GENETIC DIVERSITY ANALYSIS OF ELEPHANT FOOT YAM

[*Amorphophallus paeoniifolius* (Dennst.) Nicolson]

by

KRISHNARAJ S.

(2011-09-107)

THESIS

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DECLARATION

I hereby declare that this thesis entitled **"GENETIC DIVERSITY ANALYSIS OF ELEPHANT FOOT YAM [*Amorphophallus paeoniifolius* (Dennst.) Nicolson]"** 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.

Place: Vellayani
Date: 18/03/2017

KRISHNARAJ S.
(2011-09-107)

Produce tuber reduce hunger

भाकृअनुप-केन्द्रीय कन्द फसल अनुसंधान संस्थान
(भारतीय कृषि अनुसंधान परिषद)
श्रीकार्यम, तिरुवनन्तपुरम -६९५०१७, केरल,भारत
ICAR-CENTRAL TUBER CROPS RESEARCH INSTITUTE
(Indian Council of Agricultural Research)
Sreekariyam, Thiruvananthapuram-695 017, Kerala, India

ISO 9001:2008

CERTIFICATE

Certified that this thesis entitled **"GENETIC DIVERSITY ANALYSIS OF ELEPHANT FOOT YAM [*Amorphophallus paeoniifolius* (Dennst.) Nicolson]"** is a record of research work done by **Mr. Krishnaraj S. (2011-09-107)** under my guidance and supervision and that this has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to him.

Place: Thiruvananthapuram
Date: 18/03/2017

(Asha Devi A)
(Major Advisor, Advisory Committee)
Principal Scientist, Division of Crop Improvement
ICAR-Central Tuber Crops Research Institute
Sreekariyam, Thiruvananthapuram – 695 017

डॉ.आशा देवी. ए
Dr. ASHA DEVI. A
Principal Scientist (Genetics)
ICAR - Central Tuber Crops Research Institute
Sreekariyam, Thiruvananthapuram - 695 017

Phone : 91-0471-2598551 to 2598554
Director (Per.) : 91-0471-2598431
(Res.) : 91-0471 - 2441957
Admn.Officer : 91-0471-2598193

Fax : 91-0471-2590063
E-Mail: ctcritvm@yahoo.com
ctcritvm@gmail.com
Web : http://www.ctcri.org

CERTIFICATE

We, the undersigned members of the advisory committee of **Mr. Krishnaraj S. (2011-09-107)**, a candidate for the degree of **Master of Science (Integrated) in Biotechnology**, agree that the thesis entitled "**GENETIC DIVERSITY ANALYSIS OF ELEPHANT FOOT YAM [*Amorphophallus paeoniifolius* (Dennst.) Nicolson]**" may be submitted by Mr. Krishnaraj S, in partial fulfillment of the requirement for the degree.

Dr. Asha Devi. A
(Major Advisor, Advisory Committee)
Principal Scientist,
Division of Crop Improvement
ICAR- Central Tuber Crops Research Institute, Sreekariyam
Thiruvananthapuram- 695 017

Dr. B. R. Reghunath
Dean, Professor and Head
Department of Plant Biotechnology
College of Agriculture, Vellayani
Thiruvananthapuram- 695 522

Dr. J. Sreekumar
Principal Scientist
Division of Social Science and Extension
ICAR- Central Tuber Crops Research Institute, Sreekariyam
Thiruvananthapuram- 695 017

Dr. Lekha Sreekantan
Professor
Department of Agronomy
College of Agriculture, Vellayani
Thiruvananthapuram-695 522

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KRISHNARAJ S.

Dedicated to my dear parents

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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	Cetyltrimethyl 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
h	Hour
ha	Hectare
ICAR-CTCRI	ICAR- Central Tuber Crops Research Institute
ICAR-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
PAGE	Polyacrylamide gel electrophoresis
PC	Principal component
PCA	Principal component analysis
PCR	Polymerase chain reaction
PEG	Polyethylene glycol

PVP	Polyvinylpyrrolidine
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)
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

INTRODUCTION

1.INTRODUCTION

The family Araceae holds more than 200 species including *Amorphophallus*, a tuberous herb (Sedayu *et al.*, 2010; Jaleel Abdul *et al.*, 2011) which can be reflected as one among nature's true marvels and a true treasure of variation (Hetterscheid and Ittenbach. 1996). It is frequently found in Africa, Madagascar, India, continental South East Asia, Malesia and North East Australia (Mayo *et al.*, 1997).

Elephant foot yam or *Amorphophallus paeoniifolius* (Dennst.) Nicolson is common to India and Srilanka with a varied range of distribution but its origin is in South-East Asia and. According to Willis (1955), the tropics hosts around 90 species of *Amorphophallus*. A total of about 100 species of *Amorphophallus* are distributed across Africa, India and Malaysia to Australia according to Hay (1990). Wild varieties of *Amorphophallus* can be found in Sri Lanka, Philippines, Malaysia, Indonesia, and other South-east Asian countries. India has around 15 endemic species. Elephant foot yam is grown frequently in West Bengal, Kerala, Andhra Pradesh, Maharashtra and Orissa in India. It is vegetatively propagated through corm pieces and is a cross pollinated crop. Due to its production potential and acceptance as a vegetable in various appealing cuisines, it is an upcoming and chief cash crop. The area under its cultivation is growing fast and it has attained the status of a cash crop (Nedunchezhiyan et al., 2006).

Elephant foot yam has high carbohydrate, protein, minerals like calcium, iron, phosphorous, vitamin A, B, C, flavanoids, and fibre content (Kay 1987; Shilpi *et al.*, 2005). It has vital medicinal characteristics like hepatoprotective, antioxidant, uterus stimulating agent (Singh *et al.*, 2011; Laderman, 1983). *A. paeoniifolius* is widely cultivated in the states of Gujarat, Maharashtra, Kerala, Tamil Nadu and Andhra Pradesh in India, (Anonymous 1998).

The assessment of diversity present within a crop is an important step in any crop improvement programme. Descriptors are available to characterize the wide variability present in the crop, characterized by the germplasm collection. CTCRI (Central Tuber Crops Research Institute), Thiruvananthapuram, India, has been recognised as the National Active Germplasm Site (NAGS) for tuber crops. About 183 elephant foo yam accessions are kept by CTCRI in the gene bank. CTCRI collects variability from all over the country through the different centres of the All India Co-Ordinated Research Project on Tuber Crops (AICRP-TC) for conservation and future use.

There are possibilities of duplicates being present in the germplasm, due to this merging of germplasm from various parts of the country. The extent of variability and duplicates present in the collection can be measured only after characterization. A core collection comprising the maximum variability existing in a crop can be prepared from this for future utilization and breeding programmes as well as in vitro conservation. The International Plant Genetic Resources Institute (IPGRI)/National Bureau of Plant Genetic Resources (NBPGR) descriptors are two major descriptors available for characterization. The current study was thus begun with the intention to morphologically characterize elephant foot yam based on NBPGR descriptors to assess the amount of diversity present.

Characterization and cataloguing of germplasm can be done using molecular markers eventhough morpho-agronomic traits are the conventional method for germplasm characterization(Sanghani *et al.,* 2015). Closely related individuals can be precisely and promptly distinguished by using ISSR primers for analysis (Zietkiewicz, *et al.,* 1994). The ISSR procedure includes attaching of designed primers to a subset of SSRs and amplify the region among two closely spaced, oppositely oriented SSRs. Thus multiplexed

banding profiles, high frequency of polymorphism, and high throughput result can be acquired in a relatively low cost, (Kantety *et al.,* 1995). Thus, through this study, molecular characterization along with morphological characterization by ISSR primers was initiated.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

The *Amorphophallus* species are mostly found in the Eastern boundary of Polynesia, Western Africa, Japan, Philippines, Taiwan, New Guinea, Central Thailand, Southward via Sumatra, Indonesia, Malaysia and several other parts of South Asian Countries such as Sri Lanka, India and Malaya. *Amorphophallus* species can also be seen in the tropical and subtropical zones of the paleo tropics, and rich from West Africa to the Pacific islands. In India, they are distributed all around and accounts for genetic diversity within the species. (Behera *et al.,* 2014*)*

Elephant foot yam (*Amorphophallus paeoniifolius* (Dennst.) Nicolson) is an underground, unbranched plant with large stout mottled leaves. They are perennial plants with an underground stem in the form of a corm and a highly dissected umbrella-shaped leaf blade (Chua *et al.*, 2010). Whole corm or cut corm pieces (500-750 g) having part of apical meristem is mainly used as seed material (Nedunchezhiyan *et al.*, 2011). Elephant foot yam (*Amorphophallus paeoniifolius* (Dennst.) Nicolson) thrives well in warm humid climate with a mean temperature of 30-35°C during the crop growth period. It is cultivated in areas with well distributed rainfall of 1500-1800 mm over 6-8 months (Sunitha *et al.*, 2013).

Because of its higher yield potential, higher biological efficiency, culinary properties, medicinal utility and therapeutic values, it is referred to as "King of tuber crops". In India, it is grown in almost all the states but commercially cultivated mainly in Andhra Pradesh, Tamil Nadu, Gujarat, Maharashtra, West-Bengal, North-Eastern states, Kerala, Karnataka, Bihar and Uttar Pradesh (Saravaiya *et al.*, 2010). This tuber is consumed by many people as a food and widely used in many Ayurvedic preparations. The tubers are anti-inflammatory, liver tonic, anodyne, anti-haemorrhoidal, expectorant, stomachic, digestive, appetizer, carminative, aphrodisiac, anthelmintic, emmenagogue, haemostatic, rejuvenating and tonic (Dey *et al.*, 2010). The corm contains an active diastatic enzyme amylase, betulinic acid, tricontane, lupeol, stigmasterol, betasitosterol and its palmitate and glucose, galactose, rhamnose and xylose.

Being vegetatively propagated, the rate of multiplication is very low, so it takes a long time for the elite varieties to reach the end users (Nath *et al.*, 2007). Although information about genetic variation is important for breeding programs, there have been few studies on the characterization of elephant foot yam accessions (Sugiyama *et al.*, 2006). Determining the genetic diversity within a species and its distribution pattern among and within populations is important for the conservation and management of genetic resources. This information allows establishing priorities for conservation and at the same time, setting up the most appropriate sampling strategy for using and maintaining this genetic diversity. (Cruz-Nicolás *et al.*, 2011).

Non availability of quality planting material in adequate quantity is the biggest constraint in its widespread cultivation (Bairagi and Singh, 2014). Nevertheless, practical constraints (e.g. insufficient amount of easily accessible plant tissue) as well as low levels of polymorphism have brought about an increasing interest in DNA-marker based methods. Among these, Polymerase Chain Reaction (PCR)-derived markers obtained with non-species specific primers have become exceedingly popular since they do not require sequence information for the target species (Nybom, 2004).

Since the mid 1980s, genome identification and selection has progressed rapidly with the help of PCR technology. A large number of marker protocols that are rapid and require only small quantities of DNA have been developed.The choice of a molecular marker technique depends on its reproducibility and simplicity. The best markers for genome mapping, marker assisted selection, phylogenic studies, and crop conservation have low cost and labour requirements and high reliability (Bornet *et al.*, 2004).

ISSR uses the presence through-out the genome of Simple Sequence Repeats (SSR) which are ubiquitous, abundant and highly polymorphic tandem repeat motifs composed of 1 to 7 nucleotides. ISSR markers are highly polymorphic and are useful in studies on genetic diversity, phylogeny, gene tagging, genome mapping and evolutionary biology (Reddy *et al.*, 2002). ISSR

primers anneal directly to simple sequence repeats and thus, unlike SSR markers, no prior knowledge of target sequences is required for ISSRs (Godwin *et al.*, 1997).

In this chapter, literature concerning the morphological characterization, ISSR marker profiling and techniques involved in molecular and statistical analysis have been presented.

2.1 CENTER OF ORIGIN OF ELEPHANT FOOT YAM

South Eastern Asia is its centre of origin (Poddar and Mukherjee, 2015). The common elephant foot yam, *Amorphophallus paeoniifolius* (Dennst.) Nicolson, is a native of India and Sri Lanka, around 15 species are endemic to India (Devi *et al.,* 2013). The wild form of the crop can be seen commonly in the Philippines, Malaysia, Indonesia and south-east Asian countries(Srinivas and Ramanathan, 2005).

2.2 NUTRITIONAL AND THERAPUETIC VALUE

The corm is a hot carminative in the form of a pickle. It contains betulinic acid, β- sitisterol, stigmasterol, triacotane, lupeol, and β-sitosterol palmitate (Ramalingam *et al.*, 2010.). It is beneficial in liver diseases, abdominal pain, emesis, dysentery, elephantiasis, diseases due to vitiated blood, rheumatic swellings (Ansil *et al.*, 2012). Corms are rich in minerals and vitamins (Nedunchezhiyan *et al.*, 2008). The botanically modified stem 'corm' is consumed as a vegetable after boiling, baking and frying (Nedunchezhiyan *et al.*, 2002). The wild form, *A. paeoniifolius* var. *paeoniifolius* possess powerful therapeutic action against piles and gastro-intestinal disorders (Raghu *et al.*, 1999). The corms are aperient, carminative and expectorant (Anil *et al.*, 2011). Its corms contain a high level of water-soluble glucomannan; therefore, it has long been used as a medicinal herb and food source (Zheng *et al.*, 2013). The tubers are rich in starch as well as protein, calcium, (50 mg g-1), phosphorus (34 mg g-1) and vitamin A (260 IU g-1) [AICRP, 2006]. It is used as staple food in countries like Philippines, Java, Indonesia,

Sumatra, Malaysia, Bangladesh, India, China and south-eastern Asian countries (Chandra, 1984). Elephant yam has medicinal properties and is used in many Ayurvedic (traditional Hindu) preparations. The tubers are considered to have pain-killing, anti-inflammatory, anti-flatulence, digestive, aphrodisiac, rejuvenating and tonic properties. They are traditionally used in the treatment of a wide range of conditions including parasitic worms, inflammation, coughs, flatulence, constipation, anaemia, haemorrhoids and fatigue.

2.3 GENERAL PLANT MORPHOLOGY

Elephant foot yam is a Perennial herb. A single inflorescence (flowering structure) is produced, followed by a solitary leaf. The plant is deciduous, dying back to a large underground corm, weighing up to 8kg, after the growing season. After the growing season, this dies back to an underground storage organ (tuber), usually one (sometimes two) per tuber. Petiole (leaf stalk) up to 2 m tall and 20 cm in diameter with rough, warty surface. Background colour pale to dark green or blackish-green with pale blotches and numerous tiny dark dots. Leaf blade up to 3 m in diameter and deeply divided into segments. The Leaflets which are up to 35×12 cm have leaf blade, in a thick fleshy stem and is divided into hundreds of leaflets, varying among 5 and 12.5 cm long, with highly ridge ovate or oblong shape (Costa *et al.,* 2016) The solitary leaf resembles a small tree. The leaf blade is much divided into hundreds of leaflets and can reach over 1 m wide. This blade sits atop a thick fleshy stem up to 13 cm diameter and 2 m tall. The pustular surface of the stem is attractively blotched with paler shades of green.

It comprises a large spadix crowned with a bulbous purple knob, encircled by a fleshy purple and green-blotched spathe up to 50 cm wide. On successful pollination of the female flowers the spadix can extend to 2 m in length. The fresh inflorescence emits an odor reminiscent of rotting flesh to attract pollinating carrion flies and beetles. Spadix (flower spike) is up to 70 cm long. The lowermost portion of the spadix is female and is covered with pistils (female parts). Each pistil consists

of a pale green or maroon ovary with a maroon stalk (style) and two- or three-lobed yellow head (stigma). The next floral zone is male and contains tightly-packed yellowish stamens. At the tip of the spadix is a bulbous, dark maroon, rounded to deeply wrinkled appendix. Spathe (bract surrounding spadix) is bell-shaped, broader than long, up to 45×60 cm, pale green to dark brown with paler blotches on the exterior. Opening outwards to form a frilled, glossy maroon, collar-like structure around the spadix. Basal portion of interior pale green-yellow.

Fruits are about 2×1 cm, bright red when ripe, borne on a spike up to 50 cm long and 8 cm in diameter, the fruiting part is held aloft on a peduncle (stalk) 20–100 cm long. Corm is depressed-globose, up to 30 cm in diameter, flowering before leafing every year from the previous year's corm. Stem-like structure, which bears the lamina, is merely the petiole, 1 m or more in height, radically developed from the corm.

In short, it is a stout herbaceous plant with underground hemispherical depressed dark brown corm; leaves compound, large, solitary, petiole stout, mottled, 60-90 cm long, leaflets 5.0-12.5 cm long of variable width, obovate or oblong, acute, strongly and many nerved; male and female inflorescences contiguous, neuters absent, appendage of spadix sub-globose or amorphous, equal or longer than the fertile region, spathe - campanulate, pointed, strongly, closely veined, greenish-pink externally, base within purple, margins recurved, undulate and crisped, male inflorescence subturbinate, female 7.5 cm or more long; fruits obovoid 2-3 seeded red berries (Agrawal, 2002).

2.4 MORPHOLOGICAL CHARACTERIZATION

Wild form of certain plants are considered as valuable sources for improving the crop in various aspects. From time immemorial, various morphological and phenological characters were used to check the similarities and variabilities between wild and cultivated varieties of crops (Mac Key, 1988). In many cases, with large number of accessions having several characters, multivariate analysis and cluster analysis has been an important tool for characterizing, evaluating and

classifying genetic resources (Peeters and Martineelli, 1989). Also Santosa *et al.*, 2002 noted that the morphological characters varies with growth habits. This type of selection or classification based on morphological characters caused rare phenotypes to be enhanced and maintained (Casas *et al.*, 1998). Manzano *et al.* (2001) took morphological characters such as subterranean, leaf and inflorescence characteristics along with cytogenic and biochemical characteristics to describe the germplasm. Morphological characteristics can be used to form subgroups out of various species and varieties (Santosa *et al.*, 2002). Morphological traits can be quickly identified and are easily distinguishable (Wu *et al.*, 2016). This gives them an upper hand over molecular markers as molecular markers requires a lot of skill for screening and analysis and is not cost effective even though the result is precise (Pathak *et al.*, 2014).

Since conventional breeding methods for the improvement of vegetatively propagated plants like taro and *Amorphophallus* is hindered (Abraham *et al.*, 2008), the selection of superior varieties based on morphological characters will lead to the improvement of that particular crop. Selection is made through analysing variability among the accessions on the basis of morphology (Poddar *et al.*, 2015). A very few cases are there in which vegetative morphologic variations are identified in *Amorphophallus* accessions (Hetterscheid *et al.*, 1996).

Few studies of characterizing *Amorphophallus* using morphological markers are reported. A study by Ohtsuki, 1968 showed that Indonesian *Amorphophallus* can be classified using morphological descriptors like leaf color, presence or absence of bulbil, skin and flesh color of corms, and the contents of starch, glucomannan, and calcium oxalate. Widjaja and Lester (1987) showed that, on the basis of petiole characteristics, elephant foot yams can be divided into two groups. Namely, *A. campanulatus* var. *hortensis* which are having smooth petioles and *A. campanulatus* var. *sylvestris* which are those having rough petioles.

2.5 MOLECULAR CHARACTERIZATION

Molecular markers are developed for various crops which are linked to numerous traits of economic importance (Cyriac *et al.*, 2016). Genetic diversity can be studied in a good manner using appropriate marker systems. For genetic diversity studies, various markers with set parameters are available. (Tiwari *et al.*, 2016). Kumar *et al.*, (2016) suggested that for crops or varieties having genetic variation data is limited or unavailable, markers such as RAPD and ISSR can be used for diversity analysis. DNA based markers can be used as an efficient tool for analysing diversity. AAD (Arbitrarily Amplified Dominant) markers consists of a set of markers which includes RAPD, ISSR and AFLP and are used commonly for diversity studies (Karp *et al.*, 1996). The neutrality of most of these markers means that the whole genome coverage can be achieved, provided that a sufficient number of markers is employed. (Li *et al.*, 2016). Diversity among 40 accessions was studied by Zhang *et al.* (2011) using DNA markers. Molecular marker analysis can be used to obtain a more specific and precise diversity data.

Gupta *et al.* (2000) concluded that there has been significant increase in the use of molecular markers in diversity studies. As there are several gene interactions, molecular characterization can have a lot of advantages over morphological characterization (Karp *et al.*, 1997). Also, molecular data can be used in association with morphological data for decreasing the severance of accessions and in turn reducing the cost of maintaining huge number of accessions (Pissard *et al.*, 2008). The variations can be seen due to the polymorphism shown by accessions (Nguyen *et al.*, 2004).

2.5.1 ISSR in diversity analysis

Genomic polymorphism can be detected using ISSR markers as they are highly sensitive and they have a high potential for the detection of both inter- and intra-specific variation even without any sequence information (Willams *et al.*, 1990). Santosa *et al.* (2010) earlier used microsatellite markers to analyse the diversity in *Amorphophallus paeniifolius* in Indonesia. Also Xuan (2010) has done

research in *Amorphophallus konjac* grown in china and are endemic and he used ISSR markers to study the genetic diversity. The ISSR fingerprinting technique has also been studied for its use in distinguishing taro genotypes (Okpul, *et al.,* 2005). ISSR differs from the microsatellite marker approach in that no prior sequence knowledge is required. The technique can therefore be applied immediately to the germplasm, with only a small amount of optimization necessary for different taxa (Mace *et al.*, 2006).

ISSR markers can be applied to the analyse of almost any organism as it scans through the entire genome of that organism owing to the fact that ISSR uses random markers which provides a big number of multilocus markers (Karatash *et al.*, 2008). 15–35 bp-long oligonucleotide primers which may or may not be anchored at the 5′ or 3′ end and will be having one to ten degenerate nucleotides. These will be inherited in Mendelian fashion (Ratnaparkhe *et al.*, 1998)

Anil *et al.*, (2014) accessed the diversity in *Amorphophallus* Blume ex Decne. using ISSR markers. 25 accessions were collected from four biogeographic zones of India of which, 8 species and 16 accessions of wild and two accessions of cultivated *A. paeoniifolius* were used. Seventeen ISSR primers were used for characterization. They Identified seven morphotypes of *A. paeoniifolius* (G3, B1, T1, V1, P19, K3-2 and V4) from the wild and one from the cultivated (T10) accessions. The two cultivated accessions GJ and T10 evolved separately due to mutations along with anthropogenic intervention to elevate them as cultivated forms.

A similar research was done in *Chinese lentinula* in which 89 accessions were taken and genetic diversity was accessed using ISSR markers by Liu *et al.*, (2012) . And the sizes of amplification fragments ranged from 150 to 3,200 bp with an average of 74.31% polymorphism. On the basis of electrophoresis data, cluster dendrogram was formed in which *L. edodes* strains was divided into two major groups and group B was further divided into 5 sub-groups. They also concluded that the *L. edodes* cultivars possessed less genetic variations as compared to wild type.

2.5.2 DNA isolation

For most of the studies of an organism regarding its molecular systematics, high quality DNA is required (Aljanabi and Martinez, 1997). For any development of PCR based marker technique, isolation of DNA of high quality and appropriate quantity is a prior requirement (Jeeva *et al.*, 2008). Most of the DNA isolation protocols will be preferring fresh and tender leaves over dry and semi-dry leaves (Khanuja *et al.*, 1999). There are many traditional ways of isolation of DNA such as those involving phenolic extraction, protienase digestion and adsorption of DNA. But for rapid and efficient extraction of DNA, these methods will not be applicable (Chomczynski *et al.*, 1997). Usually nucleic acid will form complexes with secondary compounds such as polysaccharides or polyphenols and are released by cell disruption which confines DNA to a sticky gelatinous matrix or to brown coloured products. Such DNA cannot be used for PCR based amplification (Guillemaut *et al.*, 1992).

Using Cesium chloride gradient sedimentation, using CTAB as a detergent and many other methods for isolation of genomic have been employed over the years (Couch and Fritz, 1990; Dellaporta *et al.*, 1983; Murray and Thomson, 1980; Rogers and Bendich, 1985; Scott and Bendich, 1985; Tai and Tanksley, 1990; Taylor and Powell, 1982; Varadarajan and Prakash, 1991; Zimmer and Newton, 1982; Drabkova *et al.*, 2002; Haymes *et al.*, 2004; Ivanova *et al.*, 2008; Li *et al.*, 2001; Ribiero and Lovato, 2007; Sharma, *et al.*, 2013; Sharma *et al.*, 2000). The size, content and organization of genome varies from plant to plant and dependent on it, a suitable DNA isolation protocol should be developed (Akhtar *et al.*, 2013; Sangwan *et al.*, 1998).

One of the major problem in isolating DNA of good quality from the leaves of tuber crops is hinderance due to various reasons. These includes presence of phenolic compounds and highly viscous polysaccharides. There are chances that the phenolics may bind to proteins and nucleic acids during tissue homogenization (Loomis, 1974; Aljanabi *et al.*, 1999). This further makes availability of DNA restricted for PCR amplification and any restriction digestion as the gelatinous

material formed after binding is difficult to separate from cell organells (Porebski *et al.*, 1997). They may also mix up with polymerases, ligases, restriction endonucleases etc., and will affect their activity (Prittila *et al.*, 2001; Diadema *et al.*, 2003; Karaca *et al.*, 2005; Moyo *et al.*, 2008; Singh and Kumar, 2010). Also degradation of DNA while isolation has been reported (Kotchoni *et al.*, 2011).

As the amount of polysaccharides increases, the chances of anomalous reassociation kinetics of DNA increases (Merlo and Kemp, 1976). Thus during gel electrophoresis procedures after DNA isolation, the DNA may stick closely to the walls of agarose gel wells. CTAB has been proved as a chemical which, with at least 0.5M NaCl, can remove the polysaccharides present. In addition, tannins and polyvinyls can be removed by the addition of PVP and β-mercaptoethanol. Those forces which can break a cell wall can also cause the shearing of DNA. Thus appropriate care must be taken (Murray and Thomson, 1980).

2.5.3 Polymerase Chain Reaction

The number of copies of a specific DNA fragment can be increased exponentially by cycling between two or three different temperature. And the molecular technique is referred as Polymerase Chain Reaction (PCR) (Furutani *et al.*, 2016). Polymerase enzyme uses a defined specific strand as template for assembling a complementary strand by adding nitrogen bases (Schochetman *et al.*, 1988). PCR based markers are commonly used after the invention of Polymerase Chain Reaction by Mullis *et al.* (1986).

PCR requires a three step cycling process. Denaturation of double stranded DNA, annealing of primers and primer extension. A PCR reaction requires a mixture of buffers, nucleotides, primers, enzyme, and nucleic acid from those species. One cycle of approximately about 2-5 minutes is repeated for about 20-40 times (Mullis *et al.*, 1986). The first step is to denature the DNA to obtain single strands. The temperature usually used for denaturation varies from 90°-97°C. Denature separates the duplex by breaking the hydrogen bonds present. Usual denaturation temperature varies from 90°C- 97°C.

The next step, annealing is the most important one and the required temperature is commonly a 5°C less than the melting temperature of the primer. It is kept for the annealing process, where primers are attached to the dissociated DNA strand. It utilizes the complementarity of the nitrogen bases to that of the bases on the template strand. After the primer is annealed the enzyme DNA polymerase catalyses addition of complementary nucleotides. Final step is extension of new DNA strands from the primer and it occurs at 72°C for about 2- 5 min.

About 25-40 cycles of the three processes are repeated in a usual PCR procedure (Joshi and Deshpande, 2010). Earlier, fresh DNA polymerase were added after each cycle. Then heat stable DNA polymerase called taq polymerase from *Thermus aquaticus* was discovered which enabled continuous exponential amplification of DNA. Also taq polymerase increases specificity, length, sensitivity and yield of the DNA generated (Saiki *et al.*, 1985). Usually, in many PCR reactions, the primers used will be 15-20 bp long, have 40-60% of G-C content and the annealing temperature may be around 55°C which is somewhat less than the melting temperature (Weising *et al.*, 2005).

PCR reactions are comparatively inexpensive and less time requiring process considering the fact that the only specialized equipment required is the thermal cycler even though it is possible to do PCR without the equipment using three water baths which is very time consuming and requires manual labour (Delidow *et al.*, 1993).

2.5.4 Agarose Gel Electrophoresis

For DNA fragments having size inbetween 200bp to 20,000bp, it can be visualized using agarose gel electrophoresis. Agarose is a linear polymer that is extracted from seaweed and sold as a white powder that is melted in buffer and allowed to cool, whereby, the agarose forms a gel by hydrogen bonding. The pores formed depends on the agarose concentration. The percentage of agarose depicts its concentration and in most cases 0.3-3% agarose is used for electrophoretic purposes (Smith, 1993). The ability of nucleic acid to move electrophoretically depends on

its size and confirmation (Groot *et al.*, 1970; Grivell *et al.*, 1971). Agar and agarose are the most commonly used solidifying agents in electrophoresis (Noble, 1968).

Many designs of electrophoresis unit are available (Shinnick, 1971). The components of electrophoresis unit are a power supply which is capable of at least 100 V and currents of up to 100 mA, an electrophoresis tank, a casting plate, and a well-forming comb. The frictional drag lags the movement of larger molecules because it's difficult for larger molecules to move through the small pores created by agarose (Sambrook, 1989). The preparation of agarose gel electrophoresis is simple as it can be prepared by adding agarose powder to cold electrophoretic buffer and boiling the solution with appropriate shaking (Southern, 1979).

2.5.5 Statistical analysis

With increases in the sample sizes of breeding materials and germplasm accessions used in crop improvement programs, methods to classify and order genetic variability are assuming considerable significance. The use of established multivariate statistical algorithms is an important strategy for classifying germplasm, ordering variability for a large number of accessions, or analyzing genetic relationships among breeding materials. Multi-variate analytical techniques, which simultaneously analyze multiple measurements on each individual under investigation, are widely used in analysis of genetic diversity irrespective of the dataset (morphological, bio-chemical, or molecular marker data). Among these algorithms, cluster analysis, principal component analysis (PCA), principal coordinate analysis (PCoA), and multidimensional scaling (MDS) are, at present, most commonly employed and appear particularly useful (Johns *et al.*, 1997; Thompson *et al.*, 1998; Brown-Guedira *et al.*, 2000).

2.5.5.1 Cluster analysis

Cluster analysis is primarily performed to group anything which may be any individuals or things on the basis of assets or characters they actually have and which varies between individuals and thus those have similar characters can be grouped together (Hair *et al.*, 1995). Genotypes can be made to clusters in which homogeneity can be seen within clusters and heterogeneity can be seen between clusters (Jaynes, *et al.*, 2003). Thus, after geometrical plotting, similar individuals will belong to the same cluster and will be close on the plot. And those which have variations between them will be separated out on the plot and will have considerable distance between them as they are on different clusters (Hair *et al.*, 1995).

Clustering methods are broadly classified into distance based methods and model based methods. In distance based method, visual identification is possible by graphical representation in which the input given will be pair-wise distance matrix and a specific clustering algorithm is used for analysis. (Johnson and Wichern, 1992). In model based method, observations from each cluster is assumed as random draws from parametric model.

Distance based clustering methods can be further divided into hierarchical and non-hierarchical. Genetic analysis for crop species can be done by using hierarchical studies. These types of studies involve a series of successive mergers or a series of successive divisions of groups of individuals. At first, the number of clusters and number of individuals will be the same, then grouping is done based on similarities.

The determination of optimal number of acceptable clusters is another important aspect. The major thing to consider is to conclude where to cut the dendrogram to find original or natural groups. An acceptable cluster is defined as "A group of two or more genotypes with a within cluster genetic distance less than the overall mean genetic distance and between cluster distances greater than them within cluster distance of the two clusters involved" (Brown-Guedira *et al.*, 2000).

Aharizad *et al.* (2012) used cluster analysis using Ward's algorithm, Squared Euclidean distances. Thus he classified the 94 bread wheat inbred lines into three groups. Five molecular characters were taken by Paulauskas *et al.* (2013) and they divided 11 oilseed cultivars into two clusters and concluded that no significant correlation of genetic diversity with morphological traits was obtained. Similarly, Siopongco *et al.* (1999) made a dendrogram with 200 corn accessions and they took only molecular markers for clustering. As a result, 32 clusters were obtained in which one was having 78 accessions which was the larger one. And 16 clusters were having single accessions in them.

2.5.5.2 Analysis of variance

Analysis of variance (ANOVA) is a method for testing the hypothesis that there is no difference between two or more population means (usually at least three). ANOVA is most commonly used for genetic diversity studies using morphological traits. The major characteristic of ANOVA is that mean and variance is calculated, dividing two variances and comparing the ratio. The major purpose of comparison being able to make it determine the statistical significance. The difference between mean of the observations which receive the treatment and the general mean gives the estimation of effect of any treatment (Jacob, 1992).

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The study entitled "Genetic diversity analysis of elephant foot yam (*Amorphophallus paeoniifolius* [Dennst. Nicholson])" was carried out at the Division of Crop Improvement, ICAR-Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram during 2015-2016. In this chapter, details regarding experimental material and procedures used in the study are elaborated.

3.1 GERMPLASM COLLECTION

Amorphophallus accessions collected from various Indian states are conserved in the ICAR-CTCRI repository, from which 28 accessions were selected for the present study. The plants were raised in the field with five plants per accession spaced at 90 cm x 90 cm spacing. The corms/cormels were planted during April 2015. Weeding and intercultural operations were carried out as per standard procedure. The characteristics of the germplasm location is given in table 1. The geographical origin of selected accessions is listed out in table 2.

3.2 MORPHOLOGICAL DATA COLLECTION

Descriptors listed by the National Bureau of Plant Genetic Resources (NBPGR) were used to take morphological observations. (Abraham *et al.*, 2006). Certain changes were made according to the handiness for listing out the observations taken and for the purpose of involving additional/modified traits. 18 above ground characters were listed of which 10 are quantitative and the rest 8 are qualitative characters. The observations were made in the full growth stage that is 5-6 months after planting. The tuber characters were recorded after the harvest and there was a total of 13 characters including 8 quantitative and 5 qualitative characters.

Table 1. Characteristic of the location at which the crop was maintained

Characteristic	
Latitude	8°40' N
Longitude	77° 0'E
Minimum Night Temperature	19°C
Maximum Day Temperature	33.4°C
Rainfall	1400-1500
Day Length	11 hour 23 minutes (Shortest in December) to 12 hour 39 minutes (Longest in June)
Crop Season	June to December

Table 2. Geographical locations of the *Amorphophallus* accessions selected for the study

Sl. No.	Acc. No.	Place of collection/Status	Botanical variety
1	AmW26	Karnataka	*Amorphophallus paeoniifolius*
2	Am120	Kerala	*Amorphophallus paeoniifolius*
3	Am84	Karnataka	*Amorphophallus paeoniifolius*
4	Am22	Kerala	*Amorphophallus paeoniifolius*
5	Am62	Thrissur, NBPGR	*Amorphophallus paeoniifolius*
6	Am44	Kerala	*Amorphophallus paeoniifolius*
7	Am85	Karnataka	*Amorphophallus paeoniifolius*
8	Am1	Kerala	*Amorphophallus paeoniifolius*
9	Am140	Kerala	*Amorphophallus paeoniifolius*
10	Am10	Kerala	*Amorphophallus paeoniifolius*

Table 2 contd...

11	AmH139	Hybrid	*Amorphophallus paeoniifolius*
12	AmH6	Hybrid	*Amorphophallus paeoniifolius*
13	Am11	Kerala	*Amorphophallus paeoniifolius*
14	Am110	Kerala	*Amorphophallus paeoniifolius*
15	Sree Padma	Variety	*Amorphophallus paeoniifolius*
16	Am107	Unknown	*Amorphophallus paeoniifolius*
17	AmH13B	Hybrid	*Amorphophallus paeoniifolius*
18	Am 102×SP	Hybrid	*Amorphophallus paeoniifolius*
19	Am37	Hybrid	*Amorphophallus paeoniifolius*
20	NDA5	Selection from Uttar Pradesh	*Amorphophallus paeoniifolius*
21	Am135	Kerala	*Amorphophallus paeoniifolius*
22	Am14	Kerala	*Amorphophallus paeoniifolius*
23	S16	Selfed progeny	*Amorphophallus paeoniifolius*
24	Am29	Unknown	*Amorphophallus paeoniifolius*
25	Am130	Tamil Nadu	*Amorphophallus paeoniifolius*
26	H69	Hybrid	*Amorphophallus paeoniifolius*
27	Am133	Kerala	*Amorphophallus paeoniifolius*
28	Am97	Tamil Nadu	*Amorphophallus paeoniifolius*

3.3 MORPHOLOGICAL DATA ANALYSIS

The morphological data was recorded by measuring, weighing and by observation. Quantitative traits are recorded as numerical data, in units and qualitative traits are recorded in the form of numerical traits as shown in table 3. All the morphological data were tabulated in excel worksheet for further statistical analysis.

Table 3. Descriptors for elephant foot yam morphological characterisation as per NBPGR minimal descriptors (Abraham *et. al.*, 2006)

1	Pseudo stem height (cm): (Recorded at full foliage stage)	Quantitative	
2	Number of primary partitions:	Quantitative	
3	Number of secondary partitions / primary partition:	Quantitative	
4	Number of tertiary partitions / secondary partition:	Quantitative	
5	Length of primary partition (cm):	Quantitative	
6	Breadth of primary partition (cm):	Quantitative	
7	Number of leaflets per primary partition:	Quantitative	
8	Length of largest leaflet (cm):	Quantitative	
9	Breadth of largest leaflet (cm):	Quantitative	
10	Leaflet nature:	1 2	Entire Not entire
11	Leaflet margin:	0 1	Not undulate Undulate
12	Surface of pseudo stem:	1 2 3	Smooth Tubercled Spiny
13	Thickness of pseudo stem base (mm):	Quantitative	
14	Pseudo stem colour:	1 2	Green mottled Dark green mottled
15	Leaf spot:	0 1	Absent Present
16	Stalk leaflet:	0 1	Absent Present
17	Nature of rachis:	0 1	Not winged Winged

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Table 3 contd...

18	Shape of rachis:	1 Angled and curved 2 Cylindrical
19	Fresh weight of corm/plat (kg): (Recorded at harvest)	Quantitative
20	Corm shape:	1 Depressed and globose 2 Flat and globose 3 Pyramidal and globose
21	Colour of corm surface top:	1 Black 2 Dark brown 3 White
22	Colour of corm surface bottom:	1 Black 2 Dark brown 3 White
23	Colour of flesh:	1 Light yellow 2 Dark yellow 3 Reddish yellow
24	Height of corm (cm):	Quantitative
25	Diameter of corm (cm):	Quantitative
27	Number of cormels / corm:	Quantitative
28	Weight of cormel / plant (g):	Quantitative
29	Length of cormel (cm):	Quantitative
30	Thickness of cormel (mm):	Quantitative
31	Re-emergence:	0 Absent 1 Present
32	Biotic stress susceptibility:	1 Very lowsusceptibility 3 Low 5 Intermediate 7 High 9 Very high

3.4 ANALYSIS OF MORPHOLOGICAL DATA

The recorded data was subjected to various statistical analysis such as Cluster analysis, PCA, ANOVA.

3.4.1 Cluster Analysis

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

3.4.2 ANOVA

For all the quantitative morphological traits, analysis of variance was done. Mean and coefficient of variation was calculated, dividing two variances and comparing the ratio. Also Duncan's multiple range test was done for each of the traits in order to identify which of the traits contribute to the diversity among each individuals. The analysis was done using SAS (Statistical Analysis System).

3.4.3 Principal Component Analysis

PCA was done to obtain both cluster plot and biplot. Cluster plot was obtained to compare the clusters with that of the clusters in cluster dendrogram analysis. And biplot was obtained in order to determine which of the traits contribute separately for the diversity of the accessions.

3.5 STANDARDATION OF DNA ISOLATION PROTOCOL

Amorphophallus paeoniifolius, like other tuber crops contains a high amount of polyphenol and mucilage. So DNA isolation from elephant foot yam is somewhat difficult. Different methods were used for DNA extraction from the leaves of the plant to compare the quality as well as quantity.

3.5.1 Cota-Sanchez method of isolating genomic DNA

Genomic DNA was extracted following the method by Cota-Sanchez (2006) from 100 mg of frozen leaf tissue ground in a pre-cooled mortar and pestle with 750 µl of extraction buffer (2% CTAB) containing β-mercaptoethanol (3 µl), a pinch of PVPP and PEG. The preparation was subsequently transferred to 2 ml Eppendorf tube and incubated at 65°C for 90 minutes followed by further extraction in an equal volume of chloroform:isolamyl alcohol (24:1). The upper layer formed was transferred in to an Eppendorf tube (1.5 ml) and the process was repeated three times. The recovered extract was then diluted by adding 0.33 volume of ice-cold iso-propanol and stored overnight at -80°C. The overnight stored extract was centrifuged at 12,000 rpm for 10 minutes at 4°C. The supernatant was discarded without disturbing the pellet. The isopropanol washing was repeated till the viscosity of the aqueous phase subsides. The extract was subsequently vacuum dried and the pellet recovered and then resuspended in 100 µl TE buffer containing 1-2 µl of 10 mg/ml RNase, mixed well and incubated at 37°C.The preparation was then mixed with 0.1 volume of 2.5 M Sodium acetate and 500 µl (2-2.5 volume) ice-cold isopropanol and stored at -80°C overnight. Stored extract was centrifuged at 12,000 rpm for 15 minutes at 4°C and the supernatant was discarded without disturbing the pellet. The retained pellet was washed with 70% chilled ethanol for two times and centrifuged at 12,000 rpm for 15 minutes at 4°C.After discarding the supernatant, the pellet was recovered and vacuum dried. Further, pellet was dissolved in 100 µl TE buffer and stored at -20°C till use. Homogeneity of the DNA extract was checked by an agarose (0.8%) horizontal gel electrophoresis using 0.5X Tris acetic acid-EDTA buffer.

3.5.2 DNA isolation by modified Cota-Sanchez method.

Most of the DNA isolated using Cota-Sanchez method was identified as degraded after doing agarose gel electrophoresis. The degradation of the DNA isolated was probably due to the very low temperature provided during the intermediate steps. Thus the temperature was changed as follows.

The steps were done correctly according to Cota-Sanchez protocol for DNA isolation till the dilution of the recovered extract by adding 0.33 volume of ice-cold iso-propanol after adding equal volume of chloroform:isolamyl alcohol. Thus after adding iso-propanol, the tubes should be stored in -20°C instead of -80°C. Also, after mixing the preparation of pellet and TE buffer with 0.1 volume of 2.5 M Sodium acetate and 500 µl (2-2.5 volume) ice-cold isopropanol, it was kept at a temperature of -20°C.

3.5.3 DNA extraction using DNeasy® Plant Mini Kit (M/S Qiagen)

An approximate quantity of 100 mg freshly collected tender leaves directly from the *Amorphophallus* field were powdered using sterile pestle and mortar. 400µl of Buffer AP1 and the solution mixture was transferred into a 1.5ml Eppendorf tube. 4µl RNase A was directly added to the tube to remove any contamination from RNA. Then the tube was vortexed using a vortex machine and was incubated for 10 minutes at 65°C . The tubes were inverted 2-3 times during incubation. Care was taken to make sure that AP1 buffer and RNase A did not mix before use. Then the lysate was centrifuged for 5 minutes at 20,000×g (approx. 14,000 rpm). The lysate was pipetted into a QIAshredder spin column placed in a 2 ml collection tube followed by centrifugation at 20.000×g. The flow-through was transferred into a new tube without disturbing the pellet. And added 1-5 volumes of Buffer AW1, and mixed by gentle and gradual pipetting. 650 µl was transferred into a DNeasy Mini spin column in a 2 ml collection tube. It was centrifuged for 1 min at a speed ≥6000×g (approx. ≥ 8000 rpm). The whole flow through was discarded. The same step was repeated for the remaining samples also. Fresh2ml collection tubes were taken and the spin column was placed into it. 500µl Buffer AW2 was added and again centrifugation was done for 2 minutes at 20,000×g. The spin column from the collection tube was removed carefully so that the column didn't come into contact with the flow-through. The whole spin column was taken and transferred to a new 1.5 ml micro centrifuge tube. Finally, 100µl of buffer AE was added to it for elution. It was incubated for 5 min. at room temperature (25°C) and centrifuged for 1 minute at ≥6000 ×g. This step was repeated.

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The composition of all the buffers (AW1, AW2, AP1, AE and RNase A) and solutions provided in the extraction kit is unknown. All the centrifugation steps were done at room temperature (≈25°C). Any precipitates in Buffer AP1 and Buffer AW1 concentrates could be re-dissolved, if necessary. Prior to use, appropriate amount of ethanol was added as per directions to buffer AW1 and Buffer AW2 concentrates. Water bath was set to 65°C prior to extraction procedure.

3.6 ANALYSIS OF THE EXTRACTED DNA

3.6.1 Agarose gel electrophoresis

The quality and integrity of the extracted DNA was checked using agarose gel electrophoresis using 1% agarose. The gel was prepared by weighing out 1% of agarose in a conical flask and dissolving it using TBE buffer up to the desired level so that 1% agarose solution was obtained. Agarose was dissolved by boiling using a microwave oven. The flask was allowed to cool and when the temperature of the flask became bearable, about 1.5µl of Ethidium Bromide (EtBr) was added directly to the molten gel and gentle mixing was done. The gel was poured to the casting tray with combs and was allowed to solidify.6µl of isolated DNA sample was mixed with 2µl of 6X loading dye. And was loaded into the wells of the prepared gel.

The gel was run in horizontal gel electrophoresis unit for 30 minutes at 80V. The run was stopped after the dye front reached $3/4^{th}$ of the gel length. Then it was visualized under ultraviolet light using a gel documentation system (Alpha Imager, M/S Syngene).

3.6.2 Measurement of DNA quality and quantity using Nano drop spectrophotometer

The Thermo Scientific NanoDrop™ 1000 Spectrophotometer was used to measure the quality and quantity of samples with high accuracy and reproducibility.

3.6.2.1 Protocol for use of NanoDrop® ND-1000

Turned on the software by clicking on the icon. Chosen "User" folder for storage of data. The first panel allowed the selection of the required analysis and the "Nucleic Acid" button was on the top left of the screen. The Module startup panel came up on the screen. For the first step, the pedestals were cleaned and a water sample was loaded in order to initialize the instrument. In order to prepare a report of all readings, the "Recording" button was pressed. The report can log either 12 or 32 measurements. Before doing "DNA, "RNA" or other analysis, the "blank" measurement was chosen. Before making measurements, a blank must be measured and stored. Placed a fresh sample of RNase Free water on the pedestal and pressed "Blank". Then placed a fresh sample on the pedestal and entered "Blank" as the sample name. A straight line appeared on the screen. If this baseline is not flat repeated the "Blank" measurement until it was. Cleaned the pedestals between readings with a Kim wipe. [*The measurement cycle took about 10 seconds*]

Samples was measured and stored. The sample names were recorded in the window and was indicated on the report page. Removed the sample from the pedestals using a Kim wipe. The pedestals were wiped with a wet Kim wipe and then dried between samples. The "Show Report" button displayed all the readings associated with the current report. There were 3 options within this window. Save – saves the report as a .jpg; Print – prints the report to the default printer and Exit – returns to the specific application module. The "Re-blank" option established a new reference which is used for the calculations of all subsequent samples. The "Exit" command closed all the application modules and supporting options. Care was always taken to clean the pedestals after all samples were read and to do a measurement on a fresh replicate of the blanking solution to confirm that the pedestal is clean.

Subsequently, the concentration of the isolated DNA samples ng/µl, Absorbance in 260nm, 280 nm and the ratio of absorbance at 260 nm to 280 nm was also measured and recorded for further calculations.

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3.7 PRIMER SCREENING

The following ISSR primers were selected for primer screening:

UBC 809, UBC 825, UBC 824, UBC 811, UBC 827, UBC 850, UBC 808, UBC 860, UBC 834, UBC 847, UBC 873, UBC 845, UBC 847, UBC 849, UBC 851, UBC 880, UBC 836, UBC 807, UBC 817, UBC 861, UBC 891, UBC 864, $(ACC)_6Y$, $(GA)_9AT$ and $(GA)_9AC$

The genomic DNA of two *Amorphophallus* accessions were taken initially in which one was a wild accession and the other one a cultivar for primer screening. These were AmW26 (Wild) and Am 120 (Cultivar). The composition of the reaction mixture was as follows:

10X Taq buffer (With 15 mM $MgCl_2$)	:	2.0 µl
dNTP mix	:	0.4 µl
$MgCl_2$ (1mM)	:	0.4 µl
Primer(0.25µM)	:	0.5 µl
Taq polymerase (1u)	:	0.2 µl
Template DNA (10 ng/ µl)	:	2.0 µl
Distilled Water	:	14.5 µl
Total Volume	:	20 µl

PCR was carried out in an Eppendorf ep gradient Mastercycler® ep (M/S BioRad) programmed for an initial denaturation at 94°C for 5 minutes followed by 35 cycles with denaturation at 95°C for 1 minute, annealing at 56°C for 2 minutes and extension at 72°C for 3 minutes. 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 percent agarose gel using 100 bp ladder for checking amplification and visualized under the AlphaImager gel documentation system (M/S. Syngene).

3.7.1 Gradient PCR for T_a optimization

The selected primers were taken one by one for screening to determine the annealing temperature for each primers. Each of the primers selected was given a letter for easy labelling. The list of primers selected, its sequence and the respective labelling letters assigned are given in table 4.

Table 4. List of ISSR primers with details screened initially for characterization

Sl. No.	Primer Name	Sequence
1	UBC 809	*AGA GAG AGA GAG AGA GG*
2	UBC 825	*ACA CAC ACA CAC ACA CT*
3	UBC 824	*TCT CTC TCT CTC TCT CG*
4	UBC 811	*GAG AGA GAG AGA GAG AC*
5	UBC 827	*ACA CAC ACA CAC ACA CG*
6	UBC 850	*GTG TGT GTG TGT GTG TYC*
7	UBC 808	*AGAGAG AGA GAG AGA GC*
8	UBC 860	*TGT GTG TGT GTG TGT GRA*
9	UBC 834	*AGAGAG AGA GAG AGA GYT*
10	UBC 847	*CAC ACA CAC ACA CAC ARC*
11	UBC 873	*GAC AGA CAG ACA GAC A*
12	UBC 845	*CTC TCT CTC TCT CTC TRG*
13	UBC 847	*CAC ACA CAC ACA CAC ARC*
14	UBC 849	*GTG TGT GTG TGT GTG TYA*
15	UBC 851	*GTG TGT GTG TGT GTG TYG*
16	UBC 880	*GGA GAG GAG AGG AGA*
17	UBC 836	*AGA GAG AGA GAG AGA GYA*
18	UBC 807	*AGA GAG AGA GAG AGA GT*
19	UBC 817	*CAC ACA CAC ACA CAC AA*
20	UBC 861	*ACC ACC ACC ACC ACC ACC*

21	UBC 891	*HVH TGT GTG TGT GTG TG*
22	UBC 864	*ATG ATG ATG ATG ATG ATG*
23	$(ACC)_6Y$	*ACCACCACCACCACCACCY*
24	$(GA)_9AT$	*GAGAGAGAGAGAGAGAGAT*
25	$(GA)_9AC$	*GAGAGAGAGAGAGAGAGAC*

Gradient PCR was performed for selected primers in which the temperature ranged between 53°C to 64°C. The selected temperatures were 53.1°C, 53.4°C, 55.0°C, 56.2°C, 57.6°C, 59.1°C,60.5°C, 61.7°C, 62.8°C, 63.6°C and 63.9°C. These temperatures were represented by small alphabets from a to l, respectively. And these letters were used for labelling of PCR tubes. The amplicons were further subjected to agarose gel electrophoresis.

3.7 ISSR ANALYSIS OF *AMORPHOPHALLUS* ACCESSIONS

After primer screening, 15 ISSR primers, which gave clear and polymorphic bands were chosen for analysis. PCR was performed using standardized PCR conditions and the annealing temperature which was found to be optimum for each primers. The PCR products were allowed to run in an agarose gel of 2% concentration using a voltage of 100 V for about 2 hours.

3.7.1 Agarose Gel Electrophoresis for ISSR Analysis

Gel electrophoresis was performed with 2% agarose.

3.7.1.2 Casting tray preparation.

The casting tray which can hold a maximum of 120 ml was washed with water thoroughly to remove any remnants of the previous use. And then the tray was allowed to dry and wiped with 70% ethanol solution using a high quality tissue paper. A sticky tape was used to cover both ends of the tray in order to allow it to hold the gel while it is still liquid. Selected suitable combs for use in the tray which

allowed loading of all the 28 accessions as well as ladders of known molecular weights. The combs were placed in the tray properly.

3.7.1.3 Gel preparation

2% of agarose was used to resolve the amplicons obtained after the PCR. For preparing a total of 120 ml gel, 2.4 g of agarose (M/S. Sigma Aldrich) was weighed out using a weighing balance and was directly put into a conical flask. About 120 ml of 1X TBE buffer which was prepared from 10X TBE buffer was taken in a measuring cylinder and was poured into the conical flask. The flask was undisturbed and using a microwave oven, the gel was melted completely. Using a cotton glove, the flask was taken out and allowed to cool until the temperature became bearable with bare hands. Then, approximately 1.2µl EtBr was taken out using a micropipette. Added directly into the gel. The conical flask was rotated gently and gradually in order to distribute EtBr uniformly. After proper mixing, it was poured on to the casting tray and allowed it to solidify for about 30 minutes.

3.7.1.4 Gel loading

Initially the tapes were taken out and the tank was filled with1X TBE buffer up to the $3/4^{th}$mark of the tank so that the tray was completely immersed in the buffer. The PCR products were arranged in a tray according to the sample number for loading. About 2µl 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 and 1 kb DNA ladders (M/S. Thermo-Fisher).

3.7.1.5 Gel running

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.

3.7.1.6 PCR product detection

The gel including the tray was taken out and was viewed under the ultraviolet light in an alpha imager gel documentation system (M/S. Syngene). The image was taken under appropriate exposure and saved in JPEG format for scoring.

3.8 ANALYSIS OF MOLECULAR MARKER DATA

3.8.1 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 analysis and other statistical analysis.

3.8.2 Quantification of genetic variability

As per the requirement of the studies, genetic variability can be overviewed by the estimation of genetic diversity parameters. The resulting data can be used to study marker efficiency and for comparing groups.

3.8.3 Polymorphic Marker Ratio

After scoring of bands using molecular data, bands may be present or absent in the different accessions. If a particular band is present in some accessions and absent in some others, then the band is said to be polymorphic. The percentage of polymorphism was calculated by using the formula - No. of polymorphic bands / Total no. of bands x 100 for a particular primer. Thus the ratio of the polymorphic bands to the total number of bands was determined.

3.8.4 Cluster Analysis

For morphological clustering of data, R statistical package was used and Hierarchical clustering was done based on Euclidean distance. As a result, a dendrogram of 28 accessions was obtained which is based on morphological and molecular traits.

3.8.5 Principal Component Analysis

Using NTSyspc, PCA was done. Both 2D and 3D plots based on molecular scoring data was obtained.

3.8.6 SM matrix

In order to determine the variation between two accessions, Simple matching matrix was drawn using NTSyspc software . The similarity between two accessions was represented as a number less than 1.0.

RESULTS

4. RESULTS

The results of the study "Genetic Diversity Analysis of Elephant Foot Yam (*Amorphophallus paeoniifolius* [Dennst. Nicholson])" carried out at the Division of Crop Improvement, ICAR-Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram during 2015-2016 are presented in this chapter.

4.1 MORPHOLOGICAL DATA ANALYSIS

Twenty-eight elephant foot yam accessions from different parts of India were used for the study. A wide range of variability was observed among the accessions for some of the characters studied. The plants at the full growth stage is shown in plate 1 and the major variations shown by *Amorphophallus* accessions (both plant and tuber) is shown in plates 2 - 5.

4.1.2 Principal Component Analysis

After the cluster plot of PCA, component 1 was responsible for 25.34% of the total variation. Component 2 was responsible for 16.04% of the total variation and component 3 was responsible for 10.3% of the variation. Thus the maximum variation was explained by the 1st component. Total three clusters were formed in which, cluster I contained only the accession AmH6. Am10 was the only accession in cluster III and all the other accessions were clustered together in Cluster II.

In biplot of PCA, weight of corm was the major contributing trait which separated out Am130, Am120 , AM14 and Am29 from other accessions. Am135, Am85 and Sree Padma was separated.

Plate 1: Elephant foot yam field with plants at full growth stage.

Plate 2: Comparison of variations in pseudo stem colour and texture

Plate 3: Comparison of variation in pseudo stem surface (smooth and rough)

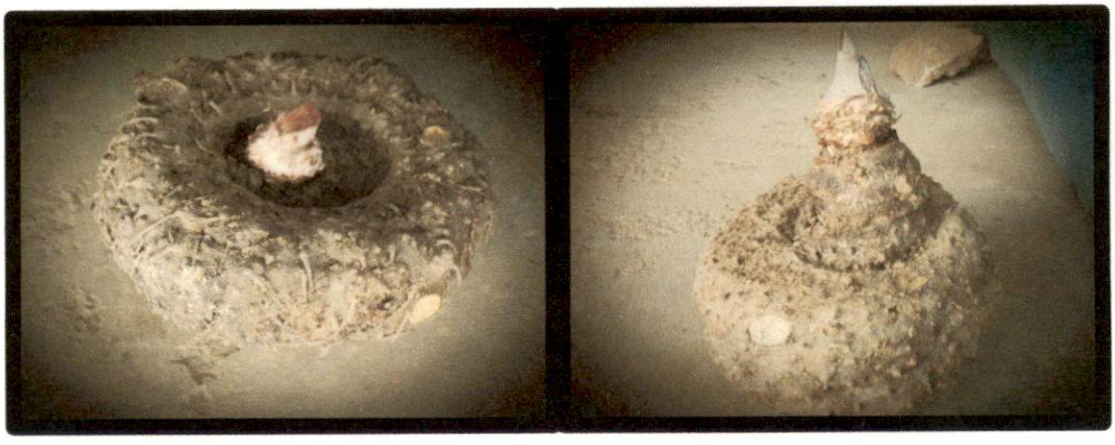

Plate 4: Comparison of variation in tuber shape

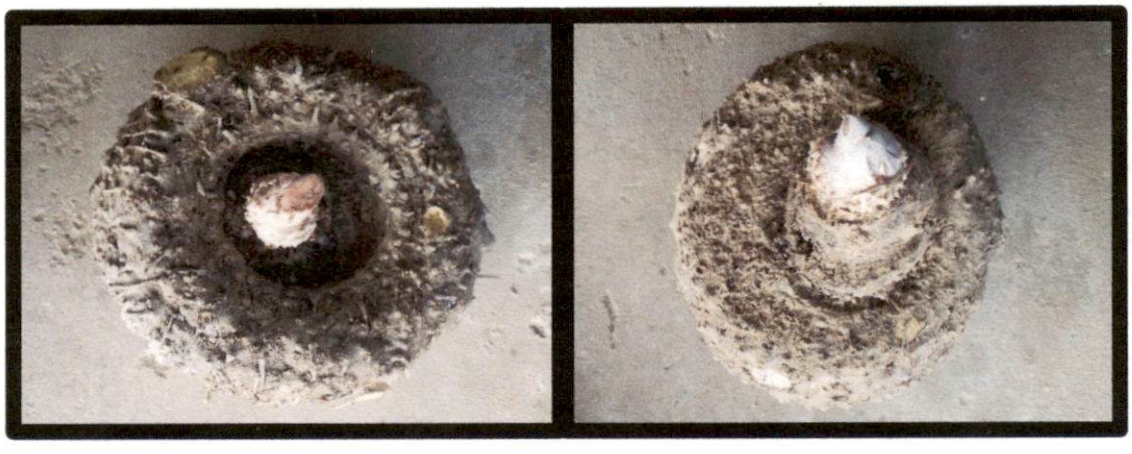

Plate 5: Comparison of corm colour (blackish brown and brown)

Table 5 : Percentage distribution for morphological traits

Sl No.	Character	Trait	Percentage (%)
1	Pseudo stem height (cm):	Dwarf=1	14.3
		Medium=2	75
		Large=3	10.7
2	Number of primary partitions	Low=1	3
		Average=2	97
		High=3	0
3	Number of secondary partitions / primary partition:	Low=1	0
		Average=2	100
		High=3	0
4	Length of primary partition	Short=1	17.8
		Medium=2	60.8
		Long=3	21.4
5	Breadth of primary partition	Thin=1	14.2
		Medium=2	71.4
		Thick=3	14.2
6	Number of leaflets per primary partition	Low=1	10.7
		Average =2	78.5
		High=3	10.7
7	Length of largest leaflet	Short=1	17.8
		Medium=2	64.2
		Long=3	14.2
8	Breadth of largest leaflet	Thin=1	14.2
		Medium=2	67.8

Table 5 Contd…

		Thick=3	17.8
9	Leaflet nature	Entire=1	100
		Not entire=0	0
10	Leaflet margin	Not undulate=0	0
		Undulate=1	100
11	Surface of pseudo stem	Smooth=2	10.7
		Tubercled=4	85.7
		Spiny=1	3
12	Thickness of pseudo stem base	Thin=1	21.4
		Medium=2	60.7
		Thick=3	17.8
13	Number of tertiary partitions / secondary partition:	Low=1	10.7
		Average=2	82.1
		High=3	7
14	Pseudo stem colour	Green mottled=1	92.8
		Dark green mottled=2	7.2
15	Leaf spot	Present=0	0
		Absent=1	100
16	Stalk leaflet	Absent=0	100
		Present=1	0
17	Nature of rachis	Not winged=0	7.2
		Winged=1	92.8
18	Shape of rachis	Angled and curved=2	96.4
		Cylindrical=1	3.6
19	Fresh weight of corm	Light=1	14.2
		Medium=2	64.2
		Heavy=3	21.4

Table 5 Contd...

20	Corm shape	Depressed and Globose=1	85.7
		Flat and Globose=2	7
		Pyramidal and Globose=3	7
21	Colour of corm surface(upper)	Blackish brown=2	21.4
		Dark brown=1	78.5
22	Colour of corm surface(lower)	Blackish brown=2	17.8
		Dark brown=1	82.2
23	Colour of flesh	Light yellow=2	7
		Dark yellow=1	71.4
		Yellow=3	17.8
		Reddish yellow=4	3
24	Height of corm	Tall=3	14.2
		Medium=2	75
		Dwarf=1	10.7
25	Diameter of corm	Big =3	21.4
		Medium=2	64.2
		Short=1	14.2
26	Number of cormels / corm	Low=1	7
		Average=2	82.1
		High=3	10.7
27	Weight of cormel	Light =1	14.2
		Medium=2	75
		Heavy=3	10.7
28	Length of cormel	Long=3	7
		Medium=2	78.5
		Short=1	14.2
29	Thickness of cormel	Thick=3	21.4
		Medium=2	53.5

60

Table 5 Contd…

		Thin=1	25
30	Pre-emergence	Absent=3	14.9
		Dried=2	3.5
		Present=1	82.1
31	Biostress	Scale=1	7.14
		Tuber rot=2	3.5
		Scale and Insect=3	7.14
		Mealy Bug=4	46.4
		Tuber rot and Mealy bug=5	10.7
		Mealy bug and Scale=5	14.2
		Tubers with holes dried up=7	3.5
		None	7.1

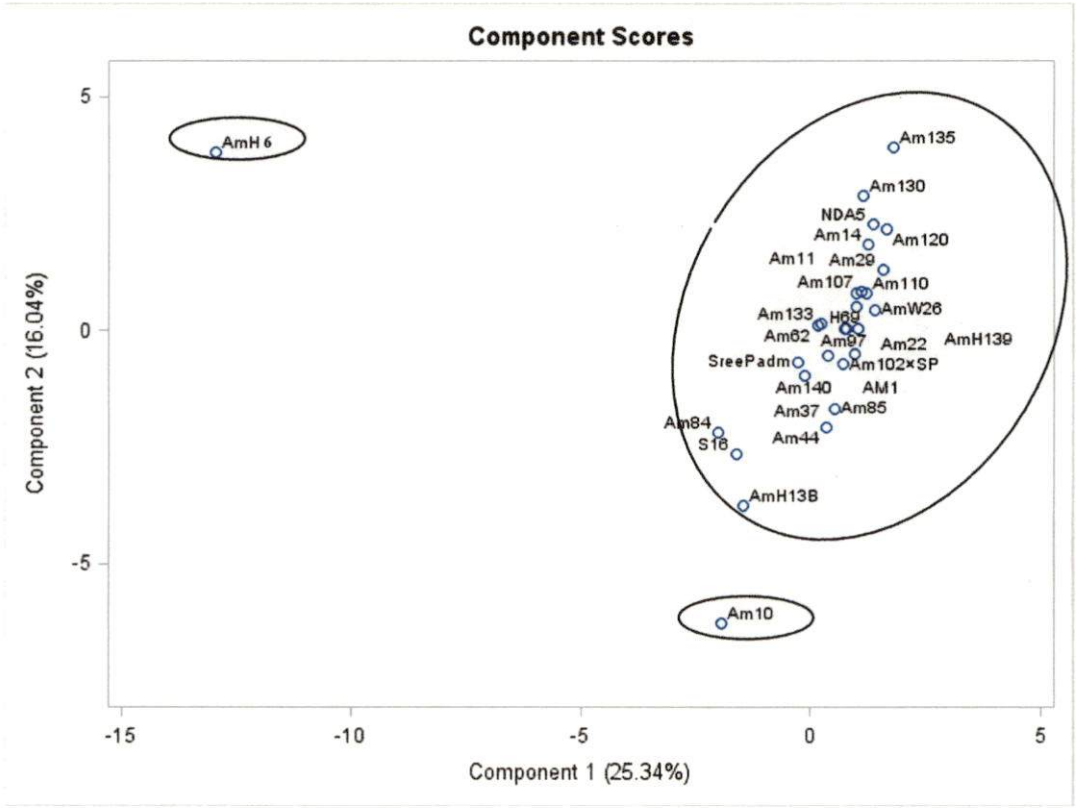

Figure 1. Cluster plot representation of 28 *A. paeoniifolius* accessions based on PCA

Table 6: Principal component analysis in 28 accessions of *A. paeoniifolius*

Character	PC1	PC2	PC3	PC4
Pseudostem height	-.311567	0.097446	0.061429	0.087036
No. pri. partition	0.229173	-.042050	-.288845	-.149393
No. sec. partition	-.334339	0.154838	-.042192	0.010365
No. ter. partition	0.110706	-.133921	-.016244	0.092897
Pri. partition (length)	0.066648	0.253311	0.291326	0.074332
Pri. partition (breadth)	0.062963	0.107178	-.279714	0.330836
No. leaflet (pri. partn.)	0.046340	0.160523	0.221582	-.340801
Largest leaflet (length)	0.205236	0.281087	-.152548	-.042694
Largest leaflet (breadth)	0.088478	0.347610	-.060470	-.180884
Pseudostem base thickness	0.005803	0.391909	-.082926	-.118360
Fresh weight of corm	0.088350	0.258139	0.100312	0.253906
Height of corm	-.030296	-.079491	0.390172	-.085191
Diameter of corm	0.086821	0.270003	-.004584	0.043123
Number of cormels	0.066739	0.288939	-.019304	0.029609
Number of corms	0.056715	0.210914	0.091208	0.277175
Weight of cormels	-.334339	0.154838	-.042192	0.010365
Length of cormels	0.000000	0.000000	0.000000	0.000000
Thickness of cormel	0.189183	-.108935	0.067500	0.016477
Leaflet nature	-.334339	0.154838	-.042192	0.010365
Leaflet margin	-.334339	0.154838	-.042192	0.010365
Pseudostem surface	0.000000	0.000000	0.000000	0.000000
Pseudostem colour	0.254740	0.051944	-.199974	-.237029
Leaf spot	0.271130	-.034488	0.107958	0.230843

Table 6 Contd…

Character	PC1	PC2	PC3	PC4
Leaf let stalk	-.173692	-.202576	-.234930	-.190671
Rachis nature	-.136287	-.108054	0.162262	-.106117
Corm shape	0.003129	-.186776	0.098956	-.003591
Corm colour	0.031639	0.004636	0.195481	0.068732
Colour of flesh	0.162431	-.076738	-.154769	0.146758
Biotic stress	-.008905	-.066793	0.344399	0.319858
Eigen values	7.603			
Percent variation	0.25			
Cumulative percentage	0.25			

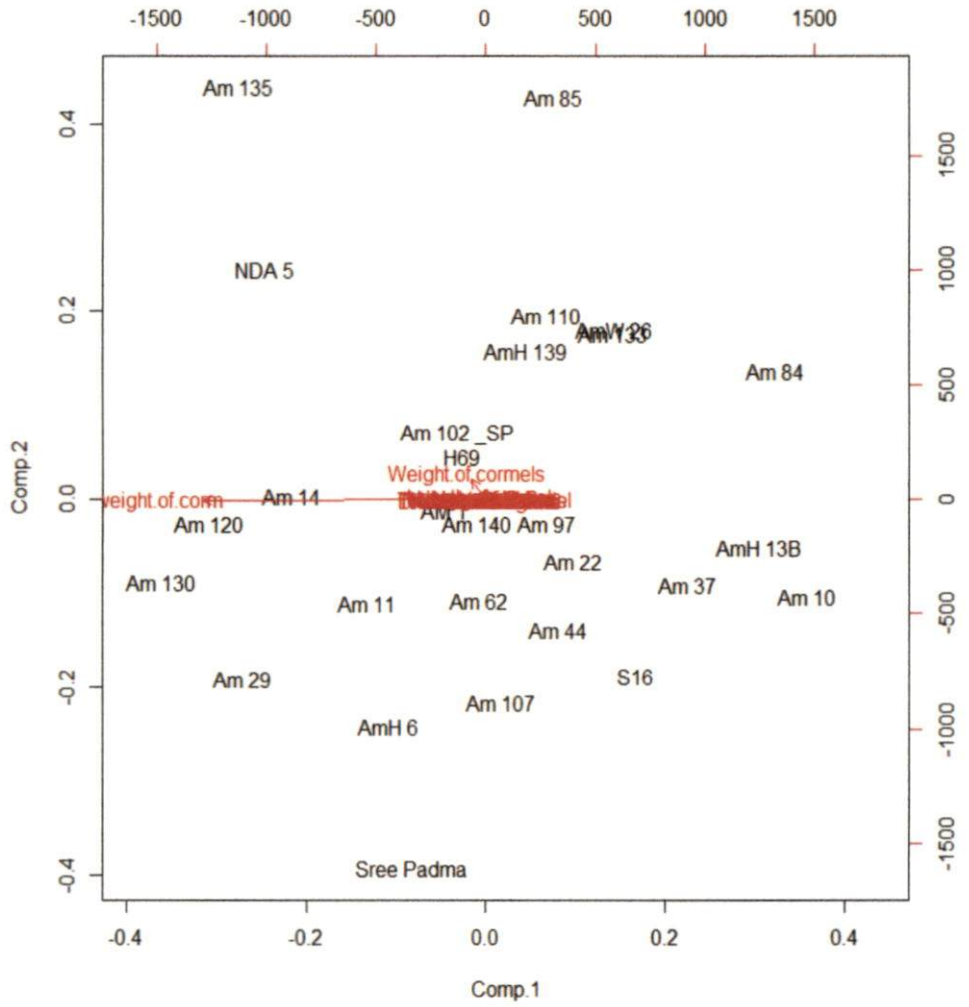

Figure 2 : Biplot showing the spatial distribution of various characters and accessions

4.1.3 Analysis of variance

There were substantial variations in certain characters when analysis of variation was done for eighteen morphological traits (Table 8). Based on the coefficient of variation, the degree of variability was found to be high for fresh weight of corm, height of corm, length of cormels, number of leaflets (pri. part.), number of corms, number of tertitiary partitions, weight of cormels and number of cormels. Duncan's multiple range test was also done for the traits and the results obtained was listed out in table 9.

4.1.4 Cluster analysis

Cluster analysis using Hierarchical clustering with the help of R program was drawn. And it revealed two major clusters. Of which, cluster I was further subdivided into Ia and Ib. Ia had three accessions on it and Ib had four accessions on it. Cluster II was found to have four subdivisions in it- IIa(6 accessions), IIb(3 accessions), IIc(6 accessions) and IId (6 accessions).

While comparing the results obtained in Dendrogram and cluster plot of PCA, Am 10 in Dendrogram was sub cluster Ia along with Am84 and AmH13B. But AmH6 was found along with Am 11 in case of Dendrogram but the same was separated out in a cluster (Cluster I) in case of PCA cluster plot. The comparison of clusters obtained after cluster analysis and Dendrogram is shown in Table 2. The Dendrogram obtained is shown in Figure 3.

Table 7: Comparison of cluster data of cluster analysis and cluster plot of PCA.

	Dendrogram			PCA		
Cluster		**Accessions**	**No. of genotypes**	**Cluster**	**Accessions**	**No. of genotypes**
Cluster I	**Ia**	Am 10, Am64, AmH13B	3	**Cluster I**	AmH 6	1
	Ib	Am 37, S16, AmW26, Am 133	4	**Cluster II**	Am 135, Am 130, NDA 5, Am 14, Am 120, Am 11, Am 29, Am 107, Am 110, Am 133, H 69, AmW26, Am 62, Am 97, Am 22, AmH139, Sree Padma, Am 102× SP, AM140, Am 1, AM 37, Am 85, S16, Am 84, Am 44, AmH 13B	26
Cluster II	**IIa**	Am 130 Am 14, NDA 5, Am 135, Am 120, Am 133	6			
	IIb	SP, AmH6, Am 11	3			
	IIc	Am 22, Am 44, Am 85, AmH 139, Am 97, Am 110	6			
	IId	Am 102×SP, Am 1, H69, Am 107, Am 62, Am 140	6	**Cluster III**	Am 10	1

Table 8. Variation in the quantitative characters for the 28 *A. peaoniifolius* accessions

Characters	Mean	Coefficient of variation	F value
Diameter of corm	38.62	19.72	**3.25****
Fresh weight of corm	695.04	54.23	**2.03***
Height of corm	8.17	22.21	1.30
Largest leaflet breadth	5.65	16.56	1.49
Length of cormels	4.92	31.28	**1.99***
Number of leaflets (pri part)	3.52	36.27	1.14
Number of pri partition	2.99	3.65	1.00
Number of corms	1.08	22.85	**1.86***
Number of sec. partitions	2.00	0.00	0.00
Number of tert. partitions	1.69	28.37	**2.17****
Primary partition (length)	9.25	18.25	**1.99***
Primary partition (breadth)	6.02	18.10	**2.19****
Psuedostem base thickness	11.82	18.79	**2.20****
Pseudostem height	32.25	18.46	**2.30****
Thickness of cormel	9.06	17.56	**2.21****
Weight of cormels	40.65	87.01	0.09
Number of cormels	3.37	61.74	**1.90***
Largest leaflet length	13.30	17.63	**1.80***

**Significant at 1% *Significant at 5%

Table 9 : Duncan's multiple range test for significant quantitative characters

Accession ID	Largest leaflet (length)	Primary partition (breadth)	Length of cormels	Pseudostem height
AmW26	15.63^{AB}	4.33^{I}	5.93^{BC}	29.50^{CDE}
Am120	13.50^{ABCDE}	6.89^{ABCDE}	5.43^{BCDEF}	34.50^{BCDE}
Am84	12.90^{ABCDE}	5.27^{EFGHI}	4.47^{CDEFG}	37.67^{BC}
Am22	13.47^{ABCDE}	$5.64^{CDEFGHI}$	4.93^{CDEFG}	31.50^{BCDE}
Am62	12.57^{ABCDE}	$6.14^{BCDEFGH}$	4.60^{CDEFG}	36.00^{BC}
Am44	13.10^{ABCDE}	5.31^{DEFGHI}	2.93^{FG}	25.50^{E}
Am85	13.67^{ABCDE}	5.16^{EFGHI}	4.60^{CDEFG}	31.93^{BCDE}
Am1	12.63^{ABCDE}	$6.12^{BCDEFGH}$	5.50^{BCDE}	32.67^{BCDE}
Am140	8.67^{F}	4.96^{GHI}	5.63^{BCDE}	29.67^{CDE}
Am10	15.40^{AB}	4.91^{GHI}	3.23^{EFG}	31.17^{CDE}
AmH139	14.40^{ABCD}	$6.32^{ABCDEFG}$	5.83^{BCD}	32.17^{BCDE}
AmH6	15.13^{ABC}	4.49^{HI}	4.80^{CDEFG}	25.50E
Am11	12.23^{ABCDEF}	7.87^{AB}	3.80^{CDEFG}	33.33^{BCDE}
Am110	15.50^{AB}	$5.80^{CDEFGHI}$	8.47^{A}	29.83^{CDE}
Sree Padma	11.00^{DEF}	$6.50^{ABCDEFG}$	2.83^{G}	50.83^{A}
Am107	12.50^{ABCDEF}	7.18^{ABC}	4.13^{CDEFG}	31.67^{BCDE}
AmH13B	14.63^{ABCD}	$6.24^{ABCDEFGH}$	3.33^{DEFG}	41.13^{AB}
Am102×SP	11.30^{CDEF}	$5.94^{CDEFGHI}$	5.77^{BCD}	25.43^{E}
Am37	10.43^{EF}	8.02^{A}	4.27^{CDEFG}	35.67^{BC}
NDA5	10.87^{DEF}	$6.57^{ABCDEFG}$	4.63^{CDEFG}	29.50^{CDE}
Am135	15.97^{A}	7.08^{ABCD}	5.80^{BCD}	29.33^{CDE}
Am14	13.73^{ABCDE}	$6.03^{CDEFGHI}$	4.60^{CDEFG}	32.80^{BCDE}
S16	11.87^{BCDEF}	$5.70^{CDEFGHI}$	4.20^{CDEFG}	30.83^{CDE}
Am29	15.40^{AB}	$6.34^{ABCDEFG}$	5.40^{BCDEF}	33.33^{BCDE}
Am130	15.00^{ABC}	6.83^{ABCDEF}	7.67^{AB}	28.17^{CDE}

Table 9 Contd…

H69	13.43^{ABCDE}	6.42^{ABCDEFG}	4.63^{CDEFG}	32.03^{BCDE}
Am133	14.97^{ABC}	5.10^{FGHI}	5.50^{BCDE}	25.83^{DE}
Am97	12.57^{ABCDE}	6.32^{ABCDEFG}	4.77^{CDEFG}	35.50^{BCD}

Table 10: Duncan's multiple range test for tuber characteristics

Accession ID	Height of corm	Fresh weight of corm	Thickness of cormel	Diameter of corm
AmW26	8.37	462.00^{DEFG}	8.03^{CDEFG}	34.23^{DEFG}
Am120	9.10	1189.33^{AB}	9.30^{BCDEFG}	48.17^{ABC}
Am84	5.63	173.33^{FG}	7.53^{EFG}	25.97^{GH}
Am22	7.70	537.33^{CDEFG}	11.13^{AB}	39.43^{BCDEF}
Am62	7.60	707.67^{ABCDEFG}	11.13^{AB}	40.00^{ABCDEF}
Am44	7.97	565.00^{CDEFG}	7.93^{CDEFG}	35.77^{CDEFG}
Am85	6.63	572.00^{CDEFG}	7.73^{DEFG}	29.03^{FGH}
Am1	7.80	768.00^{ABCDEF}	9.57^{BCDEFG}	42.13^{ABCDE}
Am140	8.63	709.00^{ABCDEFG}	9.17^{BCDEFG}	42.93^{ABCDE}
Am10	5.70	119.67^{G}	7.13^{FG}	20.20^{H}
AmH139	7.93	623.33^{BCDEFG}	9.97^{ABCDE}	37.97^{BCDEFG}
AmH6	10.13	871.33^{ABCDE}	9.07^{BCDEFG}	42.50^{ABCDE}
Am11	9.17	909.67^{ABCDE}	7.10^{G}	34.90^{DEFG}
Am110	7.40	585.00^{BCDEFG}	8.60^{BCDEFG}	39.43^{BCDEF}
Sree Padma	8.20	829.67^{ABCDE}	7.63^{DEFG}	40.93^{ABCDEF}
Am107	7.87	667.00^{ABCDEFG}	7.93^{CDEFG}	42.97^{ABCDE}
AmH13B	5.97	204.33^{FG}	9.03^{BCDEFG}	25.50^{GH}
Am102×SP	8.13	744.33^{ABCDEF}	12.40^{A}	43.20^{ABCDE}
Am37	8.23	333.00^{EFG}	7.67^{DEFG}	31.00^{EFGH}
NDA5	10.13	1090.67^{ABC}	9.47^{BCDEFG}	46.60^{ABCD}
Am135	9.60	1135.33^{ABC}	10.50^{ABC}	48.23^{ABC}

Table 10 Contd…

Am14	8.90	1043.00^{ABCD}	10.07^{ABCDE}	47.83^{ABC}
S16	9.00	426.33^{DEFG}	7.33^{FG}	31.03^{EFGH}
Am29	8.53	1132.00^{ABC}	10.50^{ABC}	49.17^{AB}
Am130	10.00	1276.33^{A}	9.73^{BCDEF}	51.97^{A}
H69	7.47	736.00^{ABCDEFG}	10.20^{ABCD}	41.23^{ABCDEF}
Am133	8.20	465.33^{DEFG}	9.10^{BCDEFG}	31.37^{EFGH}
Am97	8.70	585.00^{BCDEFG}	8.80^{BCDEFG}	37.53^{BCDEFG}
	NS			

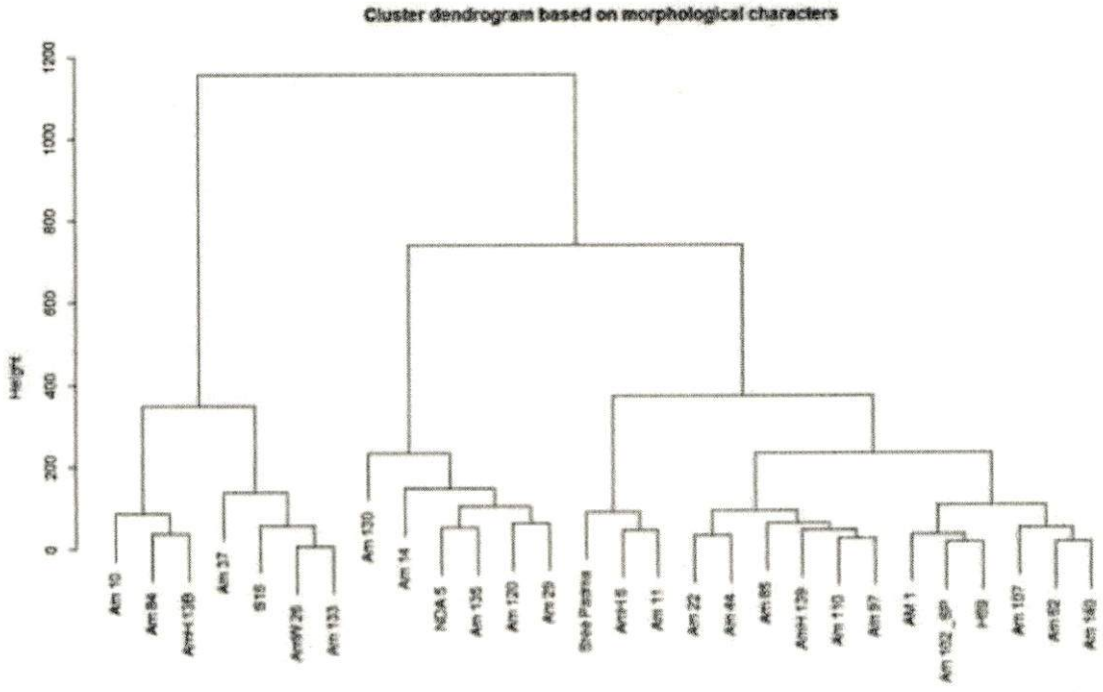

Figure 3: Cluster dendrogram of 28 accessions based on Hierrarchial clustering(R package)

4.2 STANDARDISATION OF DNA ISOLATION PROTOCOL

Kit based and manual methods for DNA isolation were carried out for the isolation of twenty-eight elephant foot yam accessions. Plate 7,8 shows the DNA profile on agarose gel using kit method and Plates 9 and 10 shows the DNA profile on agarose gel while using Cota- Sanchez and modified Cota- Sanchez method.. DNA isolated using DNeasy plant mini kit (Qiagen) was found to be intact and gave good spectrophotometric readings. Table 6 shows the spectrophotometric readings of DNA isolated using the kit method. Using the kit method, a very good quality of DNA with A_{260}/A_{280} ratio being between 1.96- 2.9 which depicts high quality DNA was obtained. The Cota-Sanchez method for isolation of DNA gave no bands for many of the accessions after resolving it in 1% agarose gel. The slight modification in the Cota-Sanchez method (changing overnight temperature from -80°C to -20°C) made an impact in the concentration of DNA. Concentration was increased several fold as compared to that of kit method of isolation of DNA. But the problem remained was that the quality of DNA measured using the nanodrop spectrophotometer was abnormal. Most of the results were showing that the A_{260}/A_{280} ratio was abnormal which shows the contamination of DNA by RNA and Protien (Table12).

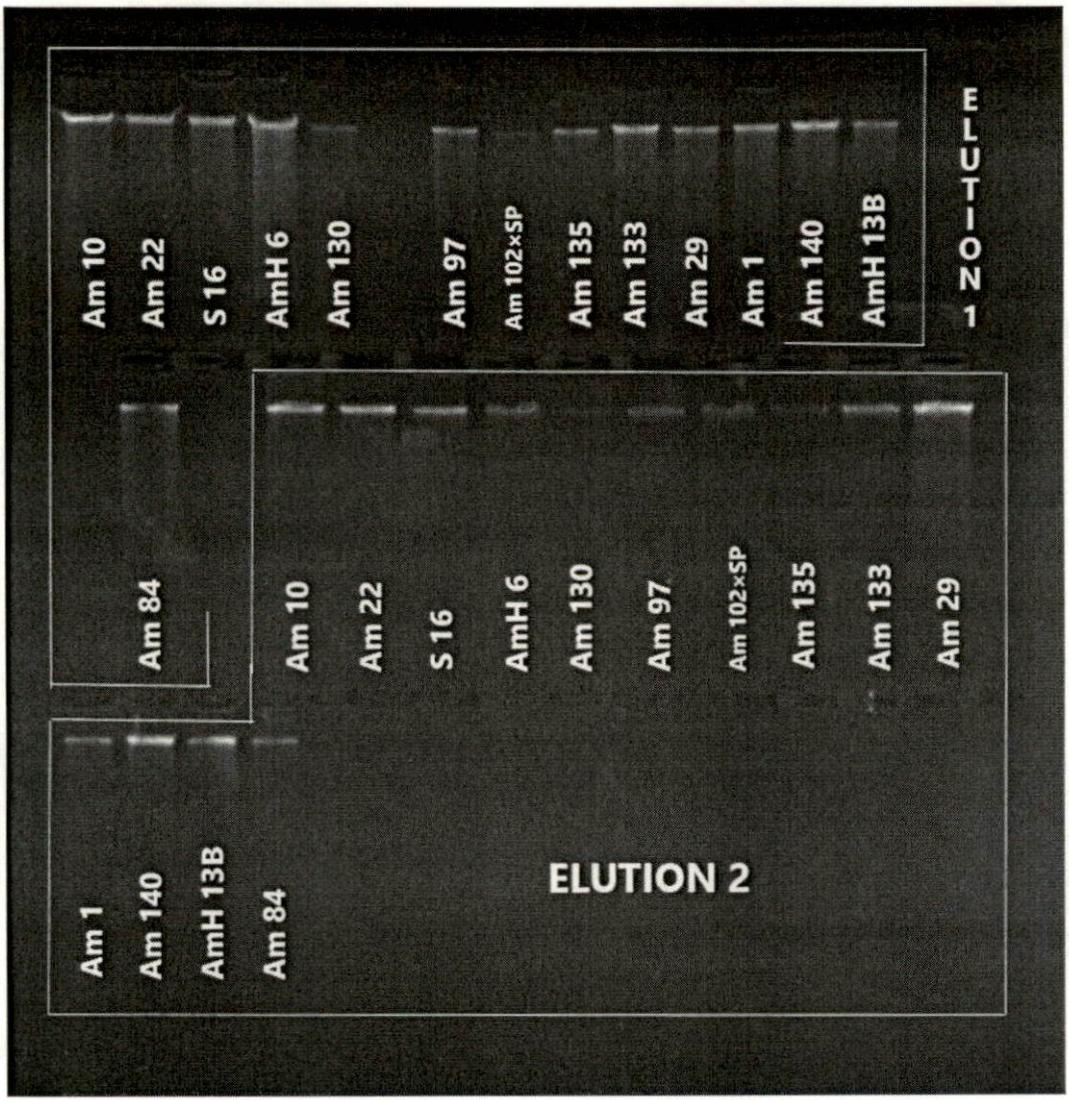

Plate 7: 1% gel profiles DNA isolated using kit method (both elutes) for 14 accessions

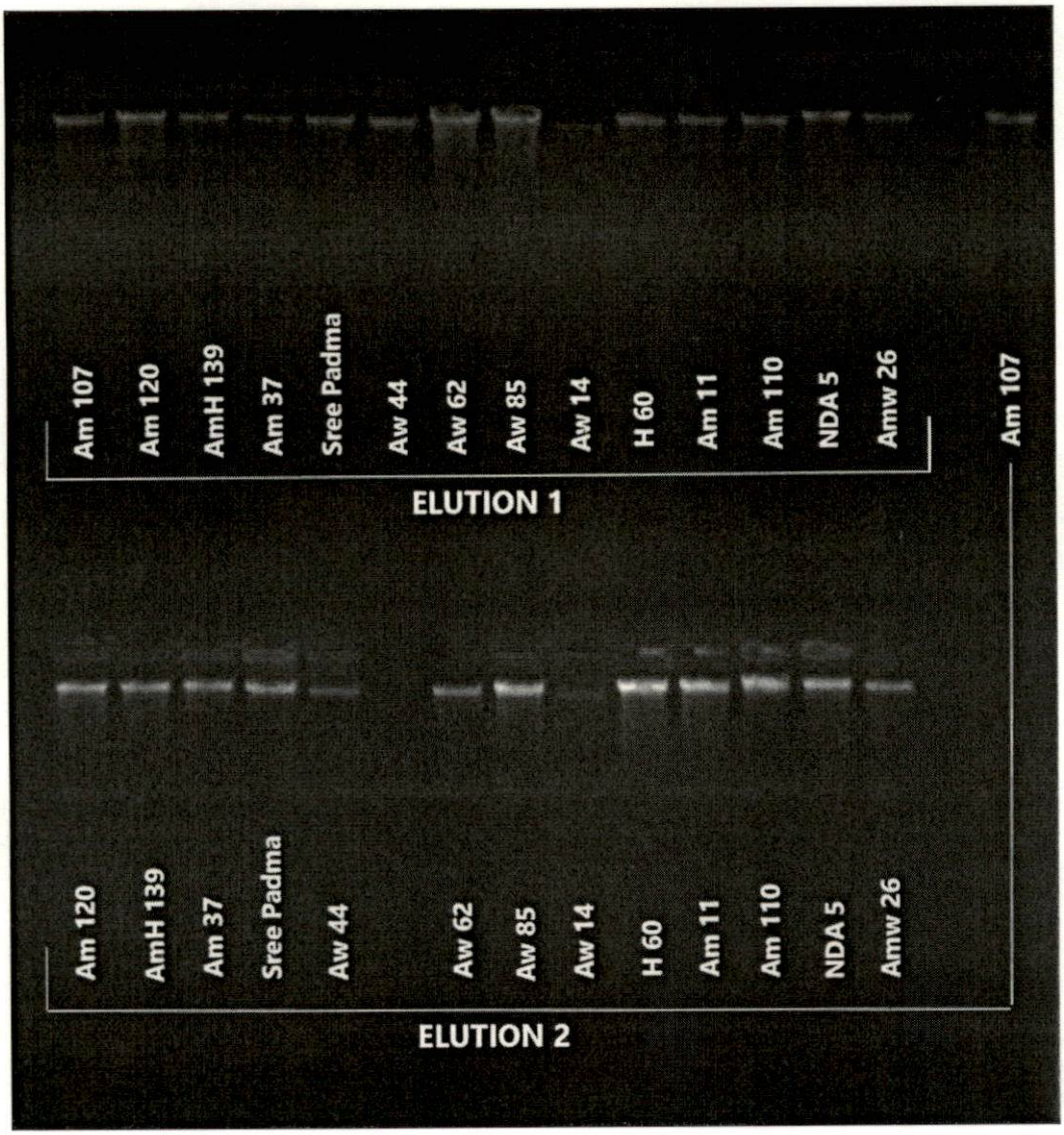

Plate 8: 1% gel profiles DNA isolated using kit method (both elutes) for 14 accessions

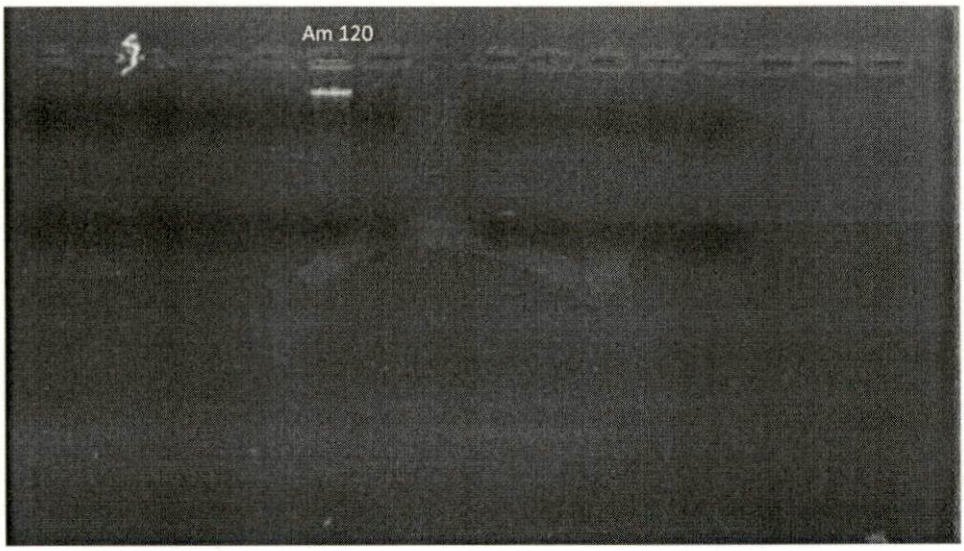

Plate 9: DNA isolation using Cota Sanchez method (2006) of isolation

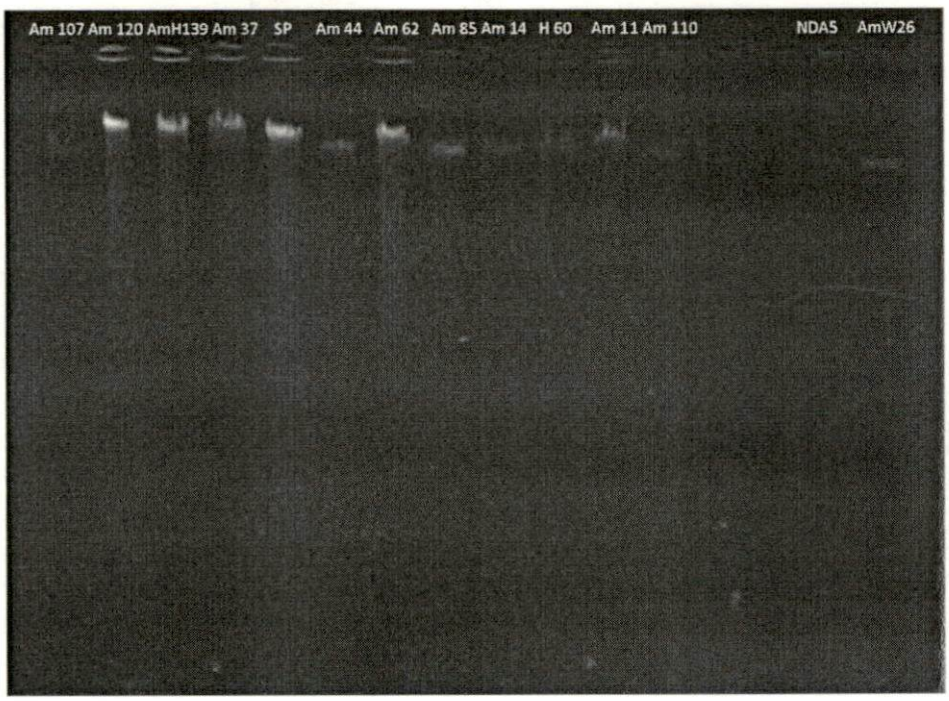

Plate 10: DNA isolation using modified Cota-Sanchez method (shearing of DNA seen)

Table 11: Spectrophotometric readings of DNA isolated using kit method.

Sample no.	Sample name	Concentration (ng/µl)	Factor	A_{260}	Path L (mm)	A_{260}/A_{280}	A_{260}/A_{230}
1	Am22	228.8	50	4.576	10	1.98	1.8
2	Am85	68.5	50	1.37	10	2.04	2.05
3	Am44	35.86	50	0.717	10	2.05	2.46
4	Am62	114.37	50	2.287	10	1.99	1.98
5	S16	287.91	50	5.758	10	1.99	1.7
6	Am14	14.63	50	0.293	10	1.9	1.79
7	AmH6	156.52	50	3.13	10	2.01	2.15
8	Am10	49.67	50	0.993	10	1.95	2.1
9	Am140	106.95	50	2.139	10	2.03	1.97
10	Am1	27.85	50	0.557	10	2.1	2.23
11	AmH139	166.1	50	3.322	10	1.99	1.91
12	Am120	394.69	50	7.894	10	1.98	1.99
13	Am110	80.47	50	1.609	10	2.06	1.74
14	Am11	32.17	50	0.643	10	1.96	2.11
15	Am84	29.14	50	0.583	10	2.15	1.95
16	Am29	32.04	50	0.641	10	2.27	2.37
17	Am135	150.67	50	3.013	10	1.97	1.29
18	NDA5	85.43	50	1.709	10	2.04	1.91
19	Sree Padma	235.32	50	4.706	10	2	1.93
20	Am107	71.27	50	1.425	10	2.09	1.98
21	Am37	39.51	50	0.79	10	2.15	1.6
22	Am102xSP	220.88	50	4.418	10	1.97	1.68
23	AmH13B	80.5	50	1.61	10	2.02	1.67
24	Am97	12.8	50	0.256	10	3	2.84
25	Am130	53.74	50	1.075	10	2.06	1.21
26	H69	240.89	50	4.818	10	2	2.07
27	AmW26	215.13	50	4.303	10	1.96	1.84
28	Am133	123.71	50	2.474	10	2	1.48

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Table 12: Spectrophotometric readings of 10 DNA samples isolated using Cota-Sanchez method (2006).

Sample Number	Sample Name	Concentration (ng/uL)	Factor	A260	Pathlength (mm)	260/280	260/230
	Blank						
1	Am 37	17.1	50	0.342	10	0.87	-0.06
2	Am 120	392.86	50	7.857	10	1.89	-14.51
3	Am 130	0.18	50	0.004	10	0.04	0
6	Am 97	-8.93	50	-0.179	10	4.16	0.03
7	Am 14	-7.86	50	-0.157	10	20.97	0.02
8	Am 85	0.33	50	0.007	10	0.11	0
9	AmW 26	2.16	50	2.463	10	1.55	-0.86
10	Am 11	0.65	50	2.456	10	1.89	-0.56

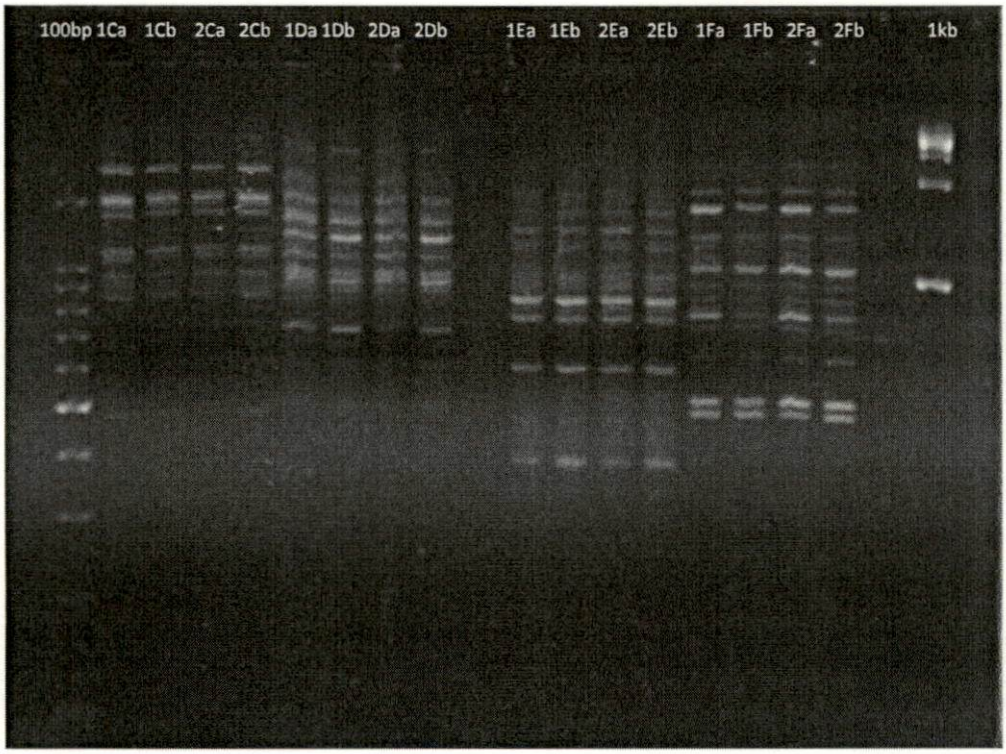
4.3 Primer screening and determination of annealing temperature (T_a)

A total of 25 ISSR primers were taken for determining its amplifying capability and the annealing temperature if amplification is possible. DNA samples of two accessions (AmW26 and Am120) were taken for screening purpose. Plates 11 to 15 show the gel profile of screening of 18 of the total 25 primers at different temperatures. A total of 10 primers were eliminated from further analysis as they gave unclear or no bands. The primers which were eliminated were: UBC 824, UBC 873, UBC 845, UBC 847, UBC 849, UBC 851, UBC 880, UBC 836, UBC 861, UBC 891 and $(GA)_9AT$.

The fifteen primers selected for ISSR analysis, its annealing temperature along with representative letters are given in the table 13.

4.4 ISSR analysis of elephant foot yam accessions

After doing PCR using the selected primers and their respective annealing temperature for amplification, the amplicons were resolved in 2% agarose gel and the bands obtained for primers are shown in Plate16-21.



Primers:

C – (ACC)$_6$Y D – (GA)$_9$AC E – (GA)$_9$AT F – UBC 811

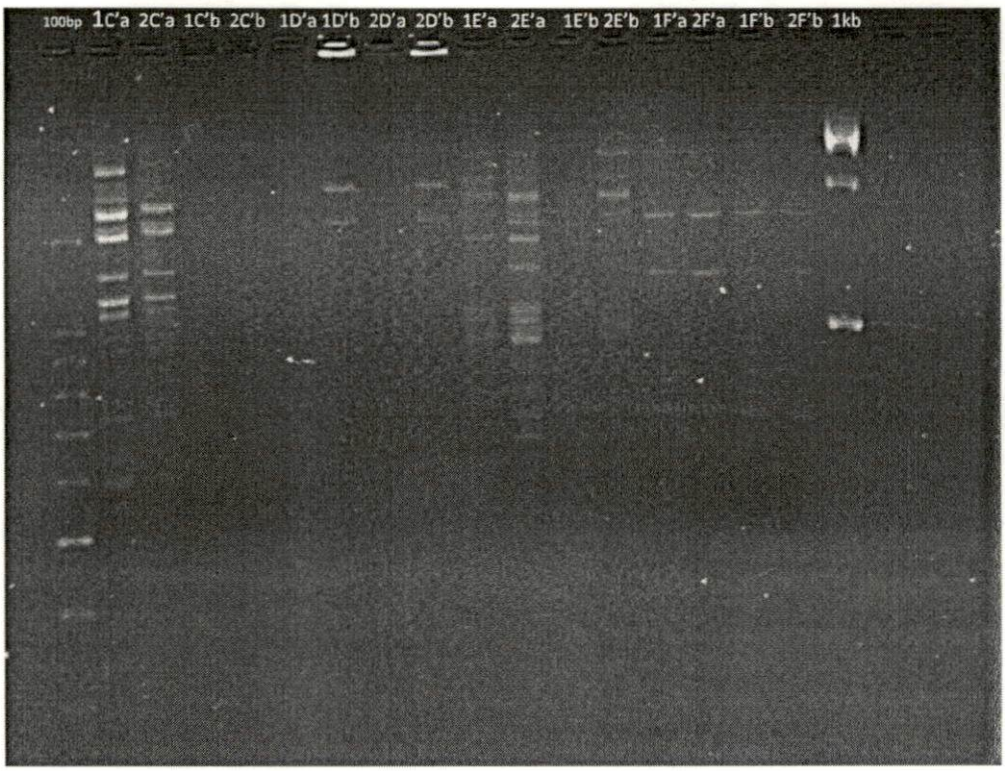
Temperature:

a – 55° C b - 56° C

DNA:

1- AmW26 2- Am 120

Plate 11: 2% agarose gel profiles of four different primers for two accessions at 55° C and 56° C



Primers:

C' – UBC 834 D' – UBC 850 E' – UBC 860 F' – UBC 808

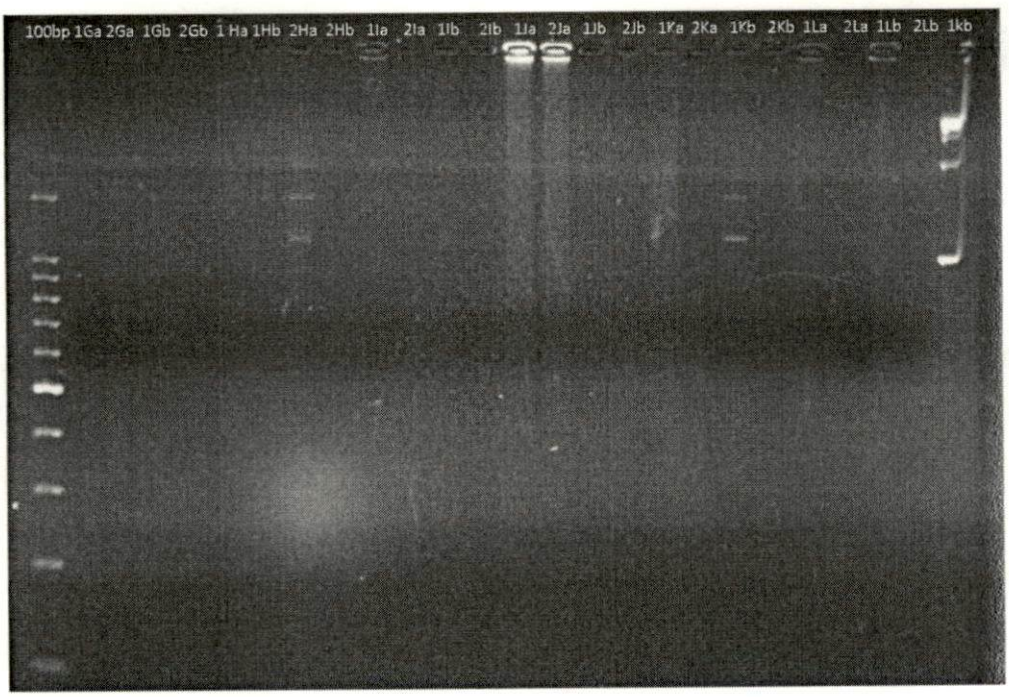
Temperature:

a – 55° C b - 56° C

DNA:

1- AmW26 2- Am 120

Plate 12: 2% agarose gel profiles of four different primers for two accessions at 55° C and 56° C



Primers:

G- UBC 845 H– UBC 847 I- UBC 849 J- UBC 851 K-UBC 857

L- UBC 880

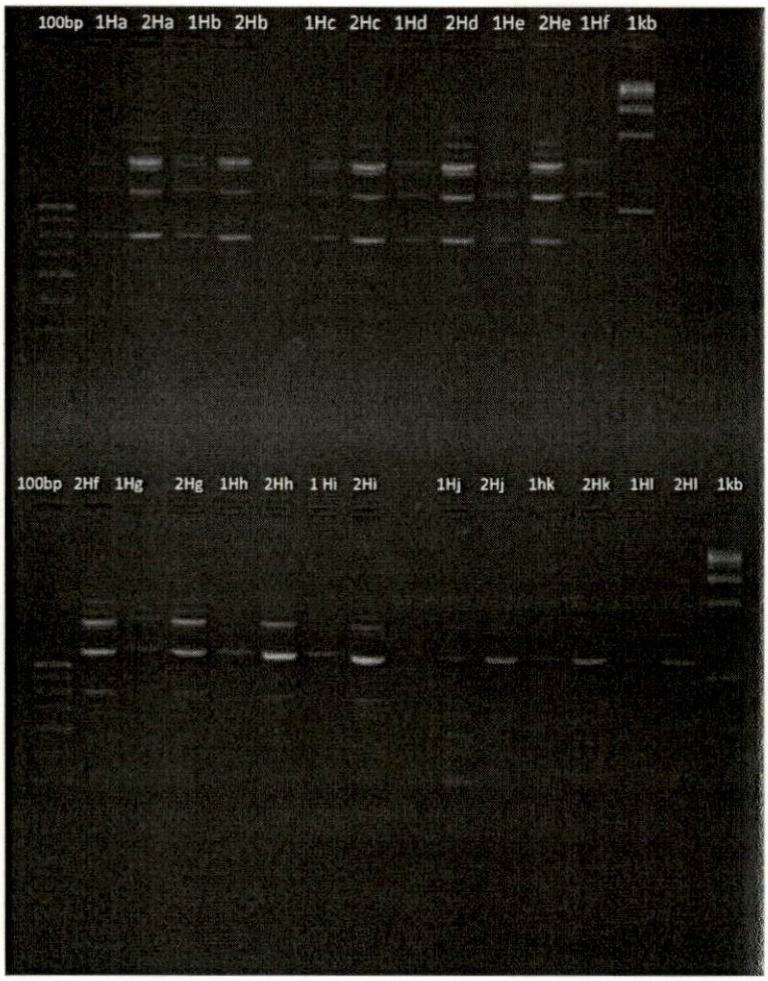
Temperature:

a – 55° C b - 56° C

DNA:

1- AmW26 2- Am 120

Plate 13: 2% agarose gel profiles of six different primers for two accessions at 55° C and 56° C



Primer:

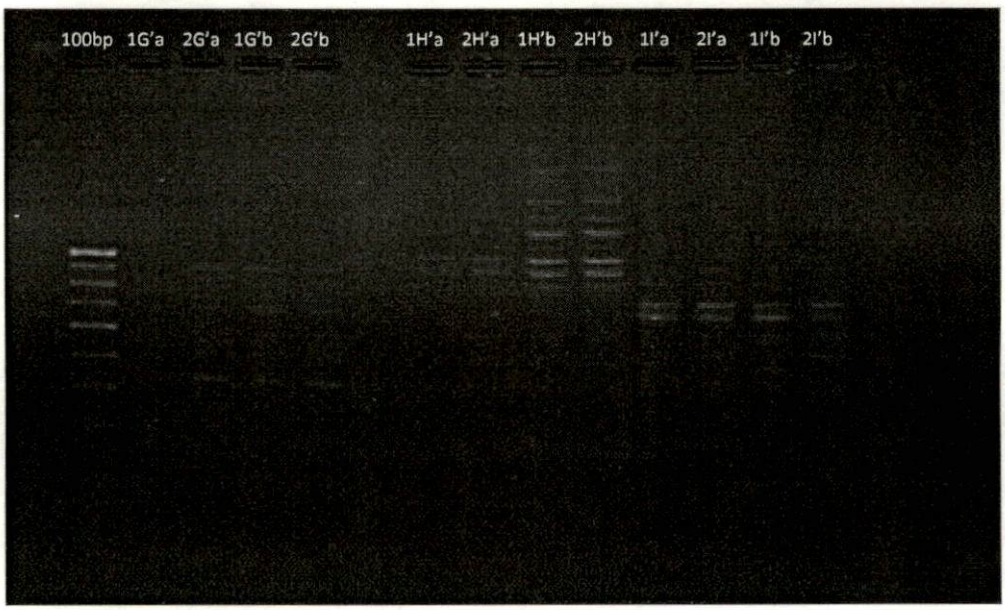
UBC 847 (H)

DNA:

1. AmW 26 2. Am 120

Temperature (in degree Celsius):
a- 53.1 b- 53.4 c- 54 d-55 e- 56.2 f- 57.6 g- 59.1 h- 60.5 i- 61.7 j- 62.8 k- 63.6 l- 63.9

Plate 14: 2% agarose gel profiles of UBC 847 for two accessions at 9 different temperatures



Primer:

G'- UBC 818 H'- UBC 836 I'- UBC 807

DNA:

1- AmW 26 2- Am 120

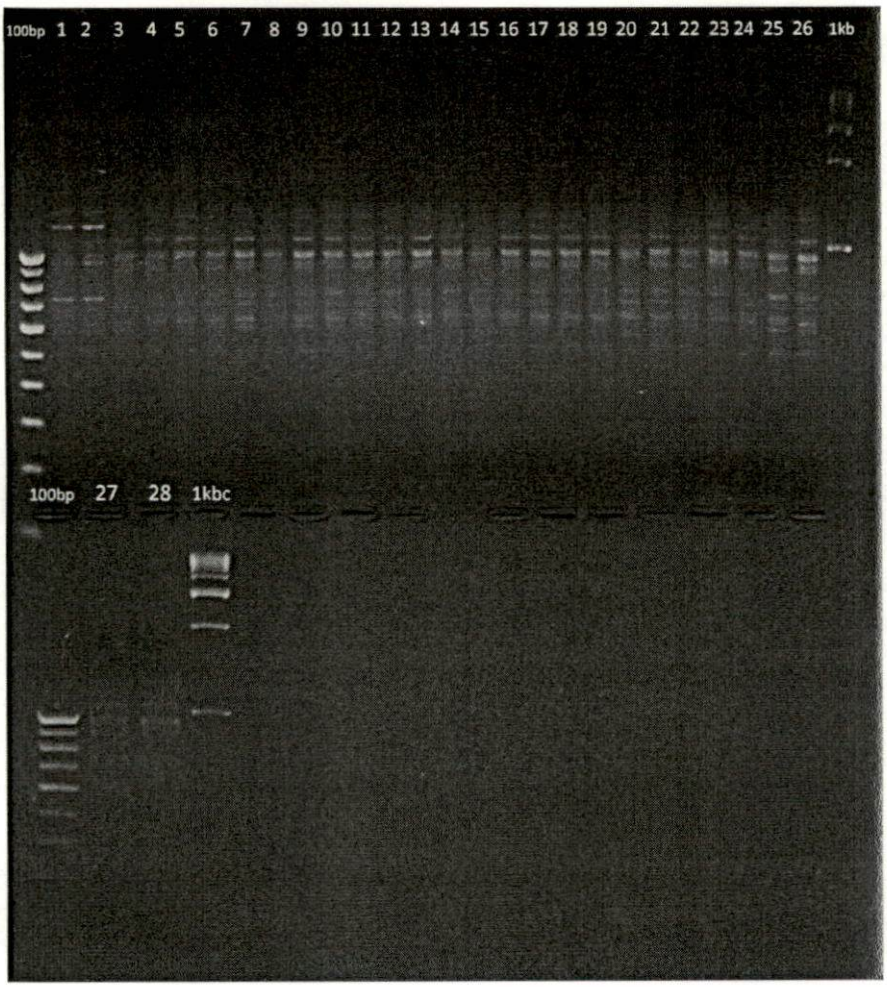
Temperature:

a- 55°C b- 56°C

Plate 15: 2% agarose gel profiles of three different primers for two accessions at 55° C and 56° C

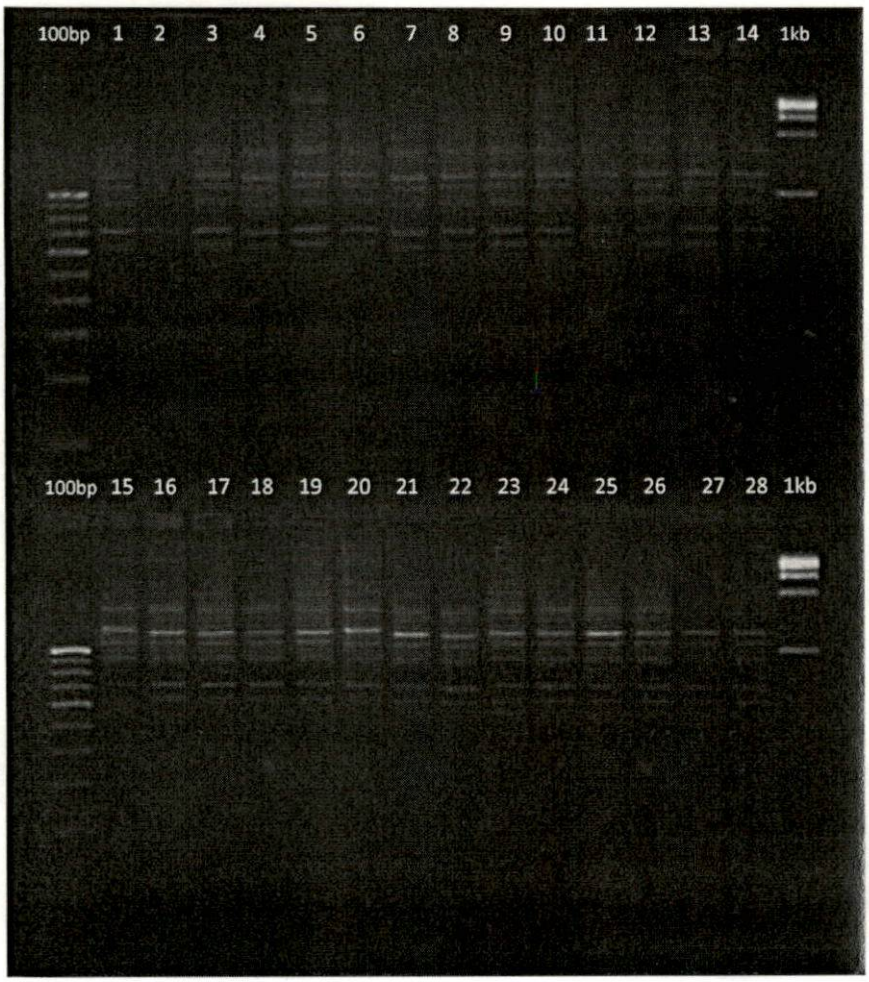
Table 13: Details of selected primers and their annealing temperature

Representative Letter	Primer	Sequence → 5' 3'	Annealing temperature (Ta)
F'	UBC808	*AGA GAG AGA GAG AGA GC*	55
C'	UBC834	*AGA GAG AGA GAG AGA GYT*	55
L'	UBC809	*AGA GAG AGA GAG AGA GG*	55
I'	UBC807	*AGA GAG AGA GAG AGA GT*	55
E'	UBC860	*TGT GTG TGT GTG TGT GRA*	55
D'	UBC850	*GTG TGT GTG TGT GTG TYC*	56
A	UBC827	*ACA CAC ACA CAC ACA CG*	56
D	$(GA)_9AC$	*GA GA GA GA GA GA GA GA GA AC*	56
C	$(ACC)_6Y$	*ACC ACC ACC ACC ACC ACC Y*	55
H'	UBC836	*AGA GAG AGA GAG AGA GYA*	55
H	UBC847	*CAC ACA CAC ACA CAC ARC*	55
F	UBC811	*GAG AGA GAG AGA GAG AC*	55
M'	UBC817	*CAC ACA CAC ACA CAC AA*	55
K'	UBC825	*ACA CAC ACA CAC ACA CT*	54
R'	UBC864	*ATG ATG ATG ATG ATG ATG*	54



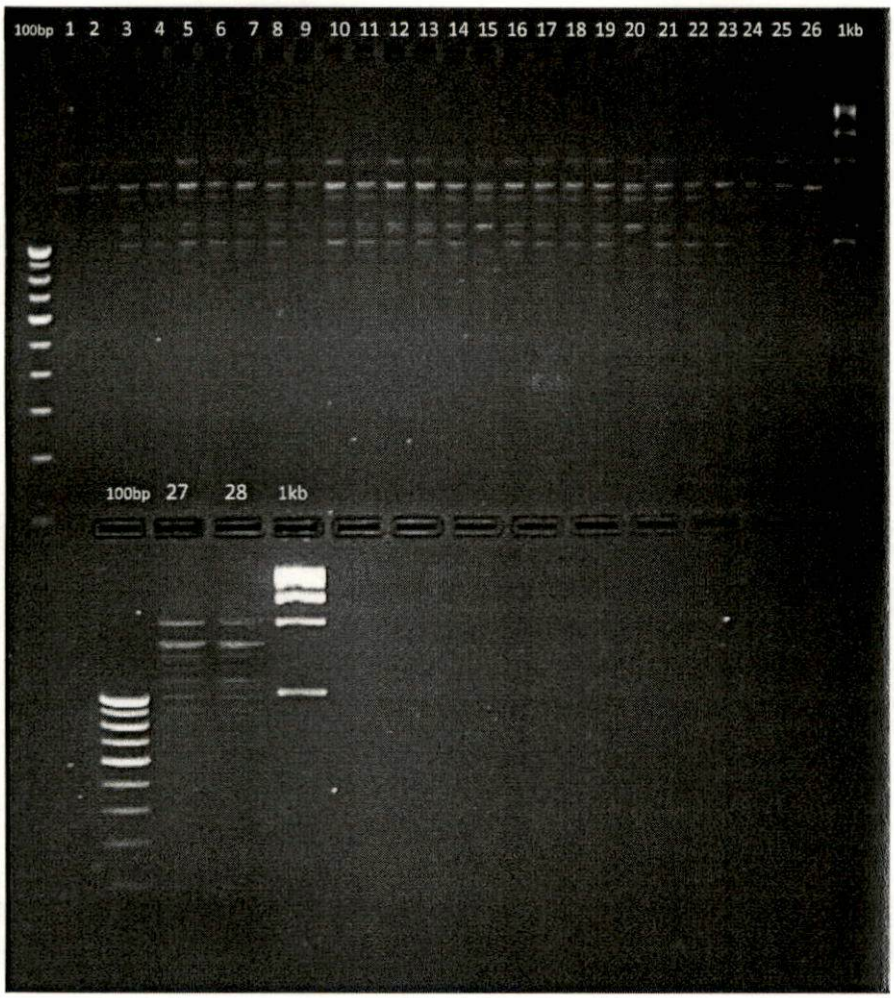
1. AmW26
2. Am120
3. Am84
4. Am22
5. Am62
6. Am44
7. Am85
8. Am1
9. Am140
10. Am10
11. AmH139
12. AmH6
13. Am11
14. Am110
15. Sree Padma
16. Am107
17. AmH13B
18. Am102×SP
19. Am37
20. NDA5
21. Am135
22. Am14
23. S16
24. Am29
25. Am130
26. H69
27. Am133
28. Am97

Plate 16: Agarose gel (2%) gel profile of 28 accessions after PCR using the primer $(GA)_9$ AC



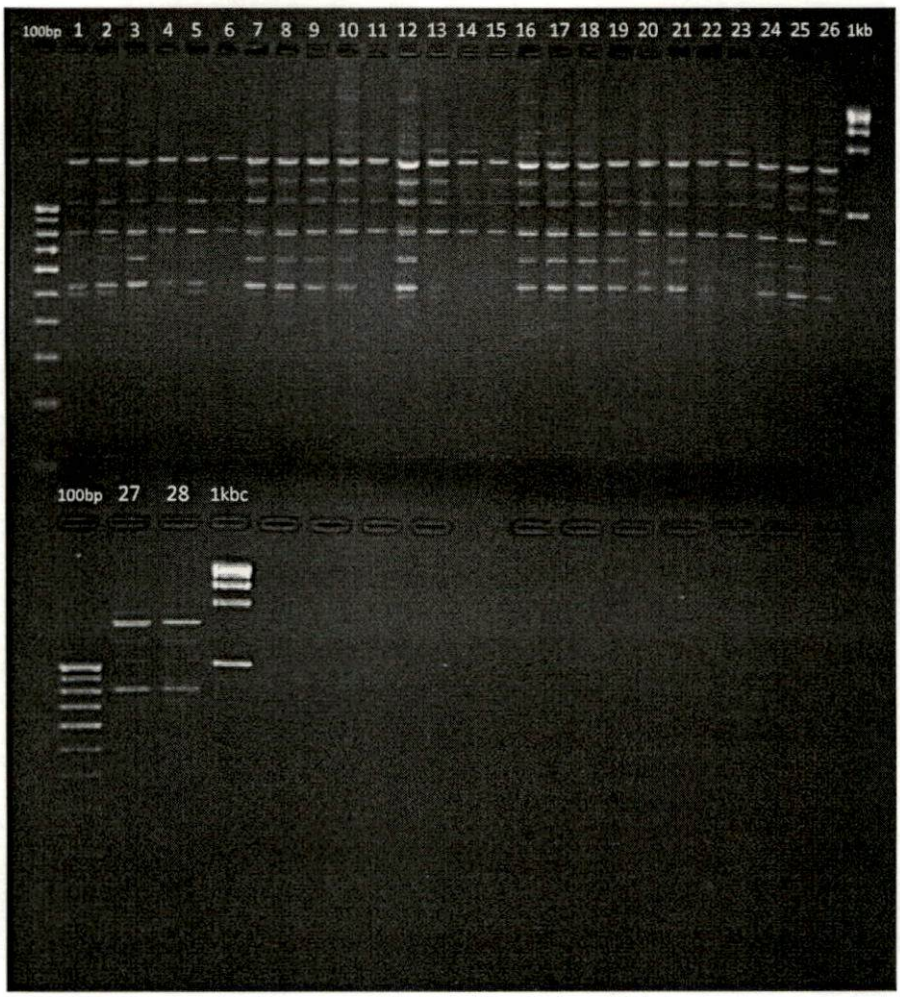
1. AmW26
2. Am120
3. Am84
4. Am22
5. Am62
6. Am44
7. Am85
8. Am1
9. Am140
10. Am10
11. AmH139
12. AmH6
13. Am11
14. Am110
15. Sree Padma
16. Am107
17. AmH13B
18. Am102×SP
19. Am37
20. NDA5
21. Am135
22. Am14
23. S16
24. Am29
25. Am130
26. H69
27. Am133
28. Am97

Plate 17: Agarose gel (2%) gel profile of 28 accessions after PCR using the primer UBC 808



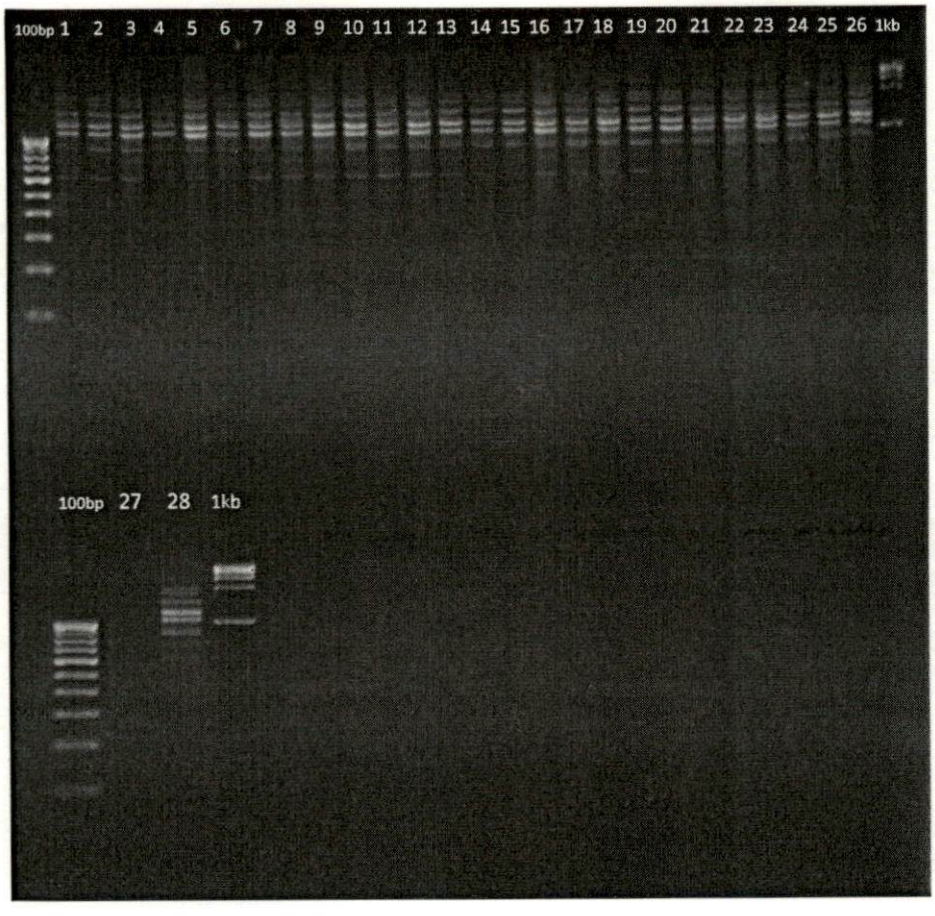
1. AmW26
2. Am120
3. Am84
4. Am22
5. Am62
6. Am44
7. Am85
8. Am1
9. Am140
10. Am10
11. AmH139
12. AmH6
13. Am11
14. Am110
15. Sree Padma
16. Am107
17. AmH13B
18. Am102×SP
19. Am37
20. NDA5
21. Am135
22. Am14
23. S16
24. Am29
25. Am130
26. H69
27. Am133
28. Am97

Plate 19: Agarose gel (2%) gel profile of 28 accessions after PCR using the primer $(ACC)_6Y$



1. AmW26
2. Am120
3. Am84
4. Am22
5. Am62
6. Am44
7. Am85
8. Am1
9. Am140
10. Am10
11. AmH139
12. AmH6
13. Am11
14. Am110
15. Sree Padma
16. Am107
17. AmH13B
18. Am102×SP
19. Am37
20. NDA5
21. Am135
22. Am14
23. S16
24. Am29
25. Am130
26. H60
27. Am133
28. Am97

Plate 20: Agarose gel (2%) gel profile of 28 accessions after PCR using the primer UBC 811



1. AmW26
2. Am120
3. Am84
4. Am22
5. Am62
6. Am44
7. Am85
8. Am1
9. Am140
10. Am10
11. AmH139
12. AmH6
13. Am11
14. Am110
15. Sree Padma
16. Am107
17. AmH13B
18. Am37
19. Am37
20. NDA5
21. Am135
22. Am14
23. S16
24. Am29
25. Am130
26. H60
27. Am133
28. Am97

Plate 21 :Agarose gel (2%) gel profile of 28 accessions after PCR using the primer UBC 864

4.5 ANALYSIS OF MOLECULAR MARKER DATA

4.5.1 Quantification of genetic variability

The various parameters estimated for the quantification of genetic variability using twenty-eight ISSR markers is shown in table 14. The primers showed 89.21% polymorphism and the number of bands ranged from 3 to 14 of which, UBC 860, UBC 850, UBC 827, UBC 847, UBC 817, UBC 825 and UBC 864 showed 100% polymorphism. The percentage polymorphism ranged from 66.67 to 100 percentage.

4.5.2 Cluster analysis

Cluster analysis was done based on Euclidean distance (Hierarchical clustering), in which the total twenty-eight accessions was grouped into eight major clusters shown as in the dendrogram (Figure 4). Maximum number of accessions was included in cluster 7 and it contained 13 of the 28 accessions. Three of the five hybrids taken for study was included in the 7^{th} cluster (AmH139, AmH6 and AmH13B). Am1, a collar rot resistant variety was found along with Am85 in a sub cluster. NDA5 was found alone in a single cluster (cluster 6).

4.5.3 Principal Component Analysis

Using, NTYSyspc, Principal component analysis was done using molecular scoring data. The 2D and 3D PCA plots are shown in Figures 5 and 6 respectively.

4.5.4 Simple Matrix Diversity Index

Variations between the accessions was obtained by determining the SM coefficient. The similarity between the accessions was represented by a number. As the value increases, the diversity decreases between the accessions. The SM coefficient of the twenty-eight accessions is shown in table 15.

Table 14: Parameters estimated for the quantification of genetic variability using twenty-eight ISSR markers

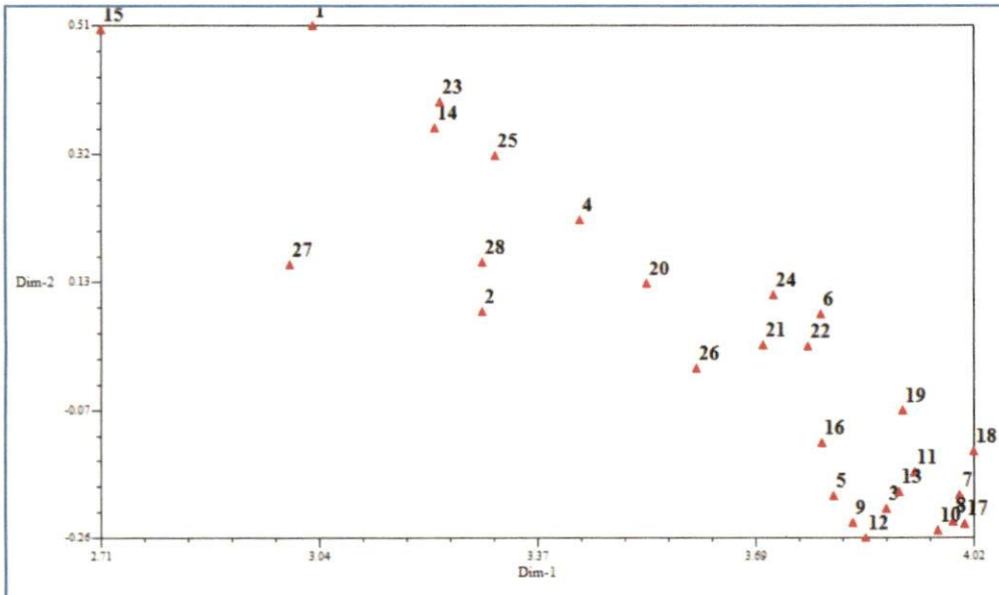
Primer used	Annealing temperature (°C)	No. of bands	Number of polymorphic bands	% polymorphism
UBC808	55	13	11	84.61
UBC834	55	6	6	100.00
UBC809	55	9	6	66.67
UBC807	55	8	6	75.00
UBC860	55	14	14	100.00
UBC850	56	4	4	100.00
UBC827	56	11	11	100.00
$(GA)_9AC$	*56*	10	9	90.00
$(ACC)_6Y$	*55*	9	7	77.77
UBC836	55	7	5	71.42
UBC847	55	8	8	100.00
UBC811	55	11	8	72.72
UBC817	55	3	3	100.00
UBC825	54	10	10	100.00
UBC864	54	4	4	100.00
Mean				89.21

Table 15: Simple matching coefficient of 28 accessions using NTYSysPC

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
1	1																											
2	.7	1																										
3	.6	.7	1																									
4	.7	.6	.8	1																								
5	.6	.6	.8	.8	1																							
6	.7	.7	.8	.8	.8	1																						
7	.7	.7	.8	.7	.8	.8	1																					
8	.6	.7	.8	.7	.8	.8	.9	1																				
9	.6	.7	.8	.7	.8	.7	.9	.9	1																			
10	.6	.7	.8	.7	.8	.8	.9	.9	.8	1																		
11	.6	.7	.8	.7	.8	.8	.9	.8	.8	.9	1																	
12	.6	.7	.8	.7	.8	.7	.9	.9	.8	.9	.8	1																
13	.6	.7	.9	.7	.8	.8	.8	.9	.8	.9	.9	.8	1															
14	.7	.6	.6	.7	.7	.7	.7	.7	.6	.7	.8	.6	.7	1														
15	.7	.6	.6	.7	.6	.6	.6	.6	.6	.6	.6	.6	.6	.7	1													
16	.7	.7	.8	.7	.8	.7	.8	.8	.8	.8	.8	.8	.8	.6	.6	1												

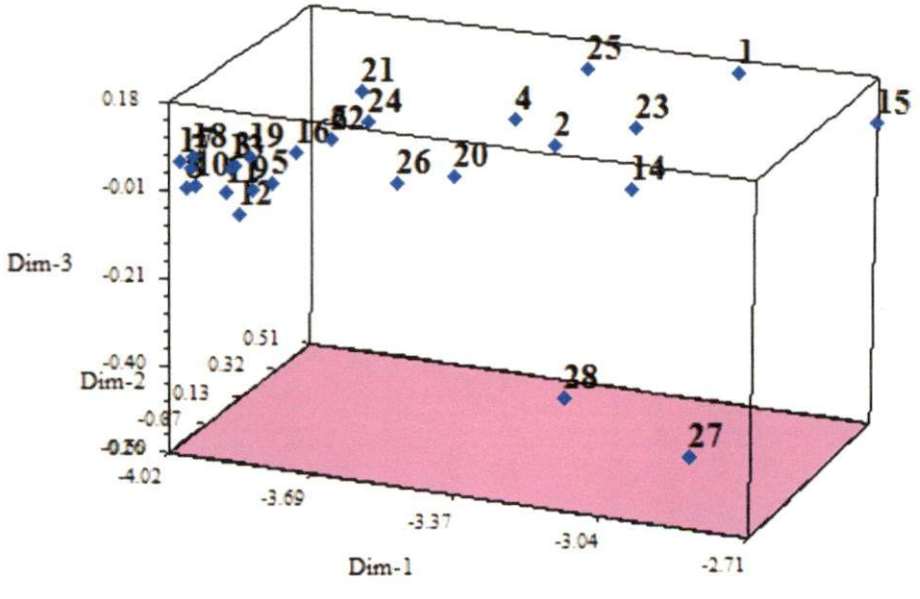
17	.6	.7	.8	.7	.8	.8	.9	.9	.8	.9	.9	.9	.9	.7	.5	.8	1											
18	.7	.7	.8	.7	.8	.8	.8	.8	.8	.8	.9	.9	.8	.7	.6	.8	.9	1										
19	.7	.7	.8	.8	.8	.8	.8	.8	.8	.8	.8	.8	.8	.7	.7	.9	.8	.9	1									
20	.6	.7	.7	.7	.7	.8	.7	.7	.7	.7	.7	.7	.7	.6	.7	.7	.7	.7	.8	1								
21	.7	.7	.8	.7	.8	.8	.8	.8	.7	.8	.8	.8	.8	.7	.6	..8	.8	.8	.8	.7	1							
22	.7	.7	.8	.8	.8	.8	.8	.8	.8	.8	.8	.8	.8	.7	.7	.8	.8	.8	.8	.8	.8	1						
23	.7	.5	.6	.7	.6	.7	.6	.7	.7	.7	.7	.6	.7	.7	.8	.7	.7	.7	.7	.7	.7	.7	1					
24	.8	.7	.8	.7	.7	.8	.8	.8	.8	.8	.8	.7	.8	.7	.6	.8	.8	.8	.8	.7	.8	.8	.7	.1				
25	.8	.7	.7	.7	.7	.7	.7	.7	.7	.7	.7	.7	.7	.7	.7	.7	.7	.7	.7	.7	.7	.7	.7	.8	1			
26	.6	.7	.7	.7	.7	.8	.7	.7	.7	.8	.8	.7	.8	.7	.6	.7	.8	.8	.8	.7	.8	.8	.7	.8	.7	1		
27	.6	.6	.6	.6	.6	.6	.6	.6	.6	.6	.7	.6	.6	.7	.5	.6	.6	.7	.7	.6	.6	.7	.6	.7	.6	.6	1	
28	.7	.7	.7	.7	.7	.7	.7	.7	.7	.7	.7	.7	.7	.7	.6	.7	.7	.7	.7	.7	.7	.7	.7	.7	.6	.7	.9	1

Figure 4 : Cluster analysis of 28 *Amorphophallus* accessions using molecular scoring data



1. AmW26
2. Am120
3. Am 84
4. Am22
5. Am62
6. Am44
7. Am85
8. Am1
9. Am140
10. Am10
11. AmH139
12. AmH6
13. Am11
14. Am110
15. Sree Padma
16. Am107
17. AmH13B
18. Am102x SP
19. Am37
20. NDA5
21. Am135
22. Am14
23. S16
24. Am29
25. Am130
26. H69
27. Am133
28. Am97

Figure 5: 2 Dimensional PCA of accessions using molecular data



1. AmW26
2. Am120
3. Am84
4. Am22
5. Am62
6. Am44
7. Am85
8. Am1
9. Am140
10. Am10
11. AmH139
12. AmH6
13. Am11
14. Am110
15. Sree Padma
16. Am107
17. AmH13B
18. Am102xSP
19. Am37
20. NDA5
21. Am135
22. Am14
23. S16
24. Am29
25. Am130
26. H69
27. Am133
28. Am97

Figure 6: 3D PCA of accessions using molecular scoring data

DISCUSSION

5. DISCUSSION

Amorphophallus paeoniifolius (Dennst.) Nicolson is widely, seen, distributed and cultivated in Indonesia and other Asian countries. In India, it is widely found in West Bengal, Kerala, Maharashtra and Orissa. One of the major characteristic of the crop is its huge variations commonly seen in case of petiole colour, i.e., the colour may vary from light green to dark green or it may also be other colours such as grey, reddish or pinkish white. Variations can also be seen in case of petiole structure mainly rough and smooth (Kurniawan *et al.,* 2011). Large variations can be seen in case of spathe characters, appendix, tuber characters, petiole and individual flowers (Hetterscheid *et al.*,1996) The crops in the tropic region is prone to ultraviolet radiations, which in turn can result in inducing mutation in the crops (Singh *et al.,* 2011).

But, the usage of molecular markers in analysing diversity was found to be very rare. Only a few number of cases was seen such as in case of characterization by Santosa *et al.* (2007). They used microsatellite markers for determining genetic diversity of *Amorphophallus paeoniifolius* (Dennst.) Nicolson. Also Zheng *et al.*(2013) developed microsatellite markers by transcriptome sequencing in *Amorphophallus*. *Amorphophallus paeoniifolius* in West Java was characterized by Santosa *et al.* (2010) using microsatellites. Thus the diversity researches on elephant foot yam was limited prior to the study. In this study, combination of morphological characteristics with that of molecular characters was taken for analysis in order to analyse maximum diversity among accessions within the species.

In this study, the genetic diversity among 28 elephant foot yam accessions collected from all across India was determined using 32 morphological descriptors which was listed out by NBPGR and molecular characterization using 15ISSR markers. Agarose gel electrophoresis was used to resolve the amplicons followed by statistical analysis.

5.1MORPHOLOGICAL ANALYSIS

Many of the morphological characters taken for the study were not uniformly distributed throughout the accessions taken. The characters except number of primary partitions, number of secondary partitions, number of corms, leaflet nature, leaflet margin, pseudostem colour, leaf spot, leaf stalk, rachis nature and rachis shape, showed considerable variations between accessions.

5.1.1 Principal Component Analysis

The cluster plot obtained revealed three clusters, in which Cluster I and Cluster III contained only one accession each. AmH6 and Am10, respectively. The first component was responsible for maximum variation accounting for about 25.34% of the total variability.

The PCA done by Anil *et al.* (2011) for 17 wild *Amorphophallus* accessions showed that corm, cormel and foliar characters such as petiole surface pattern varied among the accessions and is considered as a general observation that corm characters are important for providing diversity.

Of both the plots, the biplot showed a visual representation of how the genotypes are related with the morphological traits. Thus, it was observed that the variation of accessions namely, Am14, Am120 and Am130 is clearly distinguished from others based on the weight of the corm. And all other characters were found to be equally important in differentiating the accessions.

5.1.2 Analysis of variance

ANOVA identified significant variation (P=0.01) for 12 of the total 18 quantitative characters analysed, of which six were tuber characters. Six of the 12 significant characters were significant at 1% and the rest at 5%. Anil *et al.* (2011) in their studies in *Amorphophallus paeoniifolius*, observed that high degree of variability existed for cormel weight per corm, cormel length, cormel number and corm fresh weight based on the coefficient of variation. In correspondence to that

analysis, in this study also, for tuber characters such asfresh weight of corm (coefficient of variation= 54.23), height of corm (coefficient of variation= 22.21), length of cormels (coefficient of variation=31.28), weight of cormels (coefficient of variation=87.01), number of cormels (coefficient of variation = 61.74), coefficient of variation was high. The coefficient of variation ranged from 0-87.01 percent in this study, no particular variation was seen on number of secondary partitions as the coefficient of variation obtained for that trait was 0.00. While comparing the coefficient of variation of this study with that of the analysis done by Beyene (2013), the percentage was found to be very high. They obtained a range only between 0.4-2.5.

Duncan's multiple range test (α= 0.05) was performed on the basis of quantitative traits in this study and thus the accessions was differentiated based on grouping of mean values. An accession with a particular letter differs from all other accessions which lacks that specific letter representing a trait.

5.1.3 Cluster Analysis

Using hierarchical clustering in R package, Cluster analysis using all the quantitative as well as qualitative characters were done in order to group the total twenty-eight accessions into different clusters. The total accessions were categorized into six major clusters. The cluster data from dendrogram was similar to the data obtained through PCA plots. The wild was separated out in a cluster along with accession Am133. The wild forms were also separated out in different clusters. The result obtained by Zubair *et al.* (2007), Ahmad *et al.* (2008) and Ali *et al.* (2008) showed a lack of association with geographical origin and morphological traits. According to their studies, geographic habitats contribute much to diversity. The plants seen in one region will vary from those fromanother region. In the present study, the cluster obtained revealed that a hybrid, an accession collected from Kerala and Karanataka were grouped under Cluster I and Cluster II.

Thus it was concluded that geographical regions do not contribute much to morphological diversity.

5.2 STANDARDIZATION OF DNA ISOLATION PROTOCOL

The DNA extraction protocol by Cota-Sánchez *et al.* (2006) was foundvery efficient in case *Amorphophallus* in the previous studies of Samuel (2012). Doyle (1991) introduced the use of CTAB for isolation of DNA from plants and the method was used by Chiang *et al.* (1998) on different plants, Grob *et al.* (2004) etc., for Brazilian giant bromeliads. Also the Cota-Sánchez (2006) method was very efficient in isolation of DNA from frozen leaf sample of *Amorphophallus* as per this study. However, in this study, the DNA was not resolved for any accessions tried except Am120. Thus, modifications in Cota- Sanchez method was tried with a reduction in the temperature for overnight treatment from -80°C to -20°C. But, this also resulted in sheared low quality DNA.

Thus a commercial kit method was adopted. Even though a high amount of mucilage was there, good quality (1.96 - 2.09) of unsheared DNA was obtained after electrophoresis. This result was in contradiction to the fact that the presence of mucilage hinders the pipetting of DNA and will lead to volumetric errors. (Ghosh *et al.*, 2009) and the spectrophotometric readings thus obtained will be abnormal.

The DNA obtained using DNeasy Plant Mini Kit (Qiagen) showed an absorbance ratio (A_{260}/A_{280}) in the range of 1.96 - 2.09. Thus it indicated high quality DNA was obtained by using this minikit for isolation of DNA from *Amorphophallus*. Young leaves were taken for isolation as it contains low number of polysaccharides, polyphenols and other secondary metabolites. (Dabo *et al.*, 1993; Zhang and Steward, 2000). But even by using matured leaves, high quality of DNA was obtained through the method. Qiagen DNesy plant minikit was successfully used by Alexander *et al.*(2007) for Ephedra and Rhipsalis, Enan *et al.* (2012) for Wheat and by Minn *et al.* (2013) for genetic diversity analysis of teak.

5.3 PRIMER SCREENING FOR PCR

Due to the limitation in the diversity studies in elephant foot yam using molecular markers, in this study ISSR primers was taken for the amplification of the isolated DNA. Liu and Wendel (2001) used ISSR for the amplification of cotton DNA. As per Mao *et al.* (2014) 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 temperature was determined using gradient PCR. Finally, they selected only eleven out of the total primers as they gave considerable clear and good bands. Also, fourteen efficient inter-simple sequence repeat (ISSR) primers were screened and optimized for detecting the genetic diversity in wild populations of *Glycyrrhiza uralensis* Fisch. by Yao *et al.* (2008). A similar screening was done by Basha and Sujatha (2007) as they screened 100 ISSR primers. Out of which, 48 primers gave amplification products of which 29 primers generated polymorphic banding patterns. In this study, 25 ISSR primers were taken initially for screening, and 15 of the best primers were chosen for further analysis. (UBC824, UBC873, UBC845, UBC847, UBC849, UBC851, UBC880, UBC836, UBC861, UBC891 and $(GA)_9AT$). The selected primers when screened for obtaining the annealing temperature and temperatures of 54°C (UBC825, UBC864), 55°C (UBC808, UBC834, UBC809, UBC807, UBC860, $(ACC)_6Y$, UBC836, UBC847, UBC811, UBC817) and 56°C (UBC850, UBC827, $(GA)_9AC$) were found to be the best for theseselected primers. Yao *et al.* (2008) selected 52°C, 52.5°C, 53°C and 54°C for the analysis. The temperatures thus were found in the range of 50°C-57°C. Contradictory to that result Mao *et al.* (2014) obtained annealing temperatures ranging from 44°C-53°C. But a generally accepted rule is that the annealing temperature should be at least 5°C less than that of the melting temperature (Yeh *et al.,* 1999). But according to Sisea and Pamfil (2009), the value is not optimal most of the times and should determine annealing temperature manually via gradient PCR.

5.4 ISSR ANALYSIS OF ELEPHANT FOOT YAM ACCESSIONS

ISSR analysis involves PCR amplification of genomic DNA using a single primer that targets the repeat per se, with 1–3 bases that anchor the primer at the 3′ or 5′ end. In addition to freedom from the necessity of obtaining flanking genomic sequence information, ISSR analysis is technically simpler than many other marker systems.

After resolving the amplicons using selected ISSR primers under the annealing temperatures determined, clear polymorphic bands were obtained for all the primers selected. Some of the primers gave 100% polymorphism. (UBC834, UBC860, UBC850, UBC827, UBC847, UBC817, UBC825, UBC864). Previous investigators have demonstrated that ISSR analysis usually detects a higher level of polymorphism than that detected with restriction fragment length polymorphism (RFLP) or random amplified polymorphic DNA (RAPD) analyses.ISSR analysis in cotton was done by Liu and Wendel (2001) and one of the conclusions was that ISSR fingerprinting patterns are highly heritable and shows a considerable polymorphism. On the basis of molecular characters, Assessment of diversity in elephant foot yam was done by Stini (2015). She took 12 accessions of elephant foot yam and done PCR with 9 ISSR primers and obtained only an average of 51.05% polymorphism . The primers used in the study were UBC 809, UBC 810, UBC 834, UBC 845, UBC 827, UBC 857, UBC 873, $(GA)_9AC$ and $(ACC)_6Y$. These were the primers used in present study also and got a polymorphism percentage of 89.21(mean) and Krishnapriya (2014) studied the diversity of elephant foot yam using 15 accessions and 6 primers. The primers used were UBC 808, UBC 827, UBC 834, UBC 850, UBC 860 and UBC 973. These were also part of the ISSR analysis in the present study. She got an average polymorphism percentage of 82.26.

The result obtained in this study also shows the level of polymorphism by ISSR primers are very high. An average of 89.21% polymorphism was shown by the primers selected in this study.

5.5ANALYSIS OF MOLECULAR MARKER DATA

5.5.1 Cluster analysis

Using the molecular scoring data, Hierarchical clustering was done and the twenty-eight accessions were divided into eight clusters in which the 7th cluster was having the maximum number of accessions. Three of the hybrids clustered together. While analysing the obtained cluster result with that of geographical regions, a variety and a selfed progeny was clustered in Cluster I(SP and S16), Two accessions from Kerala and one from Tamil Nadu was clustered in Cluster II, Accessions from Kerala and Tamil Nadu was clustered in Cluster II.I Two hybrids were clustered in Cluster VI. Thus this research showed considerable relationship of diversity with geographical region. This is in contradiction to the research done by Lakhanpaul *et al.* (2003) on *Colocasia* accessions showed a dendrogram which did not show considerable relationship between origin and diversity. He obtained a dendrogram such that the taro accessions from different regions were clustered together.

Divergent parents can be selected from different clusters for breeding purposes. This can be used as a technique to improve a particular trait. In the present study, Am1 was clustered together with Am85. Am1 is a collar rot resistant variety. Thus there are chances that Am85 may also have a similar genetic makeup which could be resistance to collar rot. Thus screening can be done and if proved right, it can be used for further breeding purposes.

5.5.2 Similarity matrix

Similarity matrix coefficient obtained for the accessions showed its variation with each other. The coefficient of an accession with itself is 1.00 and that

with any other accession will be less than 1.00. For greater similarity between two accessions, the value was greater. The value was low when there was greater diversity present between two accessions.

The study was focused on the diversity analysis in the Indian origin and morphological as well as molecular characterization was done. Diversity was analysed with the help of Cluster analysis (Dendrogram and PCA). Not much research has been done on the diversity analysis of elephant foot yam till now. But based on morphological characteristics, *A. paeoniifolius* (Dennst.) Nicolson, was characterized by Anil *et al.* (2011) mainly due to its economic importance. Also Devi *et al.* (2013) characterized twenty-six elephant foot yam accessions based on morphological characters along with the characterization of taro. On observation of morphological characters by Sugiyama and Santosa (2008), the size of leaves was found to be comparatively larger for preflowering corms than in case of post flowering corms. Also Santosa *et al.* (2006) found that when a particular number of weeds was present, there were substantial decrease in the number of leaves, total leaf area, leaf thickness and fresh masses of corms. Pushpakumari and Sasidhar (1992) observed that corm yield decreased by 66% when the light intensity was reduced to 25% of full sunlight. Thus a wide range of morphological variations are present in elephant foot yam accessions.

SUMMARY

6. SUMMARY

The study entitled "Genetic diversity analysis of elephant foot yam [*Amorphophallus paeoniifolius* (Dennst.) Nicolson]" was carried out at the Division of Crop Improvement, ICAR-Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram during 2015-2016. The objective of the study was to analyse the genetic variation in elephant foot yam based on morphological and molecular characteristics. Twenty- eight accessions of elephant foot yam from all over the country were selected for characterization.

The study was divided into two phases -morphological and molecular characterization. Morphological characterization started with recording the observations and measurement of all the above ground characteristics of elephant foot yam and measurement of all the quantitative characters. All the traits listed out for observation were those listed as per NBPGR descriptors for elephant foot yam. 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 analysed using various statistical tools such as Cluster dendrogram, PCA and ANOVA. Cluster dendrogram was done for all the 32 traits studied and it resulted in two major clusters. Cluster I was further divided into Ia and Ib. Ia had three accessions in it, one was a hybrid (AmH13B). Ib had a total of four accessions in it. Cluster II had the remaining hybrids in it and was subdivided into four clusters- IIa (4 accessions), IIb (6 accessions), IIc (3 accessions) and IId (6 accessions). But the PCA resulted in only three clusters and only one accession was found in both Cluster I and Cluster III. Hence, Am1 and Am10 were separated out into Cluster I and Cluster III, respectively in PCA. ANOVA was done for 18 quantitative traits.Out of these, 12 quantitative characters were found significant (6 at 1% and 6 at 5%). Duncan's multiple range test was also performed. The software used for morphological analysis were both NTSyspc and R package.

After morphological analysis, molecular analysis was done by DNA isolation procedures. Young leaf samples freshly collected from the field was used for studies. DNA was isolated using three methods- Using QIAGEN DNasy® plant mini kit, using Cota-Sanchez method (2006) and by modifying the Cota-Sanchez method. DNA having abnormal quality and quantity was obtained by using Cota-Sanchez method. The modification in Cota-Sanchez method was done by changing the overnight temperature from -80°C to -20°C. But still, the quality of the obtained DNA was not of good quality, whereas, good quality DNA was obtained using the mini kit method and the extracted DNA was having a A_{260}/A_{280} ratio of 1.96–2.09 when resolved in a 1% agarose gel. Thus isolation using kit method was confirmed as a good method for isolation of genomic DNA from elephant foot yam.

Isolation of DNA from the selected accessions was followed by primer screening for ISSR analysis. A total of 25 ISSR primers were taken for the screening process. And out of which, 15 primers which gave clear and reproducible bands while resolving in 1% agarose gel were selected and their annealing temperatures were determined by doing gradient PCR. All the primers amplified in an annealing temperature of 54°C (UBC 825 and UBC 864), 55°C (UBC 817, UBC 811, UBC 847, UBC 836, $(ACC)_6Y$, UBC 860, UBC 807, UBC 809, UBC 834 and UBC 808) or 56°C (UBC 850, UBC 829and $(GA)_9AC$). After the final PCR using the primers and the determined annealing temperatures, the product was resolved in 2% agarose and clear polymorphic bands were obtained. The primers showed 89.21% polymorphism and the number of bands ranged from 3 to 14. UBC 860, UBC 850, UBC 827, UBC 847, UBC 817, UBC 825, UBC 864 showed 100% polymorphism. Using the molecular scoring data, Hierarchical clustering (R package) was done and the twenty-eight accessions were divided into eight clusters in which the VII cluster was having the maximum number of accessions. 3 of the hybrids were clustered together. Certain accessions like NDA5, AmW26 and Am120 showed high degree of variation from others. Majority of the hybrids were grouped in the same sub-clusters. Principle component analysis was done using the same scoring data. Both two dimensional

and 3 dimensional plots were drawn using PCA. To determine the similarity and diversity between two accessions, SM coefficient was also determined for all the accessions using molecular scoring data. Both NTSyspc and R-package were used for molecular analysis also.

The diversity analysis and cluster data showed high genetic divergence among the accessions at one of the centers of origin and application of other molecular tools can help to reduce further redundancy. The diversity analysis of the crop from other centers of origin, especially Sri Lanka was difficult due to the non-availability of samples. A more reliable data can be obtained by using some more polymorphic markers and later the results can be used for the formation of core collection from this center of origin. The divergent parents can be used in breeding programs for exploiting the heterosis.

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7. REFERENCES

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APPENDICES

APPENDIX I

CTAB Extraction Buffer

Tris - HCL	1M	
EDTA (pH=8)	0.25 M	
NaCl	5 M	
CTAB	2%	
β-mercaptoethanol	0.2% (v/v)	Added freshly prior to grinding
PVP	1% (w/v)	
PEG	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 1000ml with distilled water and autoclave before use.

ABSTRACT

GENETIC DIVERSITY ANALYSIS OF ELEPHANT FOOT YAM
[*Amorphophallus paeoniifolius* (Dennst.) Nicolson]

KRISHNARAJ S.

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Abstract of the thesis

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Faculty of Agriculture
Kerala Agricultural University, Thrissur

B.Sc.-M.Sc. (INTEGRATED) BIOTECHNOLOGY
DEPARTMENT OF PLANT BIOTECHNOLOGY
COLLEGE OF AGRICULTURE
VELLAYANI, THIRUVANANTHAPURAM-695 522
KERALA, INDIA
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ABSTRACT

The study entitled "Genetic diversity analysis of elephant foot yam [*Amorphophallus paeoniifolius* (Dennst.) Nicolson]" was carried out at the Division of Crop Improvement, ICAR-Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram during 2015-2016. The objective of the study was to analyze the genetic variation in elephant foot yam based on morphological and molecular characteristics. The knowledge can be exploited for crop improvement programs of elephant foot yam and form forming core collections from the region.

Twenty-eight accessions were selected for the study. The study was divided into two phases - morphological and molecular analysis. Morphological analysis started with the observation and measurement of all the above ground characteristics of the plant and measurement and observation of all the tuber characters (using NBPGR descriptors). The recorded data was analyzed using various statistical tools such as cluster dendrogram, PCA and ANOVA. Cluster dendrogram resulted in two major clusters. Cluster I was further divided into Ia and Ib. Ia had three accessions in it, one was a hybrid (AmH13B). Ib had a total of four accessions in it. Cluster II had the remaining hybrids in it and was subdivided into four clusters- IIa (4 accessions), IIb (6 accessions), IIc (3 accessions) and IId (6 accessions). After ANOVA, 12 quantitative characters were found significant (6 at 1% and 6 at 5%). Duncan's multiple range test was also performed. After morphological analysis, DNA was isolated using three methods - using QIAGEN minikit, using Cota-Sanchez method and by modifying the Cota-Sanchez method. Good quality DNA ranging from 1.96 - 2.09 was obtained while using minikit and the extracted DNA was resolved in 1% agarose gel. A total of 28 ISSR primers were taken for the screening process. And out of which, 15 were selected and their annealing temperatures were determined by doing gradient PCR. All the primers used an annealing temperature of 54°C (UBC825 and UBC864), 55°C (UBC817, UBC811, UBC847, UBC836, $(ACC)_6Y$, UBC860, UBC807, UBC809, UBC834 and UBC808) or 56°C (UBC850, UBC829 and $(GA)_9AC$). After the final PCR

using the primers, the product was resolved in 2% agarose and polymorphic bands were obtained. The primers showed 89.21% polymorphism and the number of bands ranged from 3 to 14. UBC860, UBC850, UBC827, UBC847, UBC817, UBC825 and UBC864 showed 100% polymorphism. Using the molecular scoring data, Hierarchical clustering was done and the whole twenty-eight accessions were divided into eight clusters in which the 7th cluster was having the maximum number of accessions. 3 of the hybrids were also clustered together. Accessions NDA5, AmW26 and Am120 showed high degree of variation from others. Majority of the hybrids were grouped in same sub clusters. Principle component analysis was done using the same scoring data. Coefficient was also determined for all the accessions using molecular scoring data for determining the similarity between accessions.