataxic neuropathy, respiratory problems and even death (Nartey, 1988).

2.2 Molecular genetics in cassava

Genetic markers represent genetic differences between individual organisms or species and they are located in close proximity to genes. Such markers do not affect the phenotype of the trait of interest and are located only near or linked to genes controlling the trait. There are three types of genetic markers which include morphological markers, biochemical markers and DNA markers.

2.2.1 Morphological characterization

In 1790, Goethe originated the morphological characterization as a scientific discipline (Donald, 2001). This is carried out on a representative population of an accession using a list of descriptors for the species (Benjamin *et al.*, 2008). In morphological characterization, a character is a feature of an organism that can be counted or measured (Heywood, 1967). Based on the ease for observation, availability and usefulness in classifying and identifying organisms, the characters have been selected. Some characters are not affected by the environmental factors and have a genetic basis. These types of characters are highly heritable and known as constant characters while some others are highly

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influenced by the environment (Deborah and Karamura, 1998). Morphological markers are usually visually readable phenotypic characters such as flower colour, seed shape, growth habits or pigmentation (Sumarani *et al.*, 2004). The phenotypic expression is the product of the combined effect of the environment and the genotype.

According to Donald (2001), there are four major areas in plant morphological study. The first area examines structures of different plants of the same or different species, then draws comparisons and formulates ideas about similarities. The second area of plant morphology observes both somatic and reproductive structures of plants. The third area of plant morphology studies overlap with plant anatomy i.e. it deals with the plant cell structural features. In the fourth area, plant morphology studies the plant growth pattern and development from its seedling till maturation. In the characterization of phenotypes, Stuber *et al.* (1999) proves that plant morphology would contribute to plant genetics. But there are limitations that are associated with the morphological markers. The high dependency of these morphological markers to the environment is considered as its major drawback (Stuber *et al.*, 1999). This limitations of the phenotype based genetic markers led to the development of DNA markers (Akkaya *et al.*, 1995).

Agromorphological characterization of elite cassava was done by Agre *et al.* (2015). For this morphological study, one hundred and sixteen cassava genotypes were selected and evaluated using the statistical tool such as randomized block design, cluster analysis and principal component analysis (PCA). Data assessment was done at three, six, nine and twelve months after planting. Both the qualitative and quantitative characters were observed. Quantitative characters include plant height, root length, petiole length, leaf lobe length and width etc. Principal component analysis clustered the total varieties into 4 groups with special characteristics and revealed that a large portion of the phenotypic variance was accounted for the genotypic component for all traits assessed indicating substantial genetic variability among the genotypes evaluated.

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Fifty nine cassava genotypes selected from the Southern region of Chad were subjected for the genetic variability analysis (Nadjiam *et al*., 2016). Here almost 45 agromorphological descriptors were assessed at the three, six, nine and twelve months after planting. These cassava accessions showed phenotypic variations such as color of apical leaf, leaf vein, petiole, root and pulp. Positive correlations were found between the leaflet and leaflobe numbers. Fresh root weight was also positively correlated to the root number, length and diameter. Principal component analysis on quantitative variables revealed four groups in this study.

2.2 DNA markers

DNA markers are the most widely used type of markers predominantly due to their abundance. There are different classes of DNA mutations such as rearrangements (insertions or deletion), substitutions (point mutations) or errors in replication of randomly repeated DNA (Paterson, 1996).

Molecular marker techniques are based on naturally occurring polymorphisms in DNA sequences (Wetermeier, 1993). Molecular markers are sequence of nucleic acids that makes up a segment of DNA near the DNA sequence of the desired gene. These markers are used to identify a particular sequence of DNA in a pool of unknown DNA.

2.2.1 Restriction Fragment Length Polymorphism (RFLP)

RFLP was the first described molecular marker technique in the detection of DNA polymorphism for the construction of genetic maps (Milee *et al.*, 2008). In RFLP method, the enzymes called restriction endonucleases, which has specific restriction sites (palindrome sequences) digest the DNA sequences (McClelland *et al.*, 1994). As a result mutations like inversion, translocation, transposition, in the plant DNA sequences leads to a simple or large base pair changes and which may occur a loss or gain of a recognition sites and in turn lead to restriction fragment of different lengths (Jonah *et al*., 2011). This length differences enables to screen

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the polymorphisms between different genotypes. By using hybridization technique, DNA polymorphism can be detected. In hybridization, a chemically labelled probe which is species specific single locus about 0.5 to 3.0 kb in size is used (Kurt *et al.*, 2005).

The main advantage of RFLP markers is that they are highly reproducible and codominant. For the genotype identification and percentage analysis, multi-locus RFLP markers were used. Because of its time consumption and tedious experimental procedure, this technique is not widely used (Milee *et al.*, 2008).

In a study conducted by Fregene *et al.* (1997), 132 RFLPs were developed for the genetic linkage map construction of cassava cultivars collected from Nigeria (TMS 3057) and Columbia (CM 2177-2).

2.2.2 Random Amplified Polymorphic DNA (RAPD)

RAPD were the first of the PCR based markers. In 1990s Welsh and McClelland discovered the RAPD marker system independently (Williams *et al.*, 1991). In RAPD a small amount of template DNA (5-50 ng) and a single RAPD primer is needed for the PCR reaction.

The key advantage of RAPD is that they are quick and easy to examine i.e. in the examination procedure 1.5% agarose gel is used for running the product and EtBr is added on the agarose gel and visualised under the ultraviolet rays. Here the simplicity and low cost of agarose gel electrophoresis made RAPD popular (Semagn *et al.*, 2006). Another advantage of RAPD is its high genomic abundance (Kumar *et al.*, 2009).

To characterize the elite cassava germplasm mainatined at CIAT, 32 RAPD markers were used (Gomez *et al.*, 1994; Bonierable *et al.*, 1995). RAPD markers were also used to evaluate the genetic distances between African cultivars and Brazilian cultivars (Carvalho *et al.*, 2001; Mignouna and Dixon, 1997). The genetic diversity studies in the 126 genotypes of cassava with RAPD markers resulted in important implications for genetic breeding and cassava germplasm collections (Colombo *et al.*, 2000).

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2.2.3 Amplified fragment length polymorphism (AFLP)

Keygene, a Dutch company developed the Amplified fragment length polymorphism technology. AFLP combines the RFLP and PCR techniques (Vos *et al.*, 1995; Milee *et al.*, 2008). AFLP method involve three steps i.e. (i) restriction enzyme digestion (ii) ligation of adapters and (iii) selective amplification of restricted fragments (Faroq and Azam, 2002).

Advantages of AFLP include its high genomic abundance, considerable reproducibility, generation of many informative bands per reaction and no sequence data for primer construction (Vos *et al.*, 1995; Kumar *et al.*, 2009).

Chavarriaga-Agurrie *et al.* (1999) evaluated the genetic diversity and redundancy in the cassava core collection using AFLP markers. For the construction of the genetic linkage map of cassava, 378 AFLP markers generated from 76 primers and the study was conducted in the F1 progeny. In this study 58 F1 progenies from a cross between Rayong 90 (female) and Rayong 5 (male) were conducted using AFLP (Kunkeaw *et al.*, 2010).

2.2.4 Inter Simple Sequence Repeat (ISSR)

ISSR is a marker technique which uses microsatellite sequences as primer in a PCR. It is a quick and simple method that combines most of the advantages of SSR and AFLP to the universality of RAPD.

Zayed *et al.* (2012) studied the genotype identification in the four imported varieties of cassava using 10 ISSR primers that can be used for the generation of reproducible and reliable amplicons. Here, ISSRs were used to evaluate the genetic distance and for the construction of molecular profile among selected cassava accessions.

2.2.5 Simple Sequence Repeats (SSR)

Small stretches of DNA sequences consisting of short tandem repeats of mono, di, tri, tetra, penta and hexa nucleotides motifs are collectively known as microsatellites or SSR markers (Tautz, 1989). The SSRs are widely distributed

throughout genomes and have been found in all genomes of prokaryotes and eukaryotes (Katti *et al.,* 2001). These genetic markers are first reported as microsatellite by Litt and Luty (1989) and later as SSRs by Jacob *et al.* (1991).

The SSR markers are codominant which are capable of detecting differences between and within the species. So they are known as ideal genetic markers (Jonah *et al.*, 2011) that can be used across genus and species boundaries (Qiu Hong *et al.*, 2004). The main applications of SSR markers are in genetic mapping, genotype identification, molecular tagging of genes, analysis of genetic diversity, phenotype mapping and marker assisted selection (MAS) (Tautz, 1989 and Powell *et al.*, 1996).

Nowadays, 1000 types of SSR markers are readily available for the genetic study in the cassava. New approaches are initiated to expand the multiplex ratio of SSRs. Multiplex-Ready™ Marker technology (MRT) is an innovative technology developed by the University of Adelaide. These declines the marker deployment costs for fluorescent based SSR analysis and enlarge genotyping through put (Nikky *et al.*, 2009).

2.2.5.1 SSR diversity in cassava

Characterization of cassava germplasm was done using the fourteen microsatellites containing GA repeats. Here, P^{32} labelled primers were used to estimate the heterozygosity of microsatellites (Chavarriaga-Aguirre *et al.*, 1999)

By the help of five SSR markers, Olsen and Schaal (2001) revealed the evolutionary and geographical origin of 220 accessions of cassava. The main aim of the study was to develope the population structure of the cassava germplasm and their phenotypic analysis.

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Cassava germplasm (419 accessions) collected from different parts of Brazil were subjected to genetic variability analysis by combining the morphological and molecular markers (Mezzette *et al.*, 2013). These subjected for screening by 12 morphological and 12 molecular markers showed lesser observed

heterozygosity (0.499) than the expected heterozygosity (0.642) and showed the genetic variations according to their origin.

An SSR based molecular genetic map of cassava was constructed using SSR markers and an F_2 population. In this study the F_2 population consist of 128 individuals was derived from selfing the genotype of K150 and an early genotyping F_1 progeny, from a cross between elite cassava varieties (TMS 30572 from IITA and CM 2177-2 from CIAT). Few numbers of SSR markers (472) were developed from the genomic and cDNA libraries of cassava. From these 472 markers, 120 polymorphic SSR markers were selected for the study. This is the first SSR based linkage map of cassava which leads to the quantitative trait mapping and genetic analysis of cassava genotypes. Constructed genetic map containing 100 marker spanning of 1236.7 cM were distributed among the 22 linkage groups have at an average marker distance of 17.92 cM (Okagbenin *et al.*, 2006).

Mba *et al.* (2001) investigated and developed an SSR based cassava genetic map by the combination of 36 RFLP and 172 SSR markers. From this study it is concluded that SSR marker based approach was productive because of its low cost and also helped to expand the efficiency of cassava breeding than RFLP markers.

Genetic variability studies were carried out in the 12 released varieties and 24 central Kerala collections of cassava using 36 SSR primers. Similarity coefficients were generated for the genetic diversity analysis. By using the statistical analysis, Similarity index and fixation index (F) for the released verities as well as central Kerala collections of cassava were derived. The calculated similarity index and fixation index of released varieties ranged from 60 to 93 % and 0.0688 while in case of central collections it ranged from 70 to 98 % and 0.1337 respectively (Lekha *et al.*, 2011).

A total of 58 cassava accessions were selected for SSR allele diversity and association mapping. This was done using 28 morphological and 15 SSR primers.

The study proved that accsessions like CO1, CO2, CO3 and Coo (TP)4 from TNAU and Sreeprakash and Sreepadmanabha from ICAR-CTCRI were allele specific to SSRY 324.PCA analysis was performed and 3 principal components accounted for 68% of the total variation in which PCA1 (48.1%), PCA2 (12.7%) and PCA3 (7.4%) variation respectively. The level of polymorphism using SSR was 86.56%. In this study morphological traits revealed 6 clusters of accessions while SSR showed 9 distinct clusters (Raghu *et al.*, 2007).

Thirty nine Tanzanian landraces of cassava were subjected for genetic diversity analysis using the above and below ground agromorphological characters combined with genomic traits using 13 SSR primers. Dendrogram drawn using both morphological and molecular markers separated the genotypes into 3 clusters with Euclidian distance ranging from 0.53 to 0.91 and 0.33 to 0.88 respectively, clearly showed the genetic variability among the landraces (Lyimo *et al.*, 2013).

In this study, we have undertaken to access the genetic diversity of 27 cassava accessions including germplasm and variety using both morphological and molecular markers.

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Table-1. Morphological and molecular markers used for Cassava diversity studies

Accessions used	References
Morphological	
42 landraces- Brazil	Siqueira *et al.* (2009)
43 accessions- Gahna	Asare *et al.* (2011)
Restriction Fragment Length Polymorphism	
80accessions (Cassava and wild species)	Beeching *et al.* (1997)
45 accessions	Fregene *et al.* (2000)
Randomly Amplified Polymorphic DNA	
24 accessions- Brazil	Carvalho *et al.* (2001)
50 accessions- Gahna	Asante *et al.* (2003)
24 accessions- Tanzania	Herzberg *et al.* (2004)
30 landraces- Latin America	Ferreira *et al.* (2008)
Amplified Fragment Length Polymorphism	
521 accessions	Chavarriaga-Agurrie *et al.* (1999)
8 accessions	Wong *et al.* (1999)
40 accessions	Elias *et al.* (2000)
Inter Simple Sequence Repeats	
4 cassava landraces	Zayed *et al.* (2012)
Simple Sequence Repeats	
48 landraces	Chavarriaga-Aguirre *et al.* (1998)
283 accessions using 67 SSR markers	Fregene *et al.* (2003)
38 varieties with 10 SSR	Elias *et al.* (2004)

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245 accessions- Uganda, 35 SSR	Kizito *et al*. (2005)
160 landraces	Pernoi *et al*. (2007)
36 cassava accessions- Uganda, 16 SSR markers	Moyib *et al*. (2007)
18 accessions, 3 SSR markers	Bi *et al*. (2010)
93 accessions, 14 microsatellites	Ribeiro *et al*. (2011)
162 landraces from Puerto Rica using 33 SSR primers	Montero *et al*. (2011)
Cassava verities from Africa, 96 SSR	Kabeya *et al*. (2012)
21 accessions - Tanzanian, 20 SSR markers	Elibariki *et al*. (2013)
43 accessions, 20 primers	Tetteh *et al*. (2013)
69 germplas-Kenya, 7 SSR markers	Ndung'u *et al*. (2014)

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The study entitled "Characterization of selected accessions of cassava germplasm using morphological and molecular markers" was carried out at the Division of Crop Improvement, ICAR-Central Tuber Crops Research Institute, Sreekariyam, and Thiruvananthapuram during 2016-2017. Details regarding the experimental materials and procedures used in the study are elaborated in this chapter.

3.1 SOURCE OF GERMPLASM

Twenty seven accessions of Cassava (*Manihot esculenta*) collected from different states of southern India and conserved in the ICAR-CTCRI germplasm repository were selected for the present study. Four plants per accession were raised in the field using the stem cuttings during April 2016 at a spacing of 90 cm x 90 cm. The passport data of accessions selected are listed out in Table-2.

3.2 MORPHOLOGICAL CHARACTERIZATION

Morphological characterization of the 27 accessions was done using a combination of IPGRI (2011)/NBPGR (2010) descriptors. Certain changes were made according to the handiness for listing out the observations taken and for the purpose of involving additional/modified traits. The observations were made in the full growth stage that is in the 8th month after planting and the tuber characters were recorded at the time of harvest.

Table-2: Passport data of the cassava accessions selected for the study

Sl. No.	Identity Number	Accession Number	LOCAL NAME	PLACE	DISTRICT	STATE
1	TCR 5	IC 086734	Mulanthari	Udurkara	Palghat	Kerala
2	TCR 10	IC 086736	Arumasam	Mundur	Palghat	Kerala
3	TCR 15	IC 086739	Pathinettukattan	Agali, Attappadi	Palghat	Kerala
4	TCR 20A	IC086743A	Elamuriyan	Nilambur	Malappuram	Kerala
5	TCR-31A	IC086748A	Nadan	Nadamangalam	Wynad	Kerala
6	TCR 36	IC086753	Nadankappa	Mukkam	Kozhikode	Kerala
7	TCR 45	IC086762	Kadalakkamaniyan	Thalipparamba	Kannur	Kerala
8	TCR 54	IC086774	Thonnuran	Kanhangad	Kasaragode	Kerala
9	TCR 56	IC086775	.....	Kanhangad	Kasaragode	Kerala
10	TCR 59	IC086772	Malavellakappa	Puthuppally	Malappuram	Kerala
11	TCR 66A	IC086782A	.....	Sikkanahalli	Mysore	Karnataka
12	TCR 69	IC086785	.....	Elanthur	Mysore	Karnataka

13	TCR 79	IC086895	.....	Budhavanahalli	Tumkur	Karnataka
14	TCR 80	IC086796	.....	Narayanahalli	Tumkur	Karnataka
15	TCR 93	IC086809	Ambakkadan	Vaikom	Kottayam	Kerala
16	TCR 124	IC136789	Thanduchoppan	Vanchiyoor	Thiruvananthapuram	Kerala
17	TCR 129	IC136790	Vellarose	Kallakurichi	South Arcot	Tamil Nadu
18	TCR 153	IC136792	Ramanadan	Mattur	Ernakulam	Kerala
19	TCR 187	IC136810	Ariyan	Vayalar	Alleppey	Kerala
20	TCR 207	IC136818	Divan vella	Aanathode	Kollam	Kerala
21	TCR 215	IC214011	Kolli	Pookkodu	Thrissur	Kerala
22	TCR 233	IC214029	Chuttutheeni	Chakkittapara	Kozhikkode	Kerala
23	TCR 250	IC210424	.....	Makkapuzha	Pathanamthitta	Kerala
24	TCR 254	IC210452	.....	Karuvelankulam	Tirunelveli	Tamil Nadu
25	TCR 261	IC349725	Kuchi	Mangalapuram	Namakkal	Tamil Nadu
26	TCR 266	IC349901	Singapurvella	Vanchiyoor	Thiruvananthapuram	Kerala
27	TCR 267	IC280861	Vellakappa	Nedumkandum	Idukki	Kerala

3.3 MORPHOLOGICAL OBSERVATIONS

The morphological observations were recorded by measuring and weighing using scale and weighing balance. Quantitative traits (7) are recorded as numerical data in units and qualitative traits (18) as descriptive data as shown in Table-3 and Table-4. All the morphological data observed were tabulated in excel worksheet for further statistical analysis.

Table-3: Qualitative characters for cassava morphological characterization as per IPGRI/NBPGR descriptors

Sl No.	Descriptor/Trait	Descriptor states
1	Mature stem color	1. Light brown 2. Brown 3. Ash 4. Light red 5. Red 6. Brown with yellow tint
2	Young stem color	1. Light green 2. Dark green with purplish tint 3. Dark green 4. Dark green with purplish brown angles 5. Green with light purple tint
3	Ridges on stem	1. Purple ridges 2. No ridges
4	Apical leaf color	1. Brownish green 2. Green 3. Light green
5	Mature leaf color	1. Dark green 2. Green 3. Yellow variation
6	Mature leaf vain color	1. Light purple 2. Green 3. Light green

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7	Petiole base color	1. Light green 2. Green 3. Whitish green 4. Yellowish green 5. Light pink 6. Pink 7. Light purple 8. Purple
8	Petiole middle color	1. Light green 2. Green 3. Whitish green 4. Yellowish green 5. Light pink 6. Pink 7. Light purple 8. Purple
9	Petiole color tip	1. Light green 2. Green 3. Whitish green 4. Yellowish green 5. Light pink 6. Pink 7. Light purple 8. Purple
10	Petiole joint color	1. Green 2. Light green 3. Purple 4. Light purple
11	Leaf marginal color	1. Green 2. Light green
12	Leaf marginal appearance	1. Wavy 2. Erect
13	Leaf tip color	1. Green 2. Light gree 3. Brownish green
14	Tuber shape	1. Conical 2. Conical-Cylindrical 3. Cylindrical
15	Tuber outer skin color	1. Dark brown 2. Light brown 3. Cream 4. Brown

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16	Tuber inner skin color	1. Purple 2. Cream 3. Light red 4. Pink 5. Yellow 6. Yellowish cream
17	Tuber flesh color	1. Cream 2. White 3. Light cream 4. Yellow
18	Tuber taste	1. Good 2. Good/Sour 3. Good/Sweet 4. Bitter

Table 4: Quantitative characters for cassava morphological characterization as per NBPGR minimal descriptors

Sl. No.	Descriptor/Trait	Descriptor states
1	Hairiness of leaf	1. Low 2. High
2	Prominence on leaf scar	1. Low 2. Medium 3. High
3	Cassava Mosaic Disease infection	1. Absent 2. Low (5-30) 3. Medium (30-60) 4. High (>60)
4	Total tuber weight per plant (kg)	Tuber ranges from : 1. 0-1 (Low) 2. 1-2 (Medium) 3. 2-3 (High)
5	Biggest tuber weight per plant (kg)	Tuber ranges from : 1. 0-1 (Low) 2. 1-2 (High)
6	Length of biggest tuber (cm)	1. Short ($\leq 20\ cm$) 2. Medium ($20 - 40\ cm$) 3. Long ($\geq 40\ cm$)
7	Width of biggest tuber (cm)	1. Short ($\leq 20\ cm$) 2. Medium ($20 - 30\ cm$) 3. Long ($\geq 30\ cm$)

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3.4 ANALYSIS OF MORPHOLOGICAL DATA

The recorded data was subjected to statistical analysis such as Cluster analysis, Principal Component Analysis.

3.4.1 Cluster Analysis

With the help of R software package (R Studio 3.2.5), Hierarchical clustering was done and a cluster dendrogram drawn. The aim was to group the morphologically similar accessions in same group and distinct accessions to distant groups. The whole analysis was based on Euclidean distance.

3.4.2 Principal Component Analysis (PCA)

Principal Component Analysis was done to obtain the biplot. Biplot was obtained in order to determine which of the traits contribute separately for the diversity of the accessions.

3.4.3 Shannon Diversity Index

Diversity indices are statics used to evaluate the diversity of a population in which each member belongs to a unique group. For the various triats under study, percentage distribution like Shannon Index was calculated.

Formula used for the Shannon diversity index:

$$H = \sum_{i=1}^{S} - (P_i * \ln P_i)$$

H = the Shannon diversity index

P_i = fraction of the entire population made up of species i

S = numbers of species encountered

$\sum$ = sum from species 1 to species S

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3.5 MOLECULAR MARKER ANALYSIS

Two different methods were used for DNA extraction from the leaves of the cassava accessions to compare the quality as well as quantity.

3.5.1 Glass wares and materials in molecular biology lab

1.5 And 2 ml eppendorffs, PCR tubes, mortar and pestle, micropipette tips for 2 µl, 200 µl and 1 ml were autoclaved and used. 1.5 ml tube stand, PCR tube holders, micropipettes, ice bags, bottles, spatula, polythene covers, labels, wipes and weighing pot are the other materials needed for molecular work.

3.5.2 Instruments

The equipments *viz.*, Water bath, Weighing balance, Ice machine, Dry bath, Vortexer, Cooling centrifuge, Nanodrop spectrophotometer, pH meter, Deep freezer (-20°C,-80°C), Refrigerator, Electrophoresis apparatus, UV trans illuminator, Alpha imager, PCR machine, Hot air oven, Autoclave and Distilled water unit were used for the study.

3.5.3 DNA Extraction

3.5.3.1 Manual method

DNA was extracted from fresh and tender young leaves of cassava using the CTAB method

Tender leaf samples were collected from the field, 100 mg samples weighed and homogenized to a fine powder using liquid nitrogen. Pre-warmed CTAB extraction buffer (2ml) was added to the samples and ground once more. The samples were transferred to 2 ml microfuge tubes and vortexed. The samples were incubated at 65 °C for 30 minutes with frequent swirling in water bath. Samples were centrifuged at 12000 rpm for 10 minutes at 4 °C and the supernatant was collected to a fresh microfuge tube. Equal volume of Chloroform: Isoamyl alcohol (24:1) was added and mixed by gentle inversion. Again samples were centrifuged at 12000 rpm for 10 minutes at 4 °C. The supernatant was collected to a fresh

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microfuge tube and 5 μl of RNase were added and incubated at 37 °C for 1 hour. After incubation, equal volume of chloroform: Isoamyl alcohol (24:1) was added into the sample. Samples were centrifuged at 12000 rpm for 10 minutes at 4 °C. Supernatant was collected in fresh microfuge tube and to the collected supernatant added 0.8 volume propanol and centrifuged at 12000 rpm for 10 minutes at 4 °C. Supernatant was discarded and saved the pellet. The DNA pellet was washed with 70% ethanol. Air dried the pellet until the ethanol evaporated and dissolved in appropriate amount of 1x TE buffer (100-150 μl). All samples were checked for DNA in 1% agarose gel and confirmed.

3.5.3.2 Using DNeasy® Plant Mini Kit

An approximate quantity of 100-200 mg freshly collected cassava leaves were ground using sterile mortar and pestle along with 400 μl of Buffer API and 4 μl of RNase A. Solution mixture was transferred into a 1.5 ml microfuge tube and vertexed well and incubated at 65 °C for 10 minutes. The tubes were inverted for 2-3 times during the incubation. Care was taken to make sure that AP1 buffer and RNase A did not mix before use. The sample was centrifuged for 5 minutes at 20000 xg (approx. 14000 rpm). Carefully pipetted the lysate into a QIAshredder spin column placed in a 2 ml collection tube. The tube was centrifuged at 20000 xg for 2 minutes. Transferred the flow through into a new tube without disturbing the pellet. 1-5 volumes of Buffer AW1 was added and mixed by gentle and gradual pipetting. 650 μl of the mixture was transferred into a DNeasy Mini spin column placed in a 2 ml collection tube. Centrifuged the collection tube at a speed of ≥6000 ×g (approx. ≥ 8000 rpm) for about a minute. Discarded the flow through and saved the pellet. Repeated the same step with the remaining samples.

Fresh 2 ml collection tube was taken and added 500 μl of the Buffer AW2. Centrifuged at ≥6000 ×g for 1 minute. The flow through was discarded and added another 500 μl of the Buffer AW2. The tube was centrifuged at 20000 xg for 2 minutes. The collection tube from the spin column was carefully removed so that column does not come in contact with flow through. Placed the spin column in a 1.5 ml or 2 ml collection tube. For the elution of DNA, 100 μl of AE buffer added.

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It was incubated for 5 minutes at room temperature (25°C) and centrifuged for 1 minute at ≥6000 ×g. This step was repeated and kept the sample in -20 °C refrigerator. All the samples were checked for DNA in 1% agarose gel and confirmed.

3.6 ANALYSIS OF THE EXTRACTED DNA

3.6.1 Agarose gel electrophoresis

Agarose gel electrophoresis is used for separating and analysing DNA molecules in which DNA molecules were separated based on the charge by giving an electric field to the electrophoretic apparatus. The gel can be used to look at the DNA in order to quantify the amount of DNA or to isolate a particular band of interest. By the addition of Ethidium Bromide, the intercalating agent along with agarose solution, the DNA can be visualized when exposed on UV.

1% agarose gel was used to check the quality and integrity of the extracted DNA. 1% agarose solution was prepared by weighing out 1 gm agarose in a conical flask and dissolving it using 1X TBE buffer. Agarose was dissolved by heating and after that the flask was allow`ed to cool and when the temperature of the flask became bearable, about 1.5 µl of Ethidium Bromide (EtBr) was added directly to the molten gel and gentle mixing was done. Casting tray was prepared with combs so that gel was poured into the tray and allowed to solidify. 6 µl of isolated sample mixed with 2 µl of 1X loading dye was loaded into the wells of prepared gel.

Horizontal gel electrophoresis unit was used to run the gel. The gel was run for about 30 minutes at 80V. 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.

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3.6.2 Quantification of DNA

Nanodrop spectrophotometer was used to quantify the isolated DNA. It helped to assess the yield and purity with high accuracy and reproducibility. TE buffer in which DNA was dissolved was used to calibrate the machine to blank i.e. zero absorbance. The advantage of Nanodrop is that it requires only 1 µl sample to measure its quantity and quality. The quantity of DNA was determined at OD 260 and the purity was determined by OD 260/OD 280 ratio. According to the better absorbance value/ OD value samples were selected.

3.6.3 Dilution of samples

Samples were diluted to 10 ng/ µl concentration using nuclease free water.

3.7 PRIMER SCREENING

The following 12 SSR primers were selected for primer screening:

SSRY 9, SSRY 28, SSRY 45, SSRY 100, SSRY 102, SSRY 105, SSRY 106, SSRY 147, SSRY 148, SSRY 161, SSRY 181, SSRY 182

The genomic DNA of three different cassava accessions collected from three different states were taken initially for primer screening. These randomly selected primers were TCR-5 (Kerala), TCR-69 (Karnataka) and TCR-129 (Tamil Nadu). The composition of the reaction mixture was as follows in Table-5.

Table-5: SSR reaction mixture

Ingredients	Stock concentration	Required concentration	Required volume for one reaction (20 µl)
Taq Buffer	10X	1X	2.0 µl
dNTP	10 mM each	0.2 mM each	0.4 µl
Primer (F)	10 µM	0.25 µM	0.5 µl
Primer (R)	10 µM	0.25 µM	0.5 µl
Taq DNA polymerase	5U/ µl	1U	0.2 µl
Template DNA	10 ng/ µl	20ng	2.0 µl
SDW	----	----	14.4 µl

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3.7.1 SSR PCR conditions

PCR was carried out in Proflex Thermocycler programmed for an initial denaturation at 95 °C for 5 minutes followed by 35 cycles with denaturation at 95 °C for 40 seconds, primer annealing at 56 °C for 1 minute and extension at 72 °C for 1 minute. The final extension was performed at 72 °C for 10 minutes followed by its holding at 4 °C. The amplified products were resolved in a 2% agarose gel using 100 bp ladder for checking amplification, to identify molecular weight of obtained bands and for polymorphism studies.

3.7.2 Gradient PCR for T_a optimization

The selected primers were taken one by one for screening to determine the annealing temperature for each primer. Each of the primers selected was given a letter for easy labelling. The list of primers selected, expected band size, annealing temperature and gradient temperature are given in Table-6.

Table-6: List of SSR primers with details screened initially for characterization

Sl. No	PRIMER Name	Band Size(bp)	Annealing Temperature °C (from literature)	Gradient Temperature °C	Selected Annealing Temperature °C
1	SSRY 9	273 - 320	55	47.2 - 58.1	48
2	SSRY 45	150 - 210	55	48.0 - 59.0	48
3	SSRY 100	209 - 273	55	50.5 - 59.0	52
4	SSRY 102	198 - 200	55	55.1 - 67.1	56
5	SSRY 105	145 - 230	55	55.0 - 67.1	57
6	SSRY 147	268 - 302	45	55.0 - 67.1	65
7	SSRY 148	118 - 136	45	55.0 - 67.1	59
8	SSRY 161	128 - 138	45	55.0 - 67.1	67
9	SSRY 181	192 - 216	45	55.0 - 67.1	65
10	SSRY 182	238 - 258	45	55.0 - 67.1	66

3.8 SSR ANALYSIS OF CASSAVA ACCESSIONS

After primer screening, 10 SSR primers which gave clear and polymorphic bands were chosen for further analysis. PCR was performed using standardized PCR conditions and the annealing temperature, which was found to be optimum for each primer. The list of primers selected and the primer sequences are given in Table-7.

Table-7: List of SSR primers with details

Sl.No.	Primer name	Sequence
1	SSRY 9	F-5' ACA ATT CAT GAG TCA TCA ACT 3' R-5' CCG TTA TTG TTC CTG GTC CT 3'
2	SSRY 45	F-5' TTG ACA TGA GTG ATA TTT TCT TGA A 3' R-5' TCC AGT TCA GTA GTT GGC T 3'
3	SSRY 100	F-5' ATC CTT GCC TGA CAT TTT GC 3' R-5' TTC GCA GAG TCC AAT TGT TG 3'
4	SSRY 102	F-5' TTG AAC ACG TTG AAC AAC CA 3' R-5' TTG GCT GCT TTC ACT AAT GC 3'
5	SSRY 105	F-5' TCG AGT GGC TTC TGG TCT TC 3' R-5' CCA ACA TTC GCA CTT TTG GC 3'
6	SSRY 147	F-5' AGA GCG GTG GGG CGA AGA GC 3' R-5' GTA CAT CAC CAC CAA CGG GC 3'
7	SSRY 148	F-5' CAA TGC TTT ACG GAA GAG CC 3' R-5' GGC TTC ATC ATG GAA AAA CC 3'
8	SSRY 161	F-5' CCA GCT GTA TGT TGA GTG AGC 3' R-5' AAG GAA CAC CTC TCC TAG AAT CA 3'
9	SSRY 181	F-5' CAA TCG AAA CCG ACG ATA CA 3' R-5' GGT AGA TCT GGA TCG AGG AGG 3'
10	SSRY 182	F-5' TTC CTT TAC AAT TCT GGA CGA 3' R-5' GGA ATT CTT TGC TTA TGA TGC C 3'

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3.8.1 Agarose Gel Electroporesis for SSR Analysis

Gel electrophoresis was performed with 4% agarose.

3.8.1.1 Gel preparation

4% of agarose was used to resolve the amplicons obtained after the PCR. For preparing a total of 120 ml of 4% gel, 4.8 g of agarose was weighed out and dissolved in 120 ml of 1X TBE buffer. The flask was undisturbed and using a microwave oven, the gel was melted completely. Added approximately 1.2 µl EtBr into the conical flask containing the melted agarose. Then gently and gradually rotated the conical flask for the uniform distribution of EtBr. After proper mixing it was poured on to the casting tray and allowed to solidify for about 30 minutes.

3.8.1.2 Gel loading and running

The PCR products were arranged in a tray according to the sample number for loading. About 2 µl of gel loading dye was added directly to the PCR tubes. Mixed well and a short spin using centrifuge was done. The combs from the solidified gel was removed slowly without disturbing the wells and the samples were loaded in the same order along with 100 bp DNA ladders

The electrophoresis apparatus was connected to a power pack and the voltage was set at 80V. The movement of the tracking dye was noted and the run was stopped when the dye reached the bottom of the tray.

3.8.1.3 PCR product detection

The gel including the tray was taken out and was viewed under the ultraviolet light in gel documentation system. The image was taken under appropriate exposure and saved in JPEG format for scoring.

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3.9 ANALYSIS OF MOLECULAR MARKER DATA

3.9.1 Band scoring

All the images of resolved PCR products were taken. Clear and reproducible bands were taken for scoring. Binary scoring was carried out by assigning "1" for the presence of a specific band and "0" for the absence of a band. The data was entered in excel and was used as an input for cluster and principal component analyses.

3.9.2 Cluster Analysis

For molecular clustering of data, R statistical (R studio 3.2.5) package was used and Hierarchical clustering was done based on Euclidean distance. As a result, a dendrogram of 27 accessions was obtained which is based on molecular traits.

3.9.3 Principal Component Analysis

Using Hierarchical R package PCA was done. Biplot based on molecular scoring data was obtained.

3.9.10 Polymorphic Information Content

Binary scoring was done with the polymorphic bands obtained with each primer. Polymorphism information content (PIC) of each individual SSR allele was calculated according to the formula described by Tehrani *et al.* (2008):

PIC=2PiQi where Pi is the frequency of presence and Qi is the frequency of absence of a particular band. PIC values for all the polymorphic bands produced by a primer pair were averaged to calculate PIC value for a primer pair.

3.10 CORRELATION OF MORPHOLOGICAL AND MOLECULAR DATA

Mantel's test was used to analyse the correlation between the morphological and molecular data.

RESULTS

4. RESULTS

The study entitled "Characterization of selected accessions of cassava germplasm using morphological and molecular markers" was carried out at the Division of Crop Improvement, ICAR-Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram during 2016-2017 and the results of the study are depicted in this chapter.

4.1 MORPHOLOGICAL DATA ANALYSIS

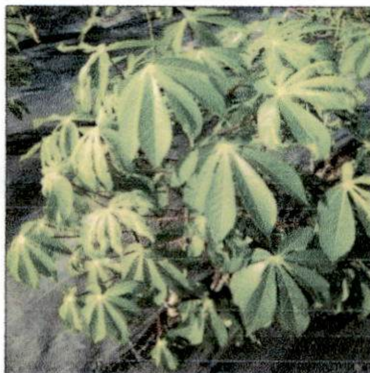
Twenty-seven accessions of cassava selected for the study collected different parts of South India viz., Kerala, Tamil Nadu and Karnataka are Shown in Plate-1. A wide range of variability was observed among the accessions for many of the characters studied. Percentage distribution of morphological data was also recorded (Table-9).

TCR-5

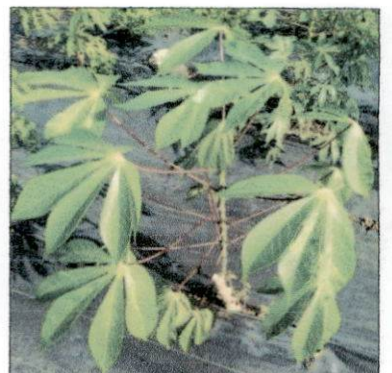
TCR-10

TCR-15

TCR-20A



TCR-31A



TCR-36

TCR-45

TCR-54

TCR-56

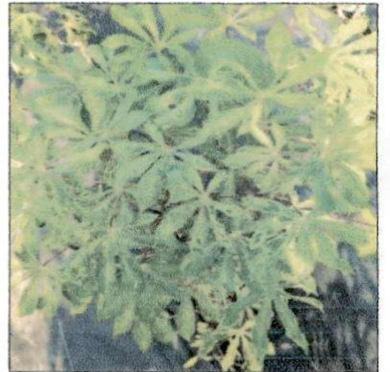
TCR-59

TCR-66A

TCR-69

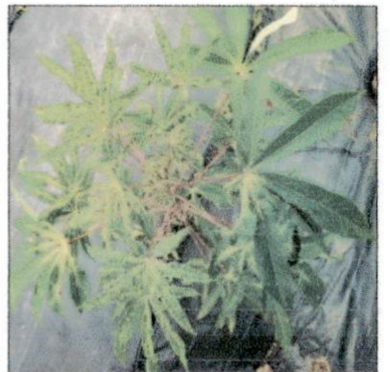
TCR-79

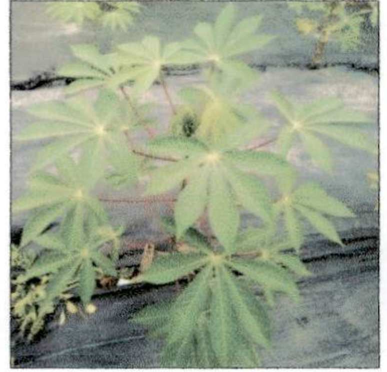
TCR-80


TCR-93

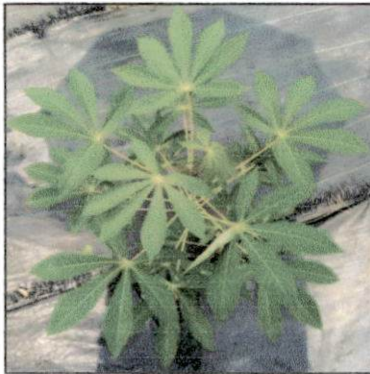
TCR-124

TCR-129

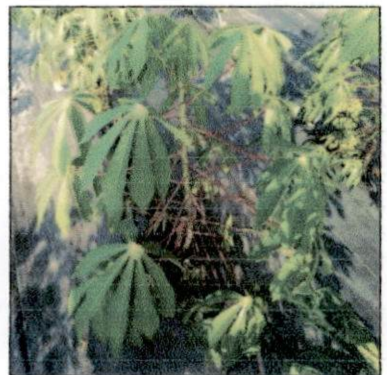

TCR-153



TCR-187 TCR-207 TCR-215



TCR-233 TCR-250 TCR-254



TCR-261 TCR-266 TCR-267

Plate-1: Juvenile stage plant pictures of 27 selected accessions of cassava

4.1.1 Cluster analysis

Cluster analysis using Hierarchical clustering with the help of R program drawn revealed the 27 selected accessions of cassava grouped into two major clusters. Of which, Cluster-I consists of 7 accessions was further subdivided into two sub clusters I A and I B. Cluster-I A consisted of four accessions while Cluster-I B of three accessions. The bigger cluster Cluster-II consisting of 20 accessions was found to have two subgroups namely II A with 12 accessions and II B with 8 accessions.

In Cluster I B, TCR-153 found to be divergent when compared with the TCR-56 and TCR-66A. But in the case of major Cluster II the number of divergent accessions are 6 (TCR-124, TCR-233, TCR-36, TCR-20A and TCR-297). Analysing the dendrogram shows that, among these accessions none were found to be completely similar. However no outliers were observed in the dendrogram. The Dendrogram obtained is shown in Fig.1.

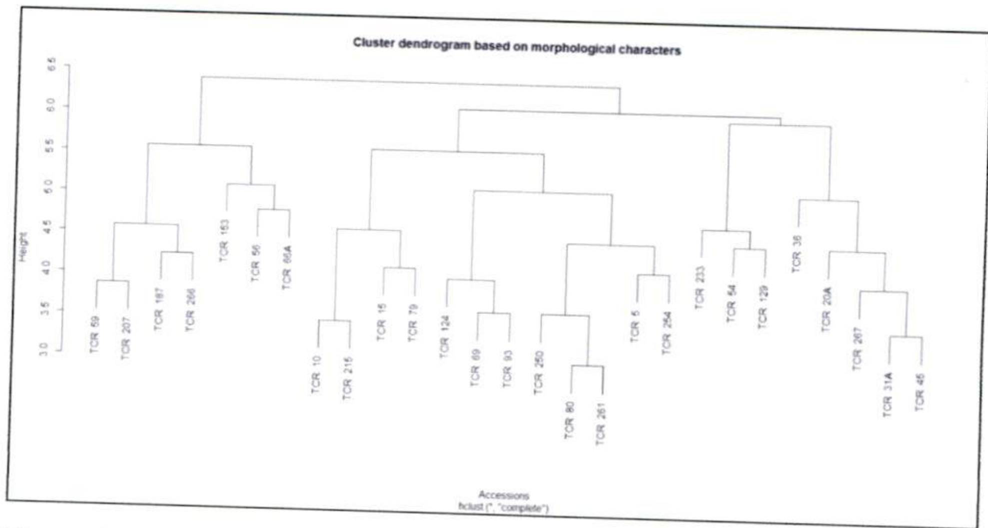


Figure-1: Dendrogram of 27 accessions of cassava based on Hierrarchial clustering based on morphological descriptors (R package)

4.1.2 Principal Component Analysis (PCA)

PCA biplot was drawn using the eigen value with R program. Morphological characters and selected accessions of 27 cassava were spatially arranged in biplot (Fig-2). In PCA, 3 main clusters were obtained. The PCA component values are shown in Table-8.

Table-8: Principal Componant Analysis in 27 accessions of cassava
4.1.3 Diversity Indices

Morphological traits/Descriptors	PCA Comp.1	PCA Comp.2	PCA Comp.3	PCA Comp.4	PCA Comp.5
Stem colour	1.205078	-0.27375	0.265336	0.104293	-0.1072
Ridges	1.353875	0.133454	0.370312	0.072624	-0.22666
Apical leaf colour	0.150597	-0.48189	1.201341	-0.33026	-0.11159
Mature leaf colour	1.303958	0.399544	0.154664	-0.40178	0.163775
Petiole base colour	0.893356	-0.34547	-1.27188	-0.55981	-0.72129
Petiole middilecolour	1.303958	0.399544	0.154664	-0.40178	0.163775
Petiole tip colour	1.401131	-0.02053	0.061349	0.125234	0.005892
Petiole joint colour	1.290799	0.595314	0.088136	-0.00458	-0.12146
Prominance on leaf scar	1.401131	-0.02053	0.061349	0.125234	0.005892
Leaf marginal appearance	1.373916	0.20914	-0.20961	-0.31919	-0.29847
Tuber shape	1.339505	0.051448	0.148112	-0.08353	0.200558
Tuber skin colour	1.207434	-0.19356	-0.00666	0.106104	-0.33972
Flesh colour	1.273092	0.472391	0.14667	0.190073	-0.25263
Eigen value	**5.312**	**5.298**	**4.976**	**4.567**	**4.231**
Percentage variation	**54.22**	**24.054**	**22.67**	**20.56**	**18.90**
Cumulative percentage	**54.22**	**63.89**	**76.98**	**83.76**	**90.75**

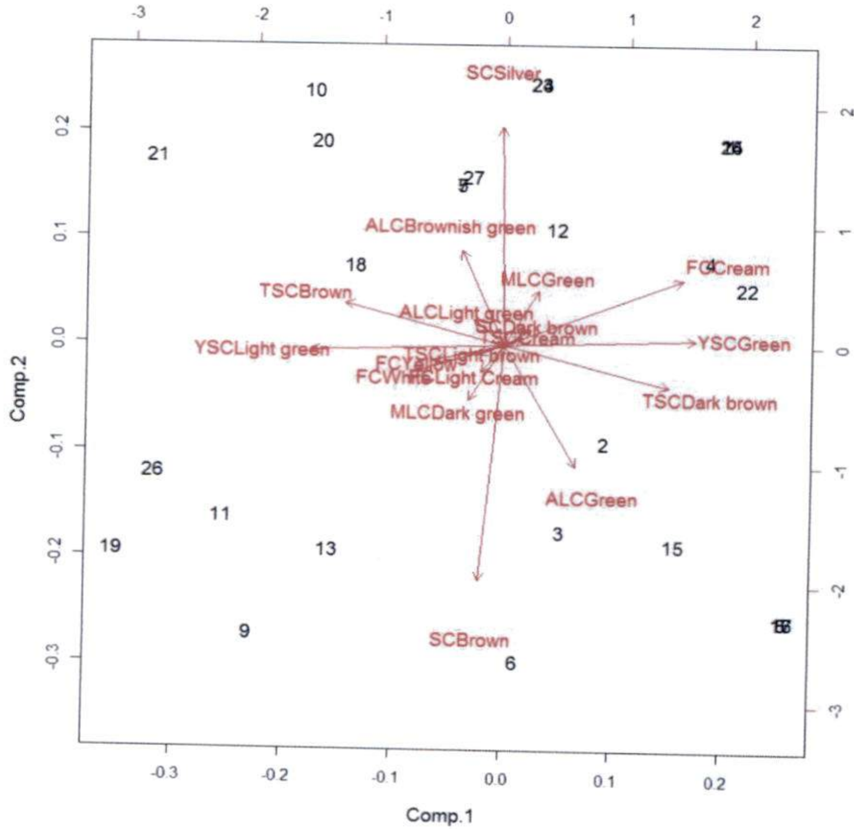


Figure-2: Biplot showing the spatial distribution of various morphological descriptors and 27 accessions of cassava

1: TCR-5
2: TCR-10
3: TCR-15
4: TCR-20A
5: TCR 31A
6: TCR 36
7: TCR 45
8: TCR 54
9: TCR 56
10: TCR 59

11: TCR 66A
12: TCR 69
13: TCR 79
14: TCR 80
15: TCR 93
16: TCR 124
17: TCR 129
18: TCR 153
19: TCR 187
20: TCR 207

21: TCR 215
22: TCR 233
23: TCR 250
24: TCR 254
25: TCR 261
26: TCR 266
27: TCR 267

The percentage distribution and Shannon diversity index was calculated across the 27 selected accessions was shown in Table-9. The percentage distributions of qualitative characters are studied. Each descriptor varies in each accessions. Shannon Index was calculated range in between 0.348832 to 1.704829. Variation in quantitative characters tabulated in Table-10.

Table-9: Percentage distribution for qualitative morphological characters

Sl. No.	Descriptor/Character	Descriptor states	Percentage (%)	Shannon diversity index (H)
1	Mature stem colour	Silver -13 Brown-13 Dark brown-1	48 48 04	0.905114
2	Young stem colour	Green-20 Light green-7	74 26	0.607693
3	Ridges on stem	Purple ridges-6 No ridges-21	22 78	0.774311
4	Apical leaf colour	Brownish green-19 Light green-2 Green-6	70 08 22	0.529706
5	Mature leaf colour	Dark green-3 Green-24	11 89	0.348832
6	Mature leaf vain colour	Light purple-8 Green-12 Light green-7	30 44 26	1.070808
7	Petiole base colour	Purple-11 Green-6 Light purple-1 Light green-9	33 41 22 04	1.160982
8	Petiole middle colour	Purple-12 Green-5	44 19	1.267901

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		Light purple-4 Light green-6	15 22	
9	Petiole tip colour	Purple-13 Green-4 Light purple-1 Light green-14	41 12 3 44	1.160982
10	Petiole joint colour	Purple-13 Green-3 Light purple-10 Light green-1	48 11 37 4	1.131581
11	Prominence on leaf scar	Low-18 Medium-8 High-1	67 29 4	0.752792
12	Leaf marginal colour	Green-26 Light green-1	96 4	0.158411
13	Leaf marginal appearance	Erect- 25 Wavy- 2	93 7	0.264052
14	Leaf tip colour	Green-25 Light green-1 Brownish green-1	92 4 4	0.315396
15	Tuber shape	Conical-11 Cylindrical-4 Conical-Cylindrical-12	41 15 44	1.054019
16	Tuber outer skin colour	Brown-10 Light brown-6 Dark brown-10 Cream-1	37 22 37 04	1.19205

17	Tuber inner skin colour	Purple-8 Light red-2 Pink-4 Yellowish cream-3 Cream-10	30 07 15 11 37	1.704829
18	Flesh colour	White-9 Cream-15 Light cream-1 Yellow-2	33 56 4 7	1.053166
19	Tuber taste	Good-13 Good/Sour-4 Good/Sweet-5 Sweet-3 Bitter-2	48 15 19 11 07	1.201823

Table-10: Quantitative character variation in 27 accessions of cassava

Sl. No.	Accessions	Number of tuber/ plant	Total tuber weight (kg)	Biggest tuber weight (kg)	Tuber length (cm)	Tuber width (cm)
1	TCR 5	3	2.15	1.68	36	28
2	TCR 10	2	0.45	0.35	20	19
3	TCR 15	2	2.1	1.00	25.7	13
4	TCR 20A	4	2.62	0.69	22	23
5	TCR-31A	2	1.4	1.00	21.7	14
6	TCR 36	3	2.0	0.21	17	16
7	TCR 45	2	1.5	1.00	33.8	15

8	TCR 54	4	1.18	0.86	32	20
9	TCR 56	2	2.9	1.00	24.5	16
10	TCR 59	4	1.08	0.67	30	24
11	TCR 66A	2	1.5	1.3	19	17
12	TCR 69	3	1.33	0.73	27	22
13	TCR 79	2	1.5	1.2	39.3	18
14	TCR 80	3	2.22	1.23	42	24
15	TCR 93	4	1.45	0.59	22	23
16	TCR 124	4	1.45	0.95	40	20
17	TCR 129	4	1.55	0.6	19	29
18	TCR 153	2	0.8	1.3	23.7	19
19	TCR 187	1	0.18	0.18	17	15
20	TCR 207	5	0.7	0.29	25	17
21	TCR 215	2	1.16	0.72	23	22
22	TCR 233	2	0.76	0.64	35	17
23	TCR 250	3	1.08	0.5	25	24
24	TCR 254	6	0.9	0.2	15	14
25	TCR 261	7	2.22	0.7	36	31
26	TCR 266	5	1.78	0.8	26	23
27	TCR 267	2	0.8	0.9	22	20
	Mean	**3.14**	**1.43**	**0.78**	**26.6**	**20.1**
	SD	**4.55**	**2.08**	**1.15**	**34.11**	**24.76**
	Coefficient of variation	**1.44**	**1.45**	**1.47**	**1.28**	**1.23**

4.2 Molecular Data Analysis

4.2.1 Standardization of DNA isolation protocol

Isolation of DNA from 27 accessions of cassava were tried by using both the kit method and manual method to find out the optimum method to extract quality DNA with better concentration. DNA isolated using DNeasy® plant mini kit (Qiagen) was found to be easy and good. Manual method (CTAB) of DNA isolation was intact and gave good spectrophotometric readings (Table-11). The concentrations of isolated DNA ranges from 195.82 ng to 2282.66 ng with high purity. Isolated DNAs checked in 1% Agarose gel is shown in Plate-2, 3 and 4.

Table-11: Spectrophotometric readings of DNA isolated using CTAB method

Sample Number	Sample Name	Concentration (ng/µl)	A_{260}	A_{260}/A_{280}
1	TCR 5	195.82	3.916	1.86
2	TCR 10	396.63	7.933	2.03
3	TCR 15	562.17	11.243	2.04
4	TCR 20	690.24	13.805	2.07
5	TCR 31A	517.23	10.344	2.06
6	TCR 36	1452.22	29.04	2.17
7	TCR 45	1873.27	37.465	2.22
8	TCR 54	504.9	10.09	2.04
9	TCR 56	676.77	13.53	2.08
10	TCR 59	1947.9	38.95	2.04
11	TCR 66A	833.64	16.673	2.09
12	TCR 69	2282.66	45.65	2.17
13	TCR 79	809.26	16.18	2.10
14	TCR 80	2226.22	44.52	2.09
15	TCR 93	1775.69	35.51	2.23
16	TCR 124	394.39	7.668	2.00
17	TCR 129	383.44	3.56	2.04

18	TCR 153	545.74	7.19	2.11
19	TCR 187	471.10	9.42	2.03
20	TCR 207	177.64	3.552	2.06
21	TCR 215	392.26	7.84	2.05
22	TCR 233	361.64	7.324	2.09
23	TCR 250	2134.38	42.6	2.07
24	TCR 254	1370.65	27.41	2.19
25	TCR 261	1078.5	21.57	2.08
26	TCR 266	1948.69	38.97	2.03
27	TCR 267	1432.90	28.65	2.65

Gel profile showing standardization of DNA isolation protocol

Plate- 2and 3: 1% gel profiles of DNA isolated using Qiagen kit method for 27 accessions of cassava

Plate 4: 1% gel profiles of DNA isolated using CTAB method for 27 accessions of cassava

Lane 1: TCR-5
Lane 2: TCR-10
Lane 3: TCR-15
Lane 4: TCR-20A
Lane 5: TCR 31A
Lane 6: TCR 36
Lane 7: TCR 45
Lane 8: TCR 54
Lane 9: TCR 56
Lane 10: TCR 59

Lane 11: TCR 66A
Lane 12: TCR 69
Lane 13: TCR 79
Lane 14: TCR 80
Lane 15: TCR 93
Lane 16: TCR 124
Lane 17: TCR 129
Lane 18: TCR 153
Lane 19: TCR 187
Lane 20: TCR 207

Lane 21: TCR 215
Lane 22: TCR 233
Lane 23: TCR 250
Lane 24: TCR 254
Lane 25: TCR 261
Lane 26: TCR 266
Lane 27: TCR 267

4.2.2 Primer screening and determination of annealing temperature (Ta)

For preliminary screening of SSR primers, 12 primers were taken for determining its amplifying capability and the annealing temperature if amplification is possible. DNA samples of three accessions namely TCR-5 (Kerala), TCR-69 (Karnataka) and TCR-129 (Tamil Nadu) were taken for screening purpose. Due to poor amplicon profile, out of 12 primers two primers (SSR 28 and SSR 106) were eliminated after preliminary screening. Gradient PCR was performed to identify the annealing temperature for the SSR primers (Plate-5 and 6).

Standardization of PCR conditions was attempted for SSR marker to obtain clear and consistent amplicons under modified annealing temperature. Changing the annealing temperature for certain SSR primers gave better results (Table-12).

4.2.3 SSR analysis of cassava accessions

After the completion of PCR using selected primers with representative annealing temperature for amplification, the amplicons were resolved in 4% agarose gel and obtained amplicons are shown in Plate-7 to 12.

Table-12: Details of selected primers and their annealing temperature

Sl. No.	Primer Name	Sequence	Annealing Temperature (Ta)
1	SSRY 9	F-5’ ACA ATT CAT GAG TCA TCA ACT 3’ R-5’ CCG TTA TTG TTC CTG GTC CT 3’	48
2	SSRY 45	F-5’ TTG ACA TGA GTG ATA TTT TCT TGA A 3’ R-5’ TCC AGT TCA GTA GTT GGC T 3’	48
3	SSRY 100	F-5’ ATC CTT GCC TGA CAT TTT GC 3’ R-5’ TTC GCA GAG TCC AAT TGT TG 3’	52
4	SSRY 102	F-5’ TTG AAC ACG TTG AAC AAC CA 3’ R-5’ TTG GCT GCT TTC ACT AAT GC 3’	56
5	SSRY 105	F-5’ TCG AGT GGC TTC TGG TCT TC 3’ R-5’ CCA ACA TTC GCA CTT TTG GC 3’	57
6	SSRY 147	F-5’ AGA GCG GTG GGG CGA AGA GC 3’ R-5’ GTA CAT CAC CAC CAA CGG GC 3’	65
7	SSRY 148	F-5’ CAA TGC TTT ACG GAA GAG CC 3’ R-5’ GGC TTC ATC ATG GAA AAA CC 3’	59
8	SSRY 161	F-5’ CCA GCT GTA TGT TGA GTG AGC 3’ R-5’ AAG GAA CAC CTC TCC TAG AAT CA 3’	67
9	SSRY 181	F-5’ CAA TCG AAA CCG ACG ATA CA 3’ R-5’ GGT AGA TCT GGA TCG AGG AGG 3’	65
10	SSRY 182	F-5’ TTC CTT TAC AAT TCT GGA CGA 3’ R-5’ GGA ATT CTT TGC TTA TGA TGC C 3’	66

Gradient PCR for identify annealing temperature

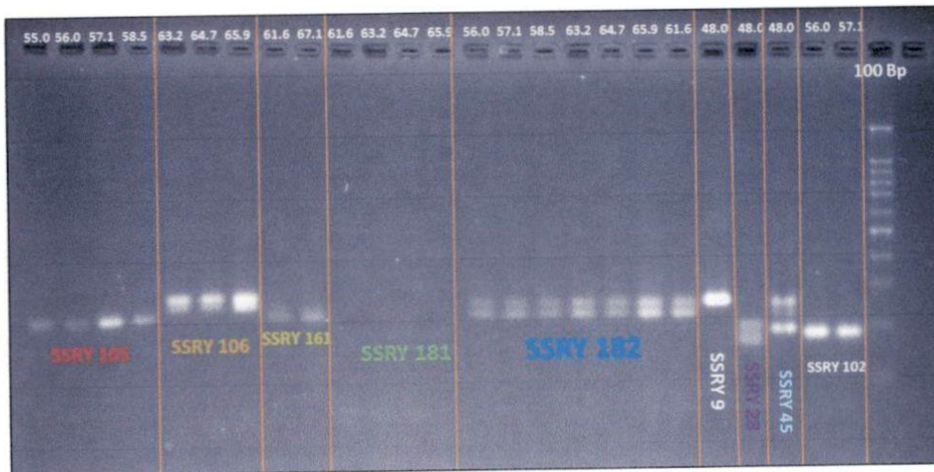

Plate-5: Agarose gel (2%) profile of the standardization of SSR primers (SSRY 105, SSRY 106, SSRY 161, SSRY 181, SSRY 182, SSRY 9, SSRY 28, SSRY 45, SSRY 102)

Gradient PCR for identify annealing temperature

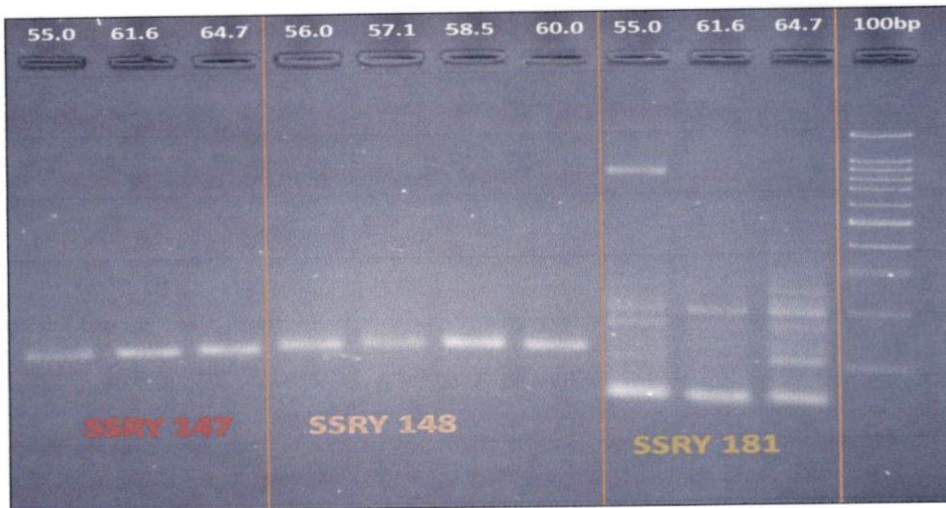

Plate-6: Agarose gel (2%) profile of the standardization of SSR primers (SSRY 147, SSRY 148, SSRY 181)

Molecular characterization using SSR markers

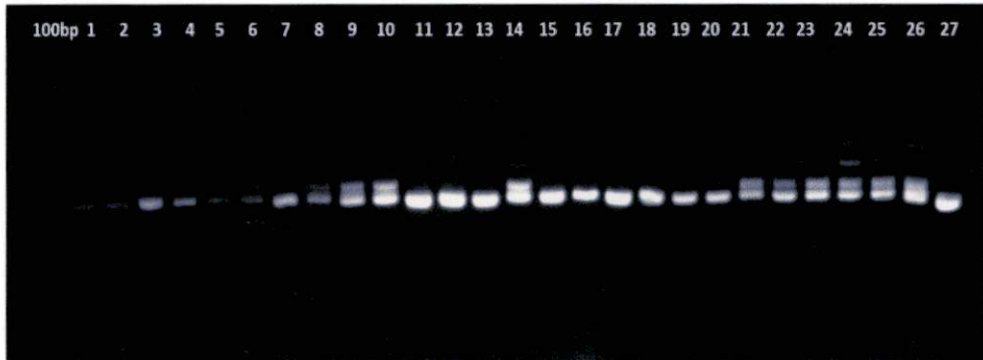

Plate-7: Agarose gel (4%) profile of the SSR primer SSRY 9 for 27 accessions of cassava

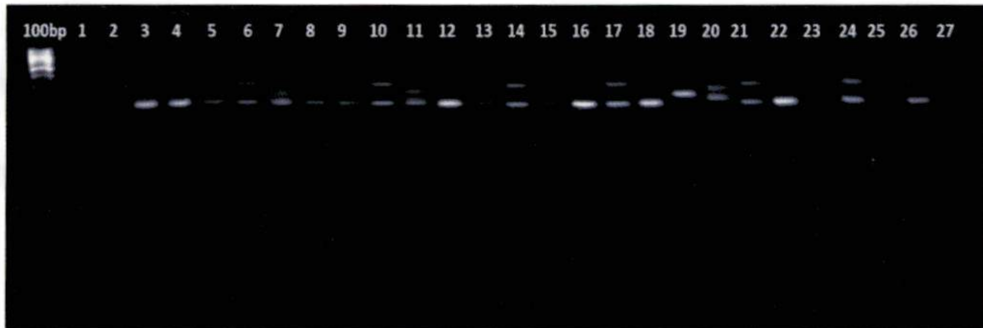

Plate-8: Agarose gel (4%) profile of the SSR primer SSRY 45 for 27 accessions of cassava

Lane 1: 100bp ladder
Lane 2: TCR-5
Lane 3: TCR-10
Lane 4: TCR-15
Lane 5: TCR-20A
Lane 6: TCR 31A
Lane 7: TCR 36
Lane 8: TCR 45
Lane 09: TCR 54
Lane 10: TCR 56

Lane 11: TCR 59
Lane 12: TCR 66A
Lane 13: TCR 69
Lane 14: TCR 79
Lane 15: TCR 80
Lane 16: TCR 93
Lane 17: TCR 124
Lane 18: TCR 129
Lane 19: TCR 153
Lane 20: TCR 187

Lane 21: TCR 207
Lane 22: TCR 215
Lane 23: TCR 233
Lane 24: TCR 250
Lane 25: TCR 254
Lane 26: TCR 261
Lane 27: TCR 266
Lane 28: TCR 267

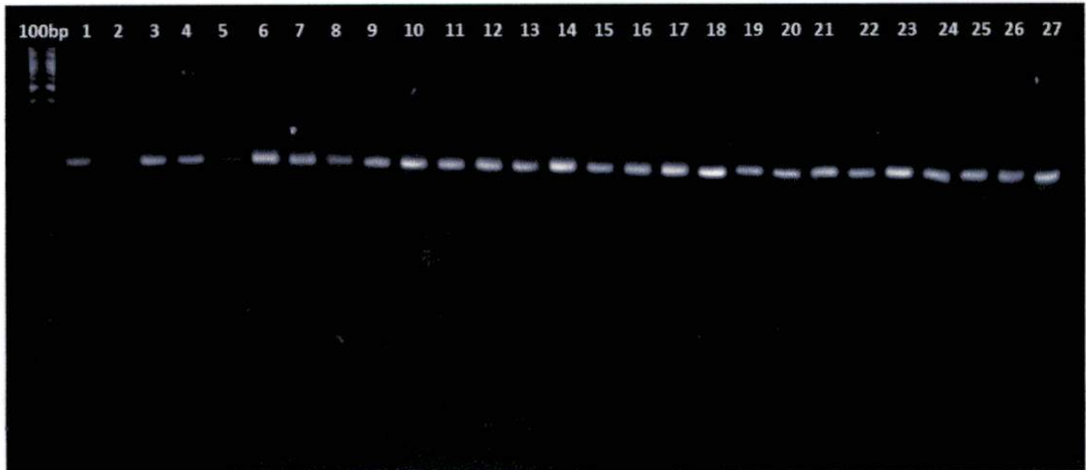

Plate-9: Agarose gel (4%) profile of the SSR primer SSRY 100 for 27 accessions of cassava

Plate-10: Agarose gel (4%) profile of the SSR primer SSRY 105 for 27 accessions of cassava

Lane 1: 100bp ladder
Lane 2: TCR-5
Lane 3: TCR-10
Lane 4: TCR-15
Lane 5: TCR-20A
Lane 6: TCR 31A
Lane 7: TCR 36
Lane 8: TCR 45
Lane 09: TCR 54
Lane 10: TCR 56
Lane 11: TCR 59
Lane 12: TCR 66A
Lane 13: TCR 69
Lane 14: TCR 79
Lane 15: TCR 80
Lane 16: TCR 93
Lane 17: TCR 124
Lane 18: TCR 129
Lane 19: TCR 153
Lane 20: TCR 187
Lane 21: TCR 207
Lane 22: TCR 215
Lane 23: TCR 233
Lane 24: TCR 250
Lane 25: TCR 254
Lane 26: TCR 261
Lane 27: TCR 266
Lane 28: TCR 267

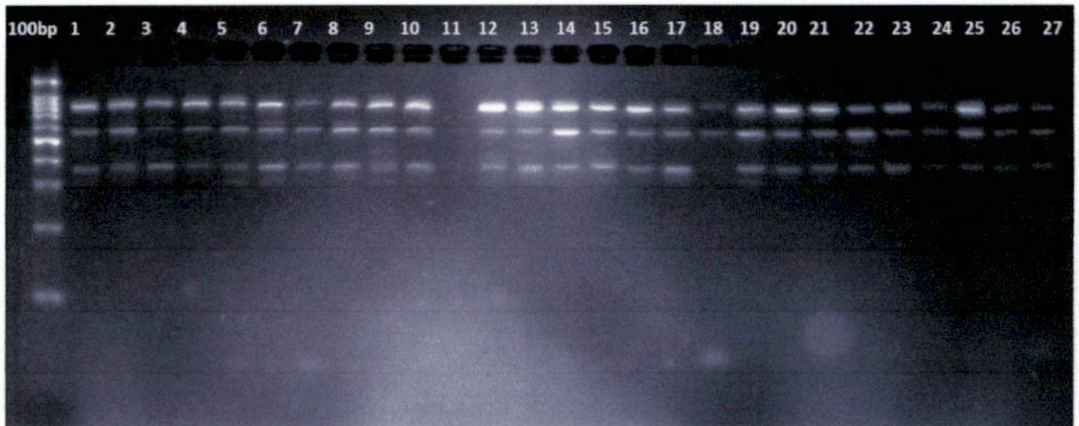

Plate-11: Agarose gel (4%) profile of the SSR primer SSRY 181 for 27 accessions of cassava

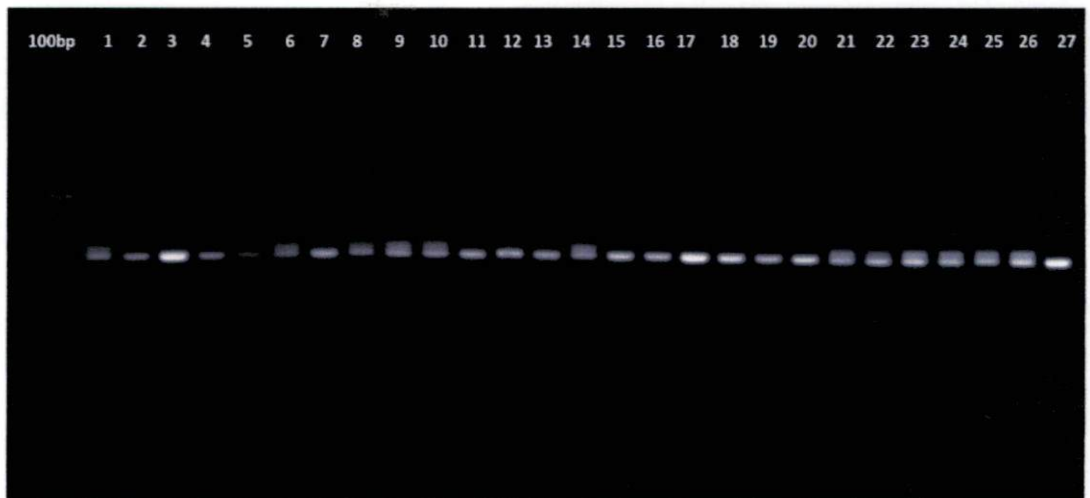

Plate-12: Agarose gel (4%) profile of the SSR primer SSRY 182 for 27 accessions of cassava

Lane 1: 100bp ladder
Lane 2: TCR-5
Lane 3: TCR-10
Lane 4: TCR-15
Lane 5: TCR-20A
Lane 6: TCR 31A
Lane 7: TCR 36
Lane 8: TCR 45
Lane 09: TCR 54
Lane 10: TCR 56
Lane 11: TCR 59
Lane 12: TCR 66A
Lane 13: TCR 69
Lane 14: TCR 79
Lane 15: TCR 80
Lane 16: TCR 93
Lane 17: TCR 124
Lane 18: TCR 129
Lane 19: TCR 153
Lane 20: TCR 187
Lane 21: TCR 207
Lane 22: TCR 215
Lane 23: TCR 233
Lane 24: TCR 250
Lane 25: TCR 254
Lane 26: TCR 261
Lane 27: TCR 266
Lane 28: TCR 267

4.3 ANALYSIS OF MOLECULAR MARKER DATA

4.3.1 Cluster analysis

Cluster analysis was done based on Euclidean distance (Hierarchical clustering), in which the total twenty-seven accessions was grouped into two major clusters shown as in the dendrogram (Fig. 3). Cluster-I contain 6 accessions was further subgrouped into I A and I B with 3 accessions each. Cluster II with 21 accessions was found to have two subgroups II A with 13accessions and II B with 8 accessions.

4.3.2 Principal Component Analysis

Using, Hierrarchial clustering (R package) Principal component analysis was done using the molecular data. Spatial arrangements of selected accessions of cassava and molecular markers are shown in Fig.4.

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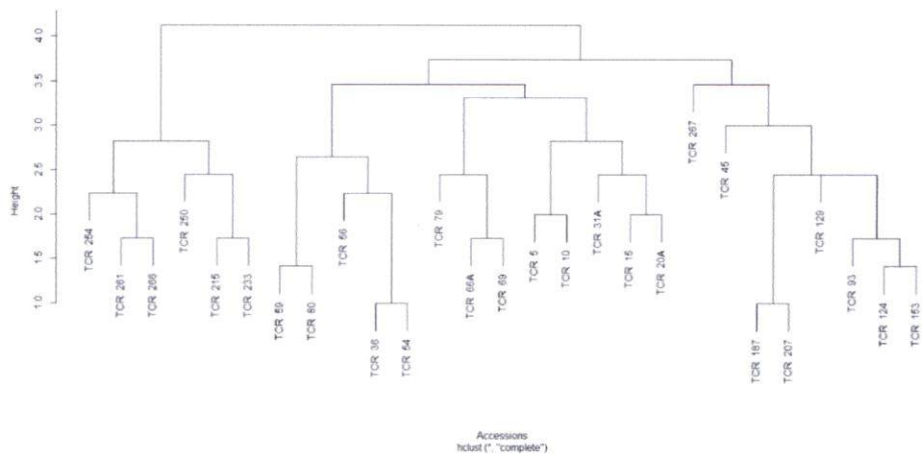

Fig. 3: Dendrogram of 27 accessions of cassava based on Hierarchical clustering using molecular markers (R package)

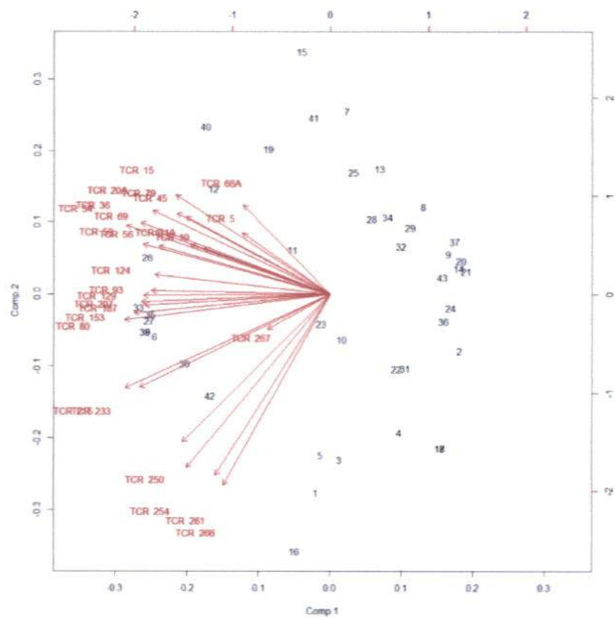

Fig. 4: PCA biplot of 27 accessions of cassava based on molecular primers

4.3.3. Polymorphic Information Content (PIC)

Polymorphic Information Content (PIC) of each individual SSR allele was calculated according to the formula described by Tehrani *et al.* (2008). PIC values that revealed the ability of each primer to distinguish the 27 selected accessions of cassava were calculated for all the 10 primers assessed. The value of heterozygosity ranged from 0.2533 (SSRY 148) to 0.8293 (SSRY 9). The average PIC value ranged from 0.2533 (SSRY 148) to 0.8091 (SSRY 9). PIC values for all the polymorphic bands produced by a primer pair were averaged to calculate PIC value for a primer pair (Table-13). High heterozygosity observed values were significant, which substantiate the heterozygote nature of most the genotypes and the fact that cassava is largely cross-pollinated (Asare *et al.*, 2011).

Table13: Polymorphic information content and expected heterozygosity of 10 SSR primers

Sl. No.	SSR Primer	Expected heterozygosity (He)	Avarage PIC
1	SSRY 9	0.8293	0.8091
2	SSRY 45	0.738	0.6943
3	SSRY 100	0.6244	0.5484
4	SSRY 102	0.449	0.449
5	SSRY 105	0.7883	0.7585
6	SSRY 147	0.5355	0.4783
7	SSRY 148	0.2975	0.2533
8	SSRY 161	0.5026	0.4644
9	SSRY 181	0.662	0.5878
10	SSRY 182	0.5417	0.4598

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4.4 CORRELATION OF MORPHOLOGICAL AND MOLECULAR DATA

Mantel's test was performed to assess if there is any correlation existed between morphological and molecular data. The values obtained in mantle's test is $r = 0.05615$ and $p = 0.208$. The analysis proved that no correlation existed between these data. But accession wise comparison of the dendrogram data (Table-14). Accessions like **TCR-5, TCR-10, TCR-15, TCR-79** and **TCR-69** are grouped together in Cluster II A of both morphological and molecular characterization.

Table-14: Accession wise comparison of morphological and molecular data

Morphological Analysis				Molecular Analysis			
Cluster		Accessions	Number of genotypes	Cluster		Accessions	Number of genotypes
Cluster I	I A	TCR-59, TCR-207, TCR187, **TCR-266**	4	Cluster I	IA	TCR-254, TCR-261, **TCR-266**	3
	I B	TCR-153, TCR-56, TCR-66A	3		IB	TCR-250, TCR-215, TCR-233	3

Cluster II	II A	**TCR-10**, TCR-215, **TCR-15**, **TCR-79**, TCR-124, **TCR-69**, TCR-93, TCR-250, TCR-80, TCR-261, **TCR-5**, TCR-254	12	Cluster II	IIA	TCR-59, TCR-80, TCR-56, TCR-36, TCR-54, **TCR-79**, TCR-66A, **TCR-69**, **TCR-5**, **TCR-10**, TCR-31A, **TCR-15**, TCR-20A	13
	II B	TCR-233, TCR-254, **TCR-129**, TCR-36, TCR-20A, TCR-267, TCR-31A, TCR-45	8		IIB	TCR-267, TCR-45, **TCR-129**, TCR-187, TCR-207, TCR-93, TCR-124, TCR-153	8

DISCUSSION

5. Discussion

Cassava is the most important tropical root crop for its starchy roots that forms a major source of dietary energy for more than 500 million people. It is known to be the highest producer of carbohydrates among staple crops. According to the United Nations Food and Agriculture Organization (FAO), cassava ranks fourth as a food crop in the developing countries, after rice, maize and wheat. The leaves are relatively rich in protein and can be consumed. Cassava can be stored in the ground for several seasons, thereby serving as a reserve food when other crops fail. Cassava is an efficient producer of carbohydrate under optimal growth conditions like uncertain rainfall, infertile soil and limited input encountered in tropical areas. This makes cassava an attractive source of food, feed and renewable industrial raw materials.

Cassava is evolving as a vital tuber crop in India. In India cassava has been used both for direct consumption and for the production of starch and sago mainly in the southern states of Andhra Pradesh, Kerala and Tamil Nadu. Genetic diversity serves as a way likely that some individuals in a population will possess variations of alleles that are suited for the environment. The genetic diversity study in cassava is very much needed for the accomplishment of breeding programs, since genetic variance produces high heterotic effect. Hence, this research work attempts to combine both the morphological and molecular data for understanding the extent of genetic variation present in the selected accessions of cassava.

In the present study, the genetic diversity among 27 accessions of cassava germplasm collected from the different states of southern India and maintained in the field genebank of ICAR-CTCRI was determined using 22 morphological descriptors which were listed out by IPGRI/NBPGR and by molecular characterization using 10 SSR markers.

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5.1 Morphological analysis

For the classification of genotypes and also for the genetic variability studies of the plant germplasm morphological descriptors are the most essential tools available in the absence of DNA markers. Morphological characterization has been used for various purposes including identification of duplicates, studies of genetic variation patterns and correlation with characteristics of agronomic importance (CIAT, 1984). Morphological characters are very useful for preliminary evaluation because they offer a fast and easy approach for assessing the extent of diversity. Here, 27 selected accessions of cassava which are used for the study were genetically different and therefore based on their close associations or relationships clustered in groups. IPGRI/NBPGR descriptors for cassava were efficient and helpful in studying the variability and in differentiating the selected accessions of cassava into different groups. Moreover some accessions coming in the same clusters were almost similar giving the result as highly related to each other. But there were no duplicates identified among these accessions with 100 % similarity based on the morphological characterization and hence no core collections identified.

Morphological traits like young and mature stem colour, apical leaf colour, young and mature leaf colour, petiole base, middle and tip colour, tuber shape, rind, cortex and flesh colour were all found to be of greater importance in distinguishing the accessions. Genetic diversity study in cassava done by Asare *et al.* (2011) also reported that morphological characters like leaf and stem colour, tuber inner and outer colour are the main traits for the characterisation of cassava germplasm.

The use of qualitative traits in the classification of crops is very essential due to their relative stability over quantitative traits. Wide range of polymorphism was observed in some of the characters namely apical and mature leaf colour, young and mature stem colour, Petiole base, middle and top colour, tuber shape, colour of tuber flesh and tuber inner and outer skin colour. Similar observations have been reported in the studies done by Elias *et al*. (2000) and Raghu *et al*. (2007).

In the study of Karuri *et al.* (2010), 22 morphological descriptors are used for the characterization of 29 cassava genotypes. This study reported that leaf colour and tubers shape of cassava helps in characterizing the selected genotypes into 3 major clusters. In our present study, the dendrogram generated gave two major clusters by the grouping associated with the morphological descriptors. From PCA analysis, it is conformed that leaf, stem and tuber colour are the major traits that contributed to the characterization of cassava accessions.

In the genetic diversity study of cassava accessions in Chad (Nadjiam *et al.*, 2016) three descriptors mainly colour of leaf, stem and tuber contributed more for depicting variability in cassava. The same studies done by Kosh-Komba *et al.* (2014) also reported that colour variation in the mature and young leaves, stem colour and differences in tuber colour including the inner and outer cortex also contributed to the genotype variation among cassava accessions.

The results obtained from Principal Component Analysis showed that the characters that most contributed to the species variability were related to plant mature and young stem colour, Petiole base, middle and tip colour, Tuber outer and inner colour. In PCA maximum variation was observed in Componant 1 (54.22%) which included descriptors like Apical leaf colour and petiole tip colour with highest PC loadings. Further morphological data clustering with tree analysis concept and tree construction based on Hierarchical clustering (R package) gave two major clusters. In the cluster formation mature and young stem colour and tuber inner and outer colour contributed maximum polymorphism. Similar observations were found in the genetic variability analysis of elite cassava collected in Benin (Agre *et al.*, 2015).

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5.2 Molecular Analysis

The DNA was isolated from the 27 selected accessions of cassava germplasm using the CTAB method (Doyle, 1991). Doyle (1991) introduced the use of CTAB for isolation of DNA from plants and the method was used by Chiang *et al.* (1998).

In present study SSR markers were selected based on the previous reports in cassava (Mba *et al.*, 2001). As per Costa *et al.* (2000) before doing the analysis, screening of primers should be done to obtain the correct annealing temperature. Thus they surveyed a total of 100 UBC primers and those primers which produced four or more bands were selected for the study and their annealing temperatures were determined using the gradient PCR. Finally, they selected only eleven out of the total primers as they gave considerably clear and good bands. Generally accepted rule is that the annealing temperature should be at least 5 °C less than that of the melting temperature (Ye *et al.,* 2001). But according to Sisea and Pamfil (2010), the value is not optimal most of the times and should determine the annealing temperature manually via gradient PCR.

In this study, 12 SSR primers were taken initially for screening the 27 accessions, and 10 of the best primers were chosen for further analysis (SSRY 9, SSRY 45, SSRY 100, SSRY 102, SSRY 105, SSRY 147, SSRY 148, SSRY 161, SSRY 181 and SSRY 182). The selected primers when screened for obtaining the annealing temperatures, temperatures of 48 ^{0}C (SSRY 9 and SSRY 45), 52 ^{0}C (SSRY 100), 56 ^{0}C (SSRY 102), 57 ^{0}C (SSRY 105), 59 ^{0}C (SSRY 148), 65 ^{0}C (SSRY 147 and SSRY 181), 66 ^{0}C (SSRY 182) and 67 ^{0}C (SSRY 161) were optimized. The temperatures thus were found be in the range of 48 ^{0}C to 67 ^{0}C.

After resolving the amplicons using the selected SSR primers under the annealing temperatures determined, clear polymorphic bands were obtained for all the selected primers. Some of the primers gave 100% polymorphism. The SSR data showed that genetic diversity is higher among accessions of cassava selected for

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the study. In present study the allele number assessed the efficiency of each primer taken for study by the calculation of Polymorphic Information Content (PIC) and observed heterozygocity. The value of heterozygocity here ranged from 0.2975 (SSRY 148) to 0.8293 (SSRY 9). The PIC value ranged from 0.2533 (SSRY 148) to 0.8091 (SSRY 9). It was already reported that the SSRs generally have high levels of polymorphism in many important crops including cassava (Chavarriaga-Aguirre *et al.*, 1999; Mba *et al.*, 2001; Raghu *et al.*, 2007). Asare *et al.* (2011) reported that 28 SSR primers were used for the genetic variability study of 128 accessions of cassava and in this the PIC value ranged from 0.19 to 0.54. Similar study conducted by Moyib *et al.* (2007) state that the PIC value of SSR markers ranges from 0.19 to 0.67.

In the present study, using the molecular scoring data, Hierarchical clustering was done and the twenty-seven accessions of cassava were divided into two major Clusters with subclusters. Cluster-I contain 6 accessions was further subgrouped into I A and I B with 3 accessions each. Cluster II with 21 accessions was found to have two subgroups II A with 13 accessions and II B with 8 accessions. The cluster data from dendrogram was agreement with data from cluster analysis done after PCA.

5.3 Comparison between morphological and molecular data

Both the morphological and genetic analyses carried out in the present study allowed separation of accessions of cassava into two main clusters in each of the method. In both the methods, the dendrogram showed similar grouping of accessions in the clusters. Lymo *et al.* (2012) reported that three main clusters were obtained in cassava which showed similar grouping by both morphological and molecular markers. Regarding cassava genetic diversity of different traits, several authors were able to reveal relationships among cassava cultivars based on morphological descriptors and SSR markers (Fragene *et al.*, 2003).

SUMMARY

6. Summary

The study entitled "Characterization of selected accessions of cassava germplasm using morphological and molecular markers" was carried out at the Division of Crop Improvement, ICAR-Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram during 2016-2017. The objective of the study was to analyze genetic variation existing within the cassava germplasm based on morphological and molecular markers. Twenty-seven accessions of cassava collected from the three different states of southern India were selected for characterization.

The characterization study was divided into two phases it morphological and molecular. Morphological characterization was done by recording the observations and measurements both qualitative and quantitative of all the above ground vegetative plant and below ground tuber characteristics of cassava. All the traits listed out for observation were those listed as per IPBGR/NBPGR descriptors for cassava. The percentage distribution of each trait for a particular character was obtained as it can be used to distinguish within species and among species differences in later studies. The recorded data were analyzed using various statistical tools such as R package and Principal Component Analysis. Cluster dendrogram was done for all the 27 accessions of cassava studied and it resulted in two major groups. Cluster-I consists of 7 accessions was further subdivided into two subclusters I A and I B. Cluster-I A consisted of four accessions while Cluster-I B of three accessions. The bigger cluster Cluster-II consisting of 20 accessions was found to have two subgroups namely II A with 12 accessions and II B with 8 accessions. In PCA, mainly three clusters were obtained. The percentage variation is high (54.22) in Component 1. The morphological descriptors like mature and young leaf colours, Petiole base colours contribute the major variations.

After morphological characterization, molecular analysis was done by DNA isolation procedures. Young leaf samples freshly collected from the field was used for studies. DNA was isolated by two methods - Using QIAGEN DNasy® plant minikit and CTAB method. Using the minikit method the quality of the obtained DNA was not of good, whereas, good quality DNA was obtained by using the CTAB method and the extracted DNA was having a A_{260}/A_{280} ratio of 1.96 - 2.09 when resolved in 1% agarose gel. Thus isolation using CTAB method was confirmed as a good method for isolation of genomic DNA from cassava.

Isolation of DNA from the selected accessions was followed by primer screening for SSR analysis. A total of 12 SSR primers were taken for the screening process and out of which, 10 primers which gave clear and reproducible bands in 1% agarose gel were selected and their annealing temperatures were determined by doing the gradient PCR. All the primers amplified in an annealing temperature of 48 °C (SSRY 9 and SSRY 45), 52 °C (SSRY 100), 56 °C (SSRY 102), 57 °C (SSRY 105), 59 °C (SSRY 148), 65 °C (SSRY 147 and SSRY 181), 66 °C (SSRY 182) and 67 °C (SSRY 161). After the final PCR using the primers and the determined annealing temperatures, the product was resolved in 4% agarose and clear polymorphic bands were obtained. In the present study, all the SSR primers in the study showed the polymorphism. The SSR primers on an average produced 9 polymorphic alleles with mean observed heterozygosity and values of Polymorphism Information Content (PIC) 0.8293 and 0.8091 respectively. The value of heterozygosity here ranged from 0.2975 (SSRY 148) to 0.8293 (SSRY 9). The PIC value ranged from 0.2533 (SSRY 148) to 0.8091 (SSRY 9). Hierarchical clustering (R package) was performed and the dendrogram obtained divided the 27 accessions into 2 major groups. Cluster-I contain 6 accessions was further sub grouped into I A and I B with 3 accessions each. Cluster II with 21 accessions was found to have two subgroups II A with 13 accessions and II B with 8 accessions. Principle component analysis was done using the same scoring data. PCA using molecular markers closely related to the dendrogram.

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The present study revealed good variability among the 27 accessions of cassava using morphological and molecular analysis. There were no duplicates found among the selected accessions of cassava based on both the studies but similarities among the accessions were noticed in the grouping by the statistical tools. The similar accessions can be maintained as core collections and the morphological traits and molecular markers used in the study can be very effectively used as tools for future germplasm characterization and diversity studies in the crop cassava.

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APPENDICES

APPENDIX I

CTAB Extraction Buffer

Tris HCl (pH 8.0)	100mM
EDTA (pH 8.0)	25mM
NaCl	1.5 M
CTAB	2.5%
β-Mercaptoethanol	0.2% (v/v)
PVP	1% (w/v)
Distilled water	

APPENDIX II

TE BUFFER (10X)

Tris HCl (pH 8.0)	10 mM
EDTA	1 mM

APPENDIX III

TBE Buffer (10 X)

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

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

APPENDIX IV

Wash solution

Ammonium acetate	15mM
Ethanol	70%

APPENDIX V

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

APPENDIX VI

70% ethanol

100% ethanol	70 ml
Distilled water	30 ml

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ABSTRACT

CHARACTERIZATION OF SELECTED ACCESSIONS OF CASSAVA GERMPLASM USING MORPHOLOGICAL AND MOLECULAR MARKERS

By

ANJALI SABU C.

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Abstract of thesis

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ABSTRACT

Cassava (*Manihot esculenta* Crantz), a perennial shrub, is an important crop in many parts of the tropics.This research work attempts morphological and molecular characterization of 27 cassava germplasm collected from Southern India. In the present study27 accessions of cassava maintained in the field genebank of ICAR-Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram were characterized based on 20 qualitative and 10 quantitative traits including the major yield components. There were no duplicate accessions identified based on morphological classification and it can be maintained as core collection. The genetic diversity on molecular basis was evaluated using 10 SSR primers. Molecular markers are proved to be valuable tools in the characterization and evaluation of genetic diversity within and between species and population. All the SSR primers in the study showed the polymorphism. The SSR primers on an average produced 9 polymorphic alleles with mean observed hetrozygosity and values of Polymorphism Information Content (PIC) 0.8293 and 0.8091 respectively. The value of heterozygosity here ranged from 0.2975 (SSRY 148) to 0.8293 (SSRY 9). The PIC value ranged from 0.2533 (SSRY 148) to 0.8091 (SSRY 9). The hetrozigosity and average PIC content observed in SSRY 9. Clustering based on morphological descriptors and molecular markers was done. In morphological clustering, Cluster-I consists of 7 accessions was further subdivided into two sub clusters I A and I B. Cluster-I A consisted of four accessions while Cluster-I Bof three accessions. The bigger cluster Cluster-II consisting of 20 accessions was found to have two subgroups namely II A with 12 accessions and II B with 8 accessions. Clustering based on SSR marker analysis grouped the genotypes into 2 Clusters. Cluster-I contain 6 accessions was further sub grouped into I A and I B with 3 accessions each. Cluster II with 21 accessions was found to have two subgroups II A with 13accessions and II B with 8 accessions. By comparing the morphological and molecular clusters, In Cluster II of each dendrogram have 6 similar accessions of cassava (TCR-5, TCR-10,TCR-15,TCR-45,TCR-79,TCR-69,). Clustering and Principal Componant Analysis of the data validated the variation among the cassava accessions. In PCA of morphological

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characters the percentage variation obtained in PC component 1 (54.22). Mantel's test proves that there is no correlation between the morphological and molecular data. The present results indicated that the primers selected for the present study will be useful for future genetic variability studies and would provide breeders with a genetic base for selection of diverse parents for crop improvement programmes in cassava.

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