Genetic diversity analysis of greater yam (*Dioscorea alata* L.) landraces in Kerala

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By

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THESIS

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DECLARATION

I hereby declare that the thesis entitled **"Genetic diversity analysis of greater yam (*Dioscorea alata*) landraces in Kerala"** is a bonafide record of research done by me and that the thesis has not previously formed the basis for the award of any degree, diploma, fellowship or other similar title, of any other University or Society.

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CERTIFICATE

We, the undersigned members of the advisory committee of **Ms. Athira Jyothy**, a candidate for the degree of **Master of Science (Integrated) in Biotechnology** with major in Biotechnology, agree that the thesis entitled **"Genetic diversity analysis of greater yam (*Dioscorea alata* L.) landraces in Kerala"** may be submitted by Ms. Athira Jyothy, in partial fulfilment of the requirement for the degree.

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LIST OF ABBREVIATIONS AND SYMBOLS USED

◦C	Degree Celsius
%	Percentage
µg	Microgram
µl	Microgram
µ*M*	Micro molar
A_{260}	Absorbance at 260 nm wavelength
A_{280}	Absorbance at 280 nm wavelength
bp	Base pair
cm	Centimetre
CTAB	Cethyl trimethyl ammonium bromide
CTCRI	Central Tuber Crops Research Institute
DNA	Deoxyribo nucleic acid
EDTA	Ethylene diamine tetra acetic acid
IPGRI	International Plant Genetic Resources Institute
ISSR	Inter simple sequence repeat
KAU	Kerala Agricultural University
Kbp	Kilo base pair
Kg	Kilogram
M	Molar
Mg	Miligram
$MgCl_2$	Magnesium Chloride
Min	Minute
Ml	Millilitre
Mm	Millimetre
m*M*	Millimolar
NaCl	Sodium chloride

NaOH	Sodium hydroxide
ng	Nanogram
nm	Nanometre
OD	Optical Density
PCR	Polymerase Chain Reaction
PVP	Polyvinyl pyrrolidine
PEG	Polyethyl Glycol
RNase	Ribonuclease
rpm	Revolution per minute
RT	Room Temperature
s	Second
SSR	Simple Sequence Repeat
TBE	Tris- EDTA Buffer
T_m	Melting Temperature
Tris Buffer	Tris (Hydroxyl Methyl) aminomethane buffer
U	Enzyme unit
UV	Ultraviolet
V	Volt
v / v	volume/ volume
w / v	weight/ volume

INTRODUCTION

INTRODUCTION

Yam plant is a vine cultivated for its large and edible underground tubers. More than 600 *Dioscorea* species present in world and Africa, America, Asia and Polynesia are its main habitat, where some of the yam species are economically significant tuber crops (Coursey, 1967). Yams are cultivated for consuming by more than 100 million people especially in many developing countries like India (Lebot, 2009). It is usually related with the traditional agriculture systems and it maintain a wide genetic reservoir represented by several varieties bearing several vernacular names (Siqueira *et al*., 2014). Yam is primarily consumed for its carbohydrates (Tetchi *et al*., 2007), and also for protein, vitamins and other minerals (Baah *et al*., 2009). It also has medicinal properties such as the steroidal sapogenins which is used in the production of cortisone and synthetic steroid hormones (Narula *et al*., 2007).

FAO (2014) annual statistical data shows that more than 60.2 million tons of yams were produced world-wide in 2013 and the area harvested in the world has increased from 1.15 million hectare in 1961 to 5.05 million hectare in 2013. Yield (Kg ha) world over, also increased from 72.35 thousand metric tons in 1961 to 119.12 thousand metric tons in 2013.

There are several hundred cultivars of *Dioscorea* exists; but, only a few of them have commercial importance. Major yam tuber types grown are *Dioscorea rotundata* (white /guinea yam), *D. alata* (greater yam), *D. bulbifera* (aerial potato), *D. esculenta* (lesser yam), *D. opposita* (Chinese yam) and *D. dumenterum* (trifoliate yam).

Greater yam is maybe the cultivated species and one of the oldest cultivated yam species with the largest world distribution. It is a major staple food crop for millions of people who are living in the humid and sub humid tropics. *Dioscorea alata* is the most edible yam species in terms of yield potential (especially under low to average soil fertility), early vigour for weed suppression, ease of propagation (production of bulbils) and reliability of sprouting and storability of tubers. Wu *et*

al. (2005), analysed the ingesting of *Dioscorea alata* by post-menopausal women and reported that although these mechanisms are not fully understood yet, its consumption decreases the risk of breast cancer and cardiovascular disease.

They are one of the distinctive tropical crops which require hot, humid climates and may stop to grow when the temperature reduced below 68 ^{0}F. Greater yam is important for its high nutritional content, with crude protein content of 7.4%, starch content of 75-84%, and vitamin C content ranging from 13.0 to 24.7 mg 100 g^{-1}. Due to high amount of starch in the tubers, greater yam act as a good source of dietary carbohydrates in tropical and subtropical regions (Osagie, 1992).

The development of *Dioscorea alata* accessions with better food quality and resistance to pests and diseases would boost its extensive cultivation and significant increases in the production. Genetic improvement programs at IITA (Nigeria) and at the Central Tuber Crops Research Institute aims at rising high yielding greater yam accessions with resistance to pests and diseases to meet the requirements of farmers. Precise data on genetic diversity is essential to the accomplishment of breeding programs, meanwhile genetic divergence produces desirable segregants for selection due to high heterotic effects.

In recent years, very less progress has been reported in using molecular markers (Asemota *et al.,* 1996), morphological traits (Cruz *et al*., 1999) and isozyme (Lebot *et al.,* 1998) for *D. alata* germplasm classification. Molecular methods for evaluating genetic diversity which supply significant tools for estimating the relationship among yam accessions and species (Zannou *et al*., 2009), which include the genetic relationship between *D. alata* and other edible *Dioscorea* species (Malapa *et al*., 2005), gene mapping (Sartie and Asiedu, 2011), cytogenetic status (Arnau *et al*., 2009) and also resistance breeding (Narina *et al*., 2011). Of all the techniques, microsatellite markers are the enlightening markers with desirable genetic attributes including high variability, multiallelic nature, reproducibility, extensive genome coverage, and relative abundance, codominant inheritance (Kalia *et al*., 2011).

This research work attempts to combine morphological and molecular data for greater understanding of the distribution and extent of genetic dissimilarity present within the *Dioscorea alata* accessions collected from different regions of Kerala. The exploitation of genetic diversity so determined serves to facilitate the development of better varieties through combination and marker assisted breeding programs.

REVIEW OF LITERATURE

2. Review of literature

2.1. Root and tuber crops

Roots and tubers are the essential crops used as food in the tropics and subtropics. They belong to the class of crops that deliver energy in the form of carbohydrates. Root and tuber refer to any growing plant that stores edible material in underground root, corm, or tuber (Aidoo *et al*., 2009). These crops are related with the human survival and their socio-economic history. The Indo-Burma area is the center of origin of Asiatic edible yams. North-Eastern Himalayas and Western Ghats which are the two hotspots of global biodiversity are mainly rich in wild species of tropical root and tuber crops (Burkill, 1960). Root crops, especially cassava and yams are important energy source in the diet in many parts of Africa. Their processing into processed products with adequate taste, colour, flavour and texture add value to these crops (Sefa-Dedeh, 1995). Root crops are the main food source of the tropics, of which yams serves as the high valued crop. Though cassava and sweet potato have overtaken yams in area and production, but in best adapted regions yams remain as the dominant (O'Sullivan, 2010). Yams also have cultural significance in many parts of the world (Addy, 2012).

2.2. Yams

Yams fit to the genus *Dioscorea* and family Dioscoreaceae. They are monocots and a main food crop in Southeast Asia, tropical America, West Africa and other regions (Coursey, 1967). At least 50 of 600 known species of *Dioscorea* have been adapted for medicinal and food purposes (Hahn, 1995). Yams remain as a vital food crop, especially in Africa, and without refrigeration some of its varieties can be stored for more than three month.

Today, over 90 percent of the yam crop produced by West Africa, with Nigeria the biggest producer. Many countries in the West Indies, South America, the Pacific islands, and Asia also produce yams, though most species are grown for local consumption of people. And also some grown for small world-wide trade and some are grown for medicinal and other purposes. In different parts of the world

yam production has undergone some dramatic changes Though, the production process from cultivation, bush clearing, chemical application, harvesting and transferring to markets is still labour-intensive process (Ennin *et al.*, 2009). Maximum food production and accessibility is vulnerable where the inputs are used incompetently (Udoh and Etim, 2007). FAO (2014) annual statistical data shows that over 60.2 million tons of yams were produced in worldwide in 2013 and the area harvested in the world has increased from 1.15 million hectares in 1961 to 5.05 million hectares in 2013. Yield (Kg ha^{-1}) in the world also increased from 72.35 thousand metric tons in 1961 to 119.12 thousand metric tons in 2013.

Dioscorea alata is one of the six economically important species of yams cultivated. It has relative advantage for maintainable production due to better agronomic properties such as easy propagation and high yield, high nutritious value and can be store the fresh tubers for several months.

2.3. Botanical and agronomic characteristics

The cultivation of the most yams is very labour intensive. Usually cultivated yams do not produce seeds and so the propagation is done by manually. Some kind of framework must be given for the support of vines and at the starting of the dry season, the vines die away and the tubers are ready to be harvested (Kay, 1987).

All economically important yam species are tuberous, which produce one or more than one underground tubers, which are derived from stem tissue and are starch storing organs. The tubers deliver a means of vegetative propagation from one season to the next season. Commonly the tubers are annual crops. Though, some genotypes of yam species produce perennial tubers, which may continue to grow over some years. Many of the species yields bulbils or aerial tubers, as a means of vegetative distribution. Cultivated yams are almost entirely propagated vegetatively and as a result, cultivars are clones of the some parental plant, but spontaneous somatic mutations may have donated to the genetic difference and productivity of commercial yam cultivars (O'Sullivan, 2010).

Yams are dioecious plants and the cultivars may be male, female or sterile. Female plants produces fewer tubers and so are relatively rare among cultivated yams (Hahn, 1995) and the flowering of different genotypes at different times. But, for the most important species, promising breeding programs are ongoing, with the exception of *Dioscorea esculenta*, of which no female plants have been commonly found (O'Sullivan, 2010).

2.4. Origin and current distribution

Dioscorea is a pantropical genus and different species have been autonomously domesticated on each continent (Coursey, 1967). *D. alata* (greater yam) is the common yam species worldwide. It was believed to be originated in southern Asia, but recent genetic studies identified Melanesia as its center of origin (Lebot, 1999). It was not found in the wild and its hybridization with other family members is infrequent, although two Asian species (*D. hamiltonii* and *D. persimilis*) believed to be part of its ancestries (Burkill, 1960).

In India, it is known by different names *viz*. *Ratalu* or violet yam or the Moraga Surprise, *Pind Aaluk*, *Peruvalli kizhangu*, *Vettilavalli kizhangu*, *Kachil* and *Kavath.* In the Philippines, it is recognised as *ube* (or *ubi*) and is used as a component in many sweet desserts and in Hawaii, it is known as *uhi* and it was fetched to Hawaii by the early Polynesian settlers and it became an important crop in the 1800s when the tubers were traded to the ships as stored food supply for their journeys (White, 2003).

For the selection of species, local environmental factors also affect. Compared with *Dioscorea esculenta*, greater yam has a smaller growing season and it is more stable in wet soils and cold temperatures. It therefore has a larger range in terms of both altitude and latitude. But, it's greater susceptibility to foliar diseases, mostly the yam anthracnose caused by the fungus *Colletotrichum gloeosporioides*, reduces the use of yam in some warm and humid areas. In general, with genotypes that can completely use the available growing season, then greatest production can be achieved.

2.5. Nutritional value of yam

Yam is considered to be the greatest nutritious among the tropical root crops (Wanasundera and Ravindran, 1994). It comprises about four times as much protein as cassava, and it is the only main root crop which exceeds rice in protein content in proportion to consumable energy (Bradbury and Holloway, 1988).

Yam is also an important source of vitamins A and C, and of fibre and minerals. It has comparatively low calcium content which is related to low concentrations of calcium oxalate, an anti-nutritional factor (Bradbury and Holloway, 1988). It is also low in the anti-nutrients phytate (Wanasundera and Ravindran, 1994) and trypsin inhibitor (Bradbury and Holloway, 1988). Bhandari *et al.* (2003), done the nutritional evaluation of wild yam tubers of Nepal and for this study four wild yam species were they selected: *D. bulbifera, D. versicolor, D. deltoidea* and *D. triphylla* and the dry matter ranged from 19.8 to 30.5 % on fresh weight basis. The crude protein, ash, crude fat and crude fibre contents ranges were 1.6-3.1, 0.5-1.2, 0.2-0.3 and 0.6-1.5% of fresh weight, respectively. This study determined that nutritional characterization of wild yams were almost same to those cultivated yams in different regions of the world excluding for the high value for crude fibre found in that samples.

Baah *et al.* (2009), studied the nutritional and biochemical characterization of *D. alata* tubers. In this study they investigated the chemical and nutritional content of the 16 *D. alata* accessions. Dry matter content ranges from 22.3 to 33.8 % on a fresh weight basis with moisture content between 66.2 and 77.7 %. The ranges of crude protein, ash, sugar, starch and total dietary fibre were 4.3-8.7, 2.9-4.1, 3.6-11.0, 60.3-74.4 and 4.10-11.0 %, on dry weight basis, respectively. The results showed that these varieties could be a very good source of nutrients to its users.

In the study of characterizing diversity based on nutritional and bioactive contents of yam germplasm commonly cultivated in china Wu *et al.* (2016), used 25 yam landraces to generate an effective classification. All varieties exhibited high

amount of starch from 60.7% to 80.6% dry weight, protein 6.3-12-2% and minerals especially Mg 326.8-544.7 mg/kg on dry weight basis. The variability among the landraces was clearly differentiated in light of UPGMA clustering and principal component analysis (PCA). The results were very helpful in defining suitable application strategies for yam germplasm in China.

2.6. Morphology

The term 'yam' denotes to all members of the genus *Dioscorea* and about ten species are normally cultivated for food, while some of other species are collected from the wild in the times of food scarcity (Bhandari *et al.*, 2003).

Most yam species grow in the tropics and sub-tropic regions with proper heavy total annual rainfall, but with a certain dry season and during the rainy season, they yields one or more underground tubers to store food and water through the dry season. Many species produce bulbils, small tuber like structures, at the bases of their leaves. These can produce new plants when fallen to the ground (Kay, 1987).

D. alata commonly have non-woody vine, twining (toward the right), and glabrous, reaching 10-15 m in length and the root system is fibrous, light, mostly confined to the top 1 m of the soil. Their tubers are commonly single but varying in size and shape, often very large; cylindrical in form or globose, often variously lobed or digitated and fasciated or curved; skin brown to black; flesh white, cream or purplish. Mature stems (at the base) cylindrical and spiny. Leaves are mostly opposite, sometimes alternate on branches of rapid growth, coriaceous, broadly ovate, the apex acute or acuminate, sometimes reflexed, the base cordiform; upper surface dark green, shiny, with the venation sunken; lower surface pale green, dull, with prominent venation; petioles 4-12 cm long, 4-winged, forming an auriculate sheath at the base, with a pair of pseudostipules that clasp the stem; bulbils elongate, pendulous, attaining 15 cm long, produced when the leaves begin to wither. Inflorescences are axillary, unisexual, and pendulous. Staminate inflorescences are paniculate, 5-15 cm long, with numerous lateral and flexuous spikes that contain

many male flowers. Pistillate inflorescences are racemose, with few flowers. Fruit a 3-locular capsule, 2-3 cm wide, each locule flattened like a wing, with two seeds inside (Acevedo-Rodríguez, 2005).

Norman *et al.* (2011), studied the morphological characters of yam species from Sierra Leone. They used 53 yam genotypes and twenty-eight morphological traits measured from these genotypes. Traits that differentiated the most between the accessions were: the number of days to emergence, shoot traits and below ground traits. Factor analysis had six factors, which elucidated 75% of the total genetic variation in the requirement structure. The study exposed that wide genetic diversity be present between the yam genotypes cultivated in Sierra Leone and it could be used to breed high yielding genotypes and other wanted traits such as resistance to pest and diseases.

Anokye *et al.* (2014), determined the morphological classification of some greater yam germplasm in Ghana. This study sought to establish the genetic variability and relationships among 35 accessions with 14 accessions introduced from International Institute of Tropical Agriculture in Nigeria. PCA showed that the initial two principal components accounted for 25.27 % of overall variation from accessions. Morphological descriptors were helpful to group the accessions into different clusters which is independent of location of collection.

A study was conducted by Otoo *et al.* (2015b), to identify cultivated yam varieties, their distribution and concentration of cultivation and to document the rate and reasons of landrace loss in five yam growing societies in the forest transition agro ecological zone of Ghana. *D. rotundata* is one of the most widely cultivated yam species followed by *D. alata, D. cayenensis, D. praehensilis* and *D. bulbifera* respectively. Factors like good cooking properties, high yield, seed generation capability, good storage properties and resistance to abiotic and biotic stresses were important criteria for good varietal selection.

2.7. Molecular studies

Molecular markers are significant tools for applications such as determining the genetic diversity and phylogenetic relationships, varietal identification, mapping of important genes and QTLs, assessing population structure, selection of required genotypes in breeding programs, and for verification of progenies obtained from genetic crosses. Simple sequence repeat markers are very important and commonly used because they are codominant and multiallelic and, thus, give more information than dominant markers (Zalapa *et al.,* 2012).

Of all the techniques, microsatellite markers are the important tools with required genetic characteristics including hyper variability, codominant inheritance, multiallelic nature, relative abundance, reproducibility and widespread genome analysis (Kalia *et al.,* 2011). Based on SSRs genetic markers are used for analysing the germplasm in several yam species (Nascimento *et al.,* 2013). Only a few SSR of genomic origin, in a total of 64 primers, were developed for *D. alata* so far (Andris *et al*., 2010), which allow further studies on genetic diversity and segregation patterns (Sartie *et al.,* 2012) and hybrid documentation in mapping populations (Sartie and Asiedu, 2011) within the yam species.

Phylogenetic relations of Guinea yams with some wild families were studied by using RFLP technique of the chloroplast and nuclear ribosomal DNA (Terauchi *et al.,* 1992). In this study four different classes with *D. rotundata* and *D. cayenensis* were categorised in the same chloroplast DNA-defined group as the wild species *D. praehensilis, D. abyssinica* and *Dioscorea liebrechtsiana* de Wild. The remaining three classes recognised among the wild species contained *Dioscorea minutiflora* Engl. *Dioscorea burkilliana* J. Miège and *Dioscorea smilacifolia* de Wild and *Dioscorea togoensis* Knuth. Terauchi *et al.* (1992), suggested that based on the ribosomal DNA analysis *Dioscorea cayenensis* should be regarded as a variety of *D. rotundata*.

Tamiru *et al.* (2007), studied the genetic diversity in yam species collected from Ethiopia and their relationship with the main cultivated *Dioscorea* species

analysed by AFLP markers. For the study they used amplified fragment length polymorphism (AFLP) for determining the genetic diversity among the 48 yam accessions from Ethiopia, and to access their relatedness to yam species which are commonly cultivated in West Africa such as *D. alata* L., *D. cayenesis* Lam., *D. bulbifera* L. and *D. rotundata* Poir. From ten AFLP primer combinations 900 fragments produced, of which 97 % were polymorphic. Principal coordinate and Cluster analysis revealed that the selected Ethiopian accessions are divergent from the commonly cultivated *Dioscorea* species in West Africa.

Random amplified polymorphism DNAs were used for determining the genetic relationships between *D. alata* cultivars (Asemota *et al.,* 1996) and amplified fragment length polymorphism (AFLP) were used for studying the genetic relationships of Guinea yams (Mignouna *et al.*, 1998) and to develop genetic maps of three *Dioscorea* species (Terauchi and Kahl, 1999; Mignouna *et al.*, 2002a, b).

Mignouna *et al.* (2002a), constructed a genetic linkage map for water yam based on AFLP markers and QTL analysis for anthracnose resistance. It was made based on 469 co-dominantly recorded AFLP markers segregating in an interspecific F1 cross and the 469 markers were mapped on 20 linkage groups with 1,233 cM overall length and 2.62 cM mean spacing. QTL mapping exposed one marker E-14/M52-307 which is located on linkage group 2 and was connected to anthracnose disease, which explain 10 % of overall phenotypic variation. It was the first linkage map reported for *D. alata* which covers 65 % of the genome and provides a better tool for further genetic analysis of traits and for marker assisted selection in *D. alata* future breeding programs and QTL mapping unlocks new avenues for gathering anthracnose resistance genes in *D. alata* cultivars.

Malapa *et al.* (2005), determined the genetic diversity of the greater yam and relatedness to *D. nummularia* Lam. And *D. transversa* Br. As revealed with AFLP markers. Four consecutive studies were showed with importance on the genetic relatatedness within *D. alata* and among species of the Enantiophyllum section from Vanuatu. The total results showed that each accession can be

fingerprinted uniquely using AFLP technique. UPGMA cluster analysis exposed the presence of three major groups of genotypes within *Dioscorea alata*, each accumulating accessions from distant geographical origins and different ploidy levels.

New microsatellite loci for greater yam and its cross-amplification with other *Dioscorea* species is determined by Siqueira *et al.* (2011). Using a microsatellite-enriched genomic library technique they isolated 14 codominant polymorphic microsatellite markers. Ten microsatellite loci were taken and studied using eighty different *D. alata* accessions from different places in Brazil. The polymorphism information content (PIC) ranged from 0.39 to 0.78 and the power discrimination (PD) varied from 0.15 to 0.91. Transferability for the species *D. bulbifera*, *D. cayenensis*, *D. rotundata* and *D. trifida* were revealed by six of these markers. The resultant SSR markers can be used a great tool for studies aiming for characterizing the genetic diversity in *D. alata* and other *Dioscorea* species accessions.

In DNA fingerprinting of 36 water yam species in Brazil done by Siqueira *et al.* (2012), using nine microsatellite markers, loci resulted higher polymorphism proved by elevated PIC values (0.57-0.77), and by larger gene diversity. Several duplicates were identified in this study. But, some of these accessions were categorized in different sub groups. Results suggested the assumption of different origins for accessions currently cultivated in Brazil. Similar accessions resulted from different municipalities showed the commercialization of the same accessions at different places.

By using AFLP markers, genetic diversity and species restriction in cultivated and wild Guinea yams (*Dioscorea* spp.) from South West Ethiopia was determined. In this study, amplified fragment length polymorphism (AFLP) genetic fingerprinting was used to calculate and characterize 43 individual plants, collected from different populations of wild and cultivated Guinea yams. In the AFLP analyses the three primer combinations resulted 158 scorable bands, with a total polymorphism of 78 % (Mengesha *et al.,* 2013).

Muthamia *et al.* (2013), estimated the genetic diversity among the Kenyan yam accessions using microsatellite markers. The study was conducted with 187 accessions consisting of 166 yam landraces and also 21 yam DNA samples received from IITA, Nigeria. Twelve primers were used for analysing genetic diversity and an overall of 131 alleles were amplified with maximum of 13 alleles and minimum of two alleles per primer with a high number of polymorphic alleles. Dense clustering of accessions indicated genetic similarity. The molecular variance study revealed that most variation of 88 % was found within the selected populations. The simple sequence repeats (SSR) markers were polymorphic and able to differentiate local yam landraces.

Genetic diversity analysis of yam accessions cultivated in China using ISSR and SRAP markers is done by Wu *et al.* (2014). In that study, ISSR and sequence related amplified polymorphism (SRAP) techniques were used for determining the genetic diversity between 21 yam landraces collected from seven different cultivated populations. They detected high amount of polymorphism among landraces, specifically, 95.3 % for ISSR and 93.5 % for SRAP. The UPGMA clusters and principal component analysis for 21 landraces formed four well differentiated groups which contain landraces namely, *D. alata* L., *D. opposita* Thunb., *D. persimilis* Prain et Burkill, and *D. fordii* Prain et Burkill. Also the primers which are used for this study were highly polymorphic among the 21 landraces. The lack of genetic diversity within the individual yam species indicate that it is essential to develop long term strategies for boosting genetic diversity within various yam species.

Molecular classification of same greater yam germplasm in Ghana using SSR markers is done by Otoo *et al.* (2015a). They investigated genetic diversity and relationships between 35 *D. alata* varieties collected from different regions of Ghana including 14 samples from IITA in Nigeria. This study was conducted using 14 SSR primer pairs available for yams. From the analysis of allele frequency, the allele sizes ranged from 100 to 510 bp. Gene diversity was high which ranged from 0.87 in YM 13 to 0.94 in YM 30 with a mean of 0.92. Mostly, all primers have the

allele frequency below 0.95 shows that they were all in polymorphic character and the results of this investigation confirm that SSR molecular markers were able to identify closely related accessions within the species.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The study entitled "Genetic diversity analysis of greater yam (*Dioscorea alata*) landraces in Kerala" was carried out at the Crop Improvement division, ICAR-Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram during 2015-2016. In this chapter, detailed information of the experimental material and procedures used in the study are elaborated.

3.1. Source of plant material

The plant materials used for molecular marker for genetic diversity studies comprises of forty five accessions of *D. alata* collected from germplasm maintained in the field gene bank at ICAR-CTCRI, Thiruvananthapuram. The details of materials used for the study is given in Table 1.

The tubers of the collected accessions were planted on the field at the end of April. Tuber cuttings were planted on mounds prepared at a spacing of 1m×1m. The vines were supported on a stake of about 2m height. Harvest was done during January-February by manually digging out the tubers and the tubers were cleaned free of soil and kept in ventilated yam storage house till the next planting season.

3.2. Molecular marker analysis

In order to study the genetic diversity among greater yam accessions, the molecular characterization using ISSR and SSR markers were carried out.

3.2.1. Source of primers

The primers were selected from collected literatures showing high polymorphic values in *Dioscorea* species, ordered and shipped from Integrated DNA Technologies, Inc., as lyophilized form. The list of primers used is given in Table 2 and Table 3.

Table 1. List of Accessions of greater yam used for the study

Sl. No.	Sample No.	Accession No.	Vernacular Name	District
1	A1	Da 340	Kalakkompan	Idukki
2	A2	Da 240	Kachil	Paravatty, Thrissur
3	A3	Da 8	Nadan chappan	Thrissur
4	A4	Da 13	Nadan chappan	Malappuram
5	A5	Da 343	Kavathu	Malappuram
6	A6	Da 215	Nadan kachil	Kallada, TVPM
7	A7	Da 278	Pal kachil	Omalloor, Pattanamthitta
8	A8	Da 289	Kachil	Omalloor, Pathanamthitta
9	A9	Da 243	Kachil	Mala, Thrissur
10	A10	Da 303	Anakkomapan	TVPM
11	A11	Da 287	Pulinthodan	Sarkara, TVPM
12	A12	Da 222	Kachil	NBPGR, Thrissur
13	A13	Da 313	Kachil	Pathanapuram
14	A14	Da 312	Kachil	Pathanapuram
15	A15	Da 308	Kachil	Pathanamthitta
16	A16	Da 122	Kachil	Wayanad
17	A17	Da 209	Kachil	Nedumangad, TVM
18	A18	Da 21	Chuvanna Muramchari	Vellayani, TVM
19	A19	Da 28	Kachil	Ernakulam
20	A20	Da 52	Aanakkalan kachil	Palode, TVPM
21	A21	Da 65	Kachil	Pariyaram
22	A22	Da 40	Elivalan	Kallar

23	A23	Da 68	Bharani Kachil	Kumali
24	A24	Da 53	Pathi kachil	Arippa
25	A25	Da 33	Kachil	Ernakulam
26	A26	Da 70	Thekkan kachil	Kaladi
27	A27	Da 27	Kuda kachil	Ponkunnam
28	A28	Da 102	Nadu chuvappan	Nedumkandam, Idukki
29	A29	Da 114	Erachi Kavithu	Kunnappally, Malappuram
30	A30	Da 241	Kachil	Vadanappally,Thrissur
31	A31	Da 73	Muramchari	Chowara
32	A32	Da 58	Kolla Kachil	Aalancheri
33	A33	Da 120	Wayanad	Wayanad
34	A34	Da 56	Ezhankulam	Ezhankulam
35	A35	Da 84	Vazhakulam	Vazhakulam
36	A36	Da 113	Ottapalam	Ottapalam
37	A37	Da 88	Alapuzha	Alapuzha
38	A38	Da 85	Nedumkandam	Nedumkandam
39	A39	Da 118	Kaduvakkayyan	Wayanad
40	A40	Da 69	Kaduvakkayyan	Kumali
41	A41	Da 99	Vettu Kachil	Kaladi
42	A42	Da 81	Parisakodan	Vazhakulam
43	A43	Da 390	Kavithu	Kasargod
44	A44	Da 331	Neela Kachil	Pathanamthitta
45	A45	Da 391	Neela Kachil	Pathanamthitta

Table.2. List of ISSR primers used for molecular characterization

Sl. No.	Primer name	Sequence (5'⟷ 3')
1	UBC 808	AGAGAGAGAGAGAGAGC
2	UBC 809	AGAGAGAGAGAGAGACG
3	UBC810	GAGAGAGAGAGAGAGAT
4	UBC 811	GAGAGAGAGAGAGAGAC
5	UBC 817	CACACACACACACACAA
6	UBC 825	ACACACACACACACACT
7	UBC 827	ACACACACACACACACG
8	UBC 864	ATGATGATGATGATGATG
9	UBC 818	CACACACACACACACAG
10	UBC 836	AGAGAGAGAGAGAGAGYA
11	(GA)9AC	GAGAGAGAGAGAGAGAGAAC
12	(GA)9AT	GAGAGAGAGAGAGAGAGAAT
13	UBC 14	CGCGCGCGCGCGCGCGTG
14	(ACC)6Y	ACCACCACCACCACCACCY
15	UBC 807	AGAGAGAGAGAGAGAGT

Table. 3. List of SSR primers used for molecular characterization

Sl. No	Primer name	Sequence (5' ⟷ 3')	Sequence (3'⟷ 5')
1	Dab205	CCCATGCTTGTAGTTGT	TGCTCACCTCTTTACTTG
2	Dab2D06	TGTAAGATGCCCACATT	TCTCAGGCTTCAGGG
3	Dab2E07	TTGAACCTTGACTTTGGT	GAGTTCCTGTCCTTGGT
4	Dab2D08	ACAAGAGAACCGACATAGT	GATTTGCTTTGAGTCCTT
5	Da1A01	TATAATCGGCCAGAGG	TGTTGGAAGCATAGAGAA
6	Dpr3F04	AGACTCTTGCTCATGT	GCCTTCTTACTTTATTC
7	YM5	AATGAAGAAACGGGTGAGGAAGT	CAGCCCAGTAGTTAGCCCATCT
8	YM15	TACGGCCTCACTCCAAACACTA	AAAATGGCCACGTCTAATCCTA
9	YM26	AATTCGTGACATCGGTTTCTCC	ACTCCCTGCCCACTCTGCT

3.2.2. Glass wares and materials in Molecular Biology Lab

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

3.2.3. Instruments

The equipments *viz.* Ice machine, weighing balance, water bath, vortexer, dry bath, centrifuge, nanodrop spectrophotometer, pH meter, electrophoresis apparatus, hot air oven, autoclave, PCR machine, alpha imager, UV trans illuminator, Deep freezer(-20^{0}C, -80^{0}C), refrigerator and distilled water unit were used for the study.

3.2.4. DNA Extraction

3.2.4.1. Sample collection

Fresh tender young leaves of greater yam accessions were collected from germplasm of CTCRI, Thiruvananthapuram. Leaves were collected in plastic bags and transferred to lab under refrigeration.

3.2.4.2. Manual method

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

Destarched leaf tissues (200–250mg) were homogenized to a fine powder using liquid nitrogen. Pre-warmed extraction buffer (1ml) was added to the samples and it was ground once more. The samples were transferred to 2.0ml Microfuge tubes and 10µl Proteinase K (10mg/ml) was added. The tubes were incubated at 37°C for 30min and then at 65°C for another 30min with frequent swirling. Samples were centrifuged at 12,000rpm for 15min at RT and supernatant was transferred to fresh microfuge tube. Equal volume of Chloroform: Isoamyl alcohol (24:1) were added and mixed by gentle inversion for 30–40 times. The samples were centrifuged at 12,000rpm for 10min at RT and the supernatant was transferred to a

fresh tube. The above step was repeated again to remove any further proteins present. To the supernatant collected in a fresh tube, 150µL of 2M NaCl solutions containing 4% PEG was added. The samples were centrifuged at 12,000rpm for 10min at RT. The supernatant was transferred to a fresh tube and precipitated with 200µl of ethanol. The nucleic acids was precipitated and collected by centrifuging at 12,000rpm for 10min. The nucleic acid pellet was washed twice with wash solution, air-dried until the ethanol evaporated and dissolved in appropriate amount of TE buffer (100–150µl). The nucleic acid dissolved in TE buffer were treated with ribonuclease (RNase, 10mg/ml), incubated at 37°C for 30min and stored at -20°C until use. All samples were checked for DNA in 1% agarose gel and confirmed.

3.2.4.3. Using DNeasy® Plant Mini Kit

Young leaves collected were weighed out and about 100-200mg was pulverized in pestle and mortar along with 400 µl of Buffer AP1 and 4 µl of RNase A. Vortexed well and it is incubated for 10 min at 65°C. the tubes were inverted for 2–3 times during the incubation. It is mixed well and incubated for 5 min on ice. Centrifuged the mixture for 5 min at 20,000 x g (14,000 rpm). Pipetted the lysate into a QIAshredder spin column placed in a 2 ml collection tube. Centrifuged for 2 min at 20,000 x g. Transferred the flow-through into a new tube without disturbing the pellet if present. Added 1.5 volumes of Buffer AW1, and mixed by pipetting. Transferred 650 µl of the mixture into a DNeasy® Mini spin column placed in a 2 ml collection tube. Centrifuged for 1 min at ≥6000 x g (≥8000 rpm). Discarded the flow-through.Repeated this step with the remaining sample. Placed the spin column into a new 2 ml collection tube. Added 500 µl Buffer AW2, and centrifuged for 1 min at ≥6000 x g. Discarded the flow-through. Added another 500 µl Buffer AW2. Centrifuged for 2 min at 20,000 x g. Removed the spin column from the collection tube carefully so that the column does not come into contact with the flow-through. Transferred the spin column to a new 1.5 ml or 2 ml micro centrifuge tube. For eluting the DNA added 100 µl Buffer AE for elution and it was incubated for 5 min at room temperature (15–25°C). Centrifuged for 1 min at ≥6000 x g. Repeated the last step and kept the sample in -20^{0}C refrigerator. All the samples were checked

for DNA in 1% agarose gel and confirmed. DNeasy® Plant Mini Kit was comparatively less time consuming and DNA obtained using this method has high purity.

3.2.5. Quantification of DNA

Isolated DNA was quantified using Nanodrop spectrophotometer. It helped to assess its yield and purity. TE buffer in which the DNA was dissolved was used to calibrate the machine to blank i.e. zero absorbance. The advantage of Nanodrop is it require only 1.5 µl sample to measure its quantity and purity. The quantity of the DNA is determined at OD 260 and the purity was determined by OD 260/OD 280 ratio. According to the better absorbance value/ OD value samples are selected.

3.2.6. Dilution of samples

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

3.2.7. Primer dilution

Primers like ISSR, and SSR were selected based on their ability to show high polymorphism. They were ordered and shipped in lyophilized form. Before opening for first time, it was briefly centrifuged to avoid loss of DNA pellet. The oligos were dissolved in nuclease free water. Initially freezer stock was made at 100 micro molar concentrations by adding a volume of nuclease free water equal to ten times the number of nano moles of DNA present in the tube and stored as main stock. Working stock of 10 micro molar concentration were made by taking 10 µl from the main stock and diluted it with 90 µl of nuclease free water and stored in -20^0C refrigerator.

3.2.8. PCR amplification

The diluted samples were amplified in thermal cycler using different primers of ISSR and SSR at different conditions, temperature gradients. Screened the best primers and optimum amplifying conditions were standardized for all primers of ISSR and SSR. The reaction mixture for the primers are given in Table 4 and 5.

3.2.8.1. ISSR PCR conditions

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

Lid – 105^0C

94^0C – 5 mins (initial denaturation)

94^0C – 30 sec (denaturation)

56^0C – 1 min (annealing)

72^0C – 1 min (extension)

Cycles x 35

72^0C – 10 mins (final extension)

4^0C – hold

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

3.2.8.2. SSR PCR conditions

PCR was carried out in Proflex Thermocycler. The program for Dab2 primers are as follows;

Lid – 105^0C

94^0C – 5 mins (initial denaturation)

94^0C – 30 sec (denaturation)

56^0C – 1 min (annealing)

72^0C – 1 min (extension)

Cycles x 35

72^0C – 8 mins (final extension)

4^0C – hold

Table 4. ISSR reaction mixture

Components	Stock concentration	Required concentration	Volume for one reaction (15µl)
Emerald master mix	2x	1x	7.5 µl
Primer	100 µM	0.3 µM	0.5 µl
$MgCl_2$	50 Mm	1.3 Mm	0.4 µl
DNA	10ng/µl	20ng	2 µl
dH_2O	---	---	4.6 µl
Total		15 µl	

Table 5. SSR reaction mixture

Components	Stock concentration	Required concentration	Volume for one reaction (15µl)
Emerald master mix	2x	1x	7.5 µl
Forward Primer	10 µM	0.3 µM	0.5 µl
Reverse Primer	10 µM	0.3 µM	0.5 µl
$MgCl_2$	50 Mm	1 Mm	0.3 µl
DNA	10ng/µl	50ng	5 µl
dH_2O	---	---	1.2 µl
Total			15 µl

Annealing temperature changed to 58^0C for primers YM 5, YM 15, YM 26 for better results. The amplified products were separated on 2% agarose gel along with 1Kb and 100bp ladders to identify molecular weight of obtained bands and for polymorphism studies.

3.2.9. Agarose Gel Electrophoresis (AGE)

Agarose gel electrophoresis is used for separating and analysing DNA molecules in which DNA molecules were separated based on the charge by giving an electric field to the electrophoretic apparatus. Smaller molecules move more easily and faster than larger molecules through the pores present in the gel and this process is called sieving and increasing the concentration of agarose will also increase sieving effect. The gel can be used to look at the DNA in order to quantify the amount of DNA or to isolate a particular band of interest. By the addition of ethidium bromide the intercalating agent along with agarose solution the DNA can be visualized when exposed on UV light.

Weighed 2.4g of agarose in 250ml conical flask, added 120ml 1X TBE buffer and gently boiled the solution in microwave oven until agarose completely dissolved in buffer. Allowed it to cool and added 1µl/ml ethidium bromide carefully without spilling. Prepared the casting tray and kept combs in position. Poured the warm gel to tray and allowed to set in 20 mins. Filled the horizontal electrophoretic tank with 1X TBE buffer. After that the solidified gel is transferred to the electrophoretic apparatus and remove the combs. Loaded 10 µl samples and also added 2 µl 100 bp and 2 µl 1 Kb ladders for reference. Run the gel at 100V for 1 to1.5 hour. Visualized the band under UV trans illuminator and documented the image on alpha imager. The images were finally scored to detect polymorphism.

3.3. Morphological analysis

The plant and tuber morphology was done using descriptors of yam (IPGRI/ IITA, 1997). Thirty three descriptors were used for characterization (Table 6 and 7) RHS colour chart is used for determining the colour characters. The respective colour codes are given for each characters.

3.4. Biochemical characterization

The tuber samples were used for analysing the biochemical properties and nutritional qualities. The harvested tubers were collected and cleaned for analysing dry matter, starch, sugar and protein contents.

3.4.1. Dry matter analysis

The yam tubers were washed, peeled and sliced into cubes. Weighed 50 g of slices and dried in hot air oven at a temperature of 60°C. The samples were weighed until constant weight obtained. The dry matter percentage was calculated using the following formula.

$$\text{Dry matter percentage} = \frac{\text{Dry weight (DW)}}{\text{Fresh weight (FW)}} \times 100$$

The dried samples were floured and stored in plastic bottles. The flour was used for further biochemical analysis.

3.4.2. Starch and sugar analysis

Starch is the major polysaccharide present in most of the cereal and tuber crops. It is a major determinant of the energy value of tuber crops. Besides starch, reducing as well as non-reducing sugars are also present in tuber crops. The content of the total sugar decides the processing quality of the tubers for various food uses. A rapid titrimetric method has been standardized at CTCRI which permits precise quantification of the starch and total sugar content in fresh tubers, dry chips, flour and processed products.

Table 6. The qualitative characters used for morphological analysis

Sl. No.	Descriptor/trait	Note/Description
1	Young vine colour	1. Green (Yellow-Green 144) 2. Purplish green 3. Brownish green (Grey Brown N199) 4. Dark brown (Grey Brown 199) 5. Purple (Red Purple 59) 99. Other
2	Mature vine colour	1. Green (Green 137) 2. Purplish green 3. Brownish green (Grey Brown N199) 4. Dark brown (Grey Brown 199) 5. Light Purple (Red Purple 59) 6. Dark purple (Red Purple 59) 99. Other
3	Colour of wings	1. Green (Yellow-Green group 144) 2. Green with purple edges (Yellow Green 144) 3. Purple (Red Purple 59) 99. Other
4	Colour of young leaves	1. Yellowish (Yellow Green N144) 2. Pale green (Yellow Green 144) 3. Dark green (Green group 137) 4. Light Brown (Grey Brown N199) 5. Dark brown (Grey Brown 199) 6. Purplish green 7. Purple (Red Purple 59) 99. other
5	Young leaf vein colour	1. Yellowish (Yellow Green N144) 2. Green (Yellow Green 144) 3. Greenish purple 4. Pale purple 5. Purple (Red Purple 59) 99. Other
6	Mature leaf vein colour (upper)	1. Yellowish (Yellow Green N144) 2. Green (Yellow Green 144) 3. Greenish purple 4. Pale purple 5. Purple (Red Purple 59) 99. Other
7	Anthracnose susceptibility	3. Low 5. Intermediate 7. High

Sl. No.	Descriptor/trait	Note/Description
8	Mature leaf colour	1. Yellowish (Yellow Green N144) 2. Pale green (Yellow Green 144) 3. Dark green (Green 137) 4. Light Brown (Grey Brown N199) 5. Dark brown (Grey Brown 199) 6. Purplish green 7. Purple (Red Purple 59) 99. other
9	Leaf shape	1. Ovate 2. Cordate 3. Cordate long 4. Cordate broad 5. Sagitate long 6. Sagitate broad 7. Hastate 99. Other
10	Leaf margin colour	1. Light purple (Red Purple 62) 2. Purple (Red Purple 59) 3. Light green (Yellow Green 144) 4. Dark green (Green 137) 99. Others
11	Petiole colour	1. All green with purple base 2. All green with purple leaf junction 3. All green with purple at both ends 4. All purplish green with purple base 5. All purplish green with purple junction 6. All purplish green with purple at both ends 7. Green (Yellow Green 144) 8. Purple (Red Purple 59) 9. Brownish green 10. Brown 11. Dark brown 99. other
13	Leaf tip shape	1. Aristate 2. Acuminate
14	Flowering	0. No flowering 1. Flowering

Sl. No.	Descriptor/trait	Note/Description
15	Sex	1. Female 2. Male 3. Female &male 4. Male & female
16	Tuber shape	1. Round 2. Oval 3. Oval-oblong 4. Cylindrical 5. Conical 6. Spindle shape 7. Digitate 8. Flattened 9. Irregular 99. Other
17	Presence of scale	0. Absent 1. Present
18	Absence/presence of aerial tubers	0. Absent 1. Present
19	Shape of aerial tubers	1. Round 2. Oval 3. Elongate 4. Irregular (not uniform)
20	Tuber skin colour	1. Yellow brown 2. Pale brown 3. Medium brown 4. Blackish brown 5. Black 99. Other
21	Tuber oxidation colour	1. Grey 2. Purple 3. Orange 99. Other
22	Tuber cortex colour	1. Light purple (Red Purple 62) 2. Purple (Red Purple N74) 3. Dark purple(Purple N79) 4. Light greenish yellow 5. Light cream (Yellow White 158) 6. Light yellow (Yellow Orange 17) 99. Others

Sl. No.	Descriptor/trait	Note/Description
23	Tuber flesh colour	1. White (White 155) 2. Yellowish white or off-white (Yellow White 158) 3. Yellow (Yellow Orange 17) 4. Orange (Yellow Orange 19) 5. Light purple (Red Purple 65) 6. Purple (Purple Violet N80) 7. Purple with white 8. White with purple 9. Outer purple/ inner yellowish 99. Other
24	Tuber skin texture	1. Smooth 2. Moderately smooth 3. Warty 4. Highly warty
25	Hairiness of tuber	1. Very low 2. Low 3. Medium 4. High 5. Very high
26	Culinary quality/ cooking quality	
	a) Softness	1. Sf – Soft 2. H – Hard
	b) Stickiness	1. Non sticky 2. Sticky 3. Very sticky
	c) Colour	1. Not coloured 9. Highly coloured
	d) Taste	1. Excellent 2. Good 3. Average 4. Poor

Table 7. The quantitative characters used for morphological analysis

Sl. No.	Descriptor/trait	Note/Description
1	Mature stem diameter (cm)	At 15 cm from the base of the plant
2	Leaf length (cm)	3. Short 5. Medium 7. long Mean of five measurements recorded at fifth month
3	Leaf breadth (cm)	3. narrow 5. Medium 7. wide Mean of five measurements recorded at fifth month
4	Petiole length (cm)	3. ≤ 5 cm (short) 5. 6-9 cm (Medium) 7. ≥ 10 cm(Long) Mean of five measurements recorded at fifth month
5	Distance between leaf lobes	3. Not measurable 5. Measurable 7. Far
6	Position of the widest part in leaf	1. Third upper 2. Middle 3. Third lower
7	Length of tuber (cm)	3. ≤ 20 cm (Short) 5. 21-40 cm(Medium) 7. ≥ 41 cm (Long)
8	Girth of tuber(cm)	3. Short 5. Medium 7. long
9	Skin thickness (mm)	1. < 1 mm (Thin) 2. ≥ 1 mm(Thick)
10	Weight/tuber (Kg)	3. Low 5. Medium 7. High

3.4.2.1. Preparation of samples

Tubers washed free of dirt were cut into small pieces. 2.0 g of each sample were weighed into 100 ml Erlenmeyer flasks and 20.0 ml 80% ethanol were added to each flask and left for overnight incubation to extract the sugar content. Flour or powdered samples of food products (1.0 g) can also be taken similarly. In the case of leaf samples, initial treatment with 80% acetone to remove pigments is required and the dried leaf powder (2.0 g) can be used for starch estimation.

The extracted sugars were separated from the residue, by filtration using Whatman No.1 filter paper and the filtrate was collected separately for sugar estimation. Residue on the filter paper was washed with 2 lots (10 ml each) of distilled water to remove the adhering sugar particles and the filtrate added to the original filtrate. The residue were taken back into the Erlenmeyer flask by adding 20 ml of 2N HCl. The starch present in that residue was then hydrolysed by leaving those flasks on a hot plate at 100^0 C for 30 minutes. After that the hydrolysates were allowed to cool to room temperature and the volume was made up to 100 ml using distilled water. This supernatant was then directly used for starch estimation.

1.0 ml concentrated HCl were added and heated for 30 minutes on a hot plate at 100^0 C for treating alcoholic sugar filtrate. The volume of the extract of sugar is adjusted to 50 ml and used for the sugar estimation.

3.4.2.2. Titrimetric Assay

Pipetted out 10 ml of Potassium ferricyanide into 100 ml Erlenmeyer flasks and to this, 5.0 ml NaOH (2.5N) was added. Mixed the contents thoroughly. The flasks were then kept over the flame for boiling. When the reagent began to boil, the flame was lowered and 3 drops of dilute methylene blue was added. The solution immediately turned blue-green. The starch hydrolysate was taken in a 2.0 ml blow pipette during starch estimation, the sugar extract was taken in a 10.0 ml blow pipette. The nearing of the end point is indicated by change of colour from blue-green to violet. A few more drops were added carefully, to reach the end point,

which is indicated by the rapid disappearance of the violet colour. At this stage, the titre reading was noted. Titrations were repeated for each of the aliquots.

3.4.2.3.. Calculations

The starch content of the sample is calculated from the formula,

$$\text{Starch (g/100g FW)} = \frac{\text{Volume of ferricyanide x made up volume x 0.9 x100}}{\text{Titre value x weight of sample x 1000}}$$

For expressing starch on dry matter basis,

$$\text{Starch (g/100g DM)} = \frac{\text{Starch content (g/100g fresh weight) x 100}}{\text{Dry Matter (\%)}}$$

For calculating the total (reducing + non - reducing) sugar content:

$$\text{Sugar (g/100g fresh weight)} = \frac{\text{Volume of ferricyanide x made up volume x 100}}{\text{Titre value x weight of sample x 1000}}$$

$$\text{Sugar (g/100g DM)} = \frac{\text{Sugar (\% in FW basis) x 100}}{\text{Dry matter \%}}$$

3.4.3. Protein analysis

Kjehdahl method was used to determine crude protein content. This method involves stages of digestion, distillation and titration.

200 mg of well grinded tuber sample weighed and transferred to the digestion tube. Digestion mixture (Na/K sulphate + $CuSO_4$) and 10 ml H_2SO_4 was added to the sample. The tube was then kept in the digestion rack for 2 h at 420^0C. The sample allowed to cool and to this added 50 ml distilled water.

The tube was then kept in the distillation unit (Kelplus – classic Dx). The distillation was done with 60ml 40% NaOH for each sample. The distilled ammonia was collected in a flask containing 20 ml 4% boric acid. This was then titrated against 0.1 N H_2SO_4. Appearance of a light pink colour was the end point. The Nitrogen value obtained from the titration was multiplied with 6.25 for determining the percentage of crude protein.

The total Nitrogen by Kjeldahl method,

$$\% \text{ of } N_2 = \frac{\text{Titre value x Normality of acid x 1.4}}{\text{Weight of the sample}}$$

Crude protein concentration = % of N_2 x 6.25

3.5. Statistical Analysis

Clear and reproducible bands were only selected for scoring. Binary scoring was carried out by assigning "1" for presence of band and "0" for absence of band. A binary matrix which indicate the presence/absence of bands was obtained from agarose gels for each SSR and ISSR markers. The data matrix created in excel format was used as the input for cluster analysis. Estimation of genetic diversity parameters results in a summary of the genetic variability and it is used as a criteria for comparison of both molecular marker efficiency and clusters from different studies. (Laurentin, 2009).

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

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

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

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

PIC =2fi (1 − fi)

Where fi is the amplified allele frequency and 1 − fi is the null allele frequency.

Heterozygosity per locus was calculated according the formula:

He = 1 − p2 − q2, where

p2 =fi. Average heterozygosity per marker was determined based on:

Hav = Σ (He / L), where L = total of detected bands.

3.5.2. Cluster Analysis

A binary matrix which indicate the presence or absence of bands was got from the agarose gels for each SSR and ISSR markers. Jaccard's similarity coefficient was determined for using in cluster analysis by Unweighted Pair-group Method with Arithmetic Average (UPGMA). Based on Jaccard's similarity (J) coefficient genetic similarity between various accessions was done using SIMQUAL programme of NTSYSpc v. 2.20 (Rohlf, 2005). For constructing UPGMA dendrograms for ISSR, SSR markers and morphological qualitative using SAHN programme of NTSYSpc v. 2.20 Jaccard's similarity coefficients of different accessions were used (Rohlf, 2005). Dendrogram grouping the 45 accessions based on SSR, ISSR and morphological data was also carried out based on complete linkage method using Dice coefficient on employing bootstraps using DarWin6.0 package.

For quantitative traits, data were used for analysis of variance (ANOVA) and LSD test was done to determine genotypes that were significantly divergent from each other.

Principal component analysis (PCA) and correlation was obtained using PRINQUAL method of SAS 9.3 software (SAS, 2011)

Genetic parameters were determined to identify genetic variation among landraces and effect of genetic and environmental factors on characters. The genotypic and phenotypic components of variance, coefficients of variability, broad

sense heritability and genetic advance were estimated by adapting the formulae suggested by Allard (1960) and Singh and Chaudhary (1977).

i. Genotypic variance (σ2 g) =$(Msg - Mse)/r$, where Msg = mean square of accession, Mse = mean square of error, and r = number of replications

ii. Environmental variance (σ2e) = Mse

iii. Phenotypic variance (σ2 p) = σ2 g + σ2 e

iv. Genotypic coefficient of variation (GCV) = √(σ2 g/X)*100

v. Phenotypic coefficient of variation (PCV) = √(σ2 p/X)*100 where, X = grand mean

vi. Heritability in broad sense (h^2) = σ2 g/σ2 p

vii. Genetic advance as per cent of mean Expected genetic advance

$$GA = (K)\ \sigma A\ H^2$$

Where, K = Selection differential (2.06 at 5% selection intensity) σA= Phenotypic standard deviation

RESULT

4. RESULTS

The results of the study entitled "Genetic diversity analysis of greater yam (*Dioscorea alata* L.) landraces in Kerala" was carried out at the Crop Improvement division, ICAR-CTCRI, Sreekariyam, Trivandrum during 2015-2016 are presented in this chapter.

4.1. Molecular Data Analysis

4.1.1. Standardization of DNA isolation protocol

Isolation of DNA on *Dioscorea alata* were tried by using both kit method and manual method to identify optimum method to extract quality DNA with better concentration. DNA isolated from kit method (DNeasy® Plant Mini Kit) was of good quality and this is a rapid and easy use method. However the concentration is less. The range of DNA yield by this method is 6.63 ng – 196.21 ng. So for getting high concentration of DNA manual method of DNA isolation modified by Raj *et al.* (2014) for the yam species is performed. By using this method very good yield of DNA (90.6-2391.97) obtained with high purity. DNA gel check using 1% agarose is shown in Plate 1.

4.1.2. PCR standardization

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

4.1.3. Primer screening

For preliminary screening of ISSR, 16 primers were used of which, only 15 were selected based on their polymorphism and repeatability. ISSR UBC 848 were eliminated after preliminary screening due to poor amplification profile.

For preliminary screening of SSR, 11 primers were used of which only nine were selected for the study based on their polymorphism and repeatability. SSR primers like MT 10 and MT 13 is eliminated after preliminary screening due to poor amplification profile.

4.1.4. ISSR analysis of greater yam accessions

The amplicons obtained after ISSR analysis of accessions using the selected primers were initially resolved in two percent agarose gel (Plates 2 and 3). Genetic characteristics of the fifteen ISSR markers used to analyse greater yam accessions is given in Table 8. Total number of bands per ISSR primer ranged from 5 (UBC 817) to 12 (UBC 809 and $(GA)_9AT$). The primers UBC 14 recorded 6 bands, UBC 825 and UBC 810 recorded 8 bands, $(GA)_9AC$ and UBC 836 recorded 9 bands, UBC 808 and UBC864 recorded 10 bands followed by UBC 811, UBC 827, UBC 807, UBC 818 and $(ACC)_6Y$ recorded 11 bands.

The polymorphism of the ISSR primers studied ranged from 90.9% to 100%. $(ACC)_6Y$ and UBC 818 Recorded the lowest polymorphism (90.9%) among the primers followed by $(GA)_9AT$ (91.7%).

The Observed heterozygosity value (Hobs) of the ISSR primers ranged between 0.7389 (UBC 817) to 0.8871 (UBC 809). For most of the ISSR primers studied Hobs was found to be >0.8.

The Polymorphism Information Content (PIC) range of the primers was from 0.6918 (UBC 817) to 0.88 (UBC 807). The studied primers showed PIC value > 0.8.

In the present study, the ISSR primer produced an average of 9 polymorphic alleles with mean Hobs and PIC values of 0.8619 and 0.8472 respectively.

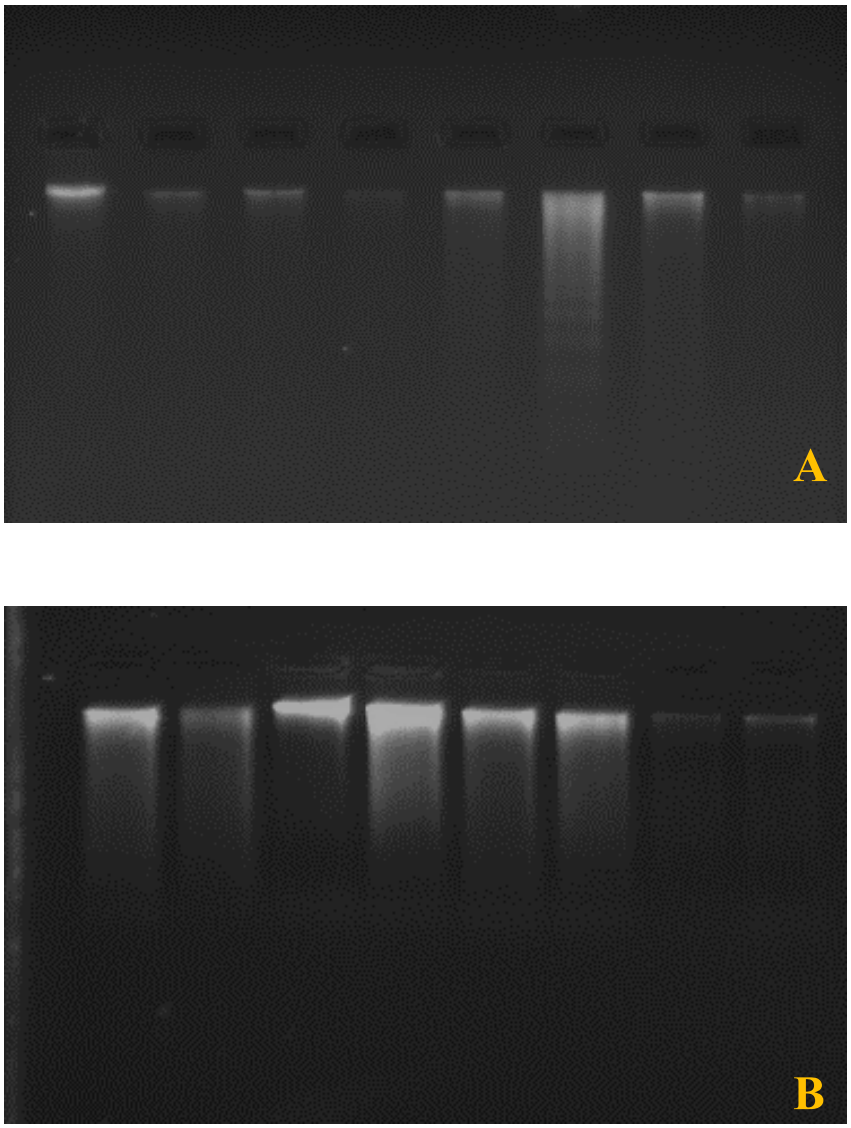
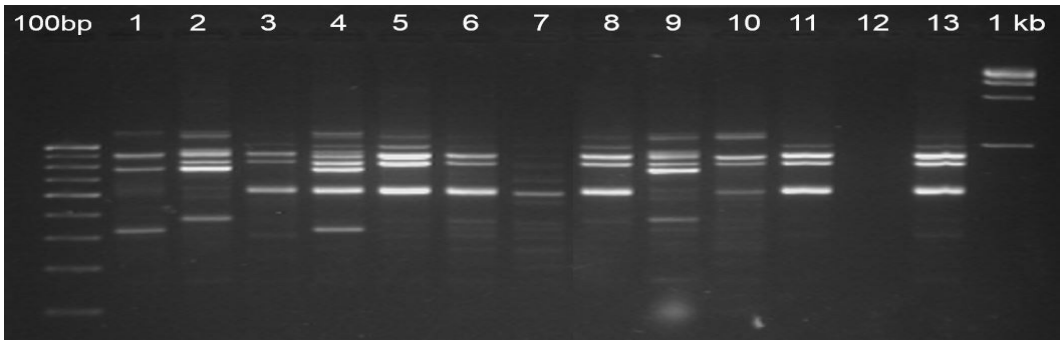


Plate 1. showing DNA bands in 1% agarose gel obtained in (A) modified CTAB (B) DNeasy® kit method

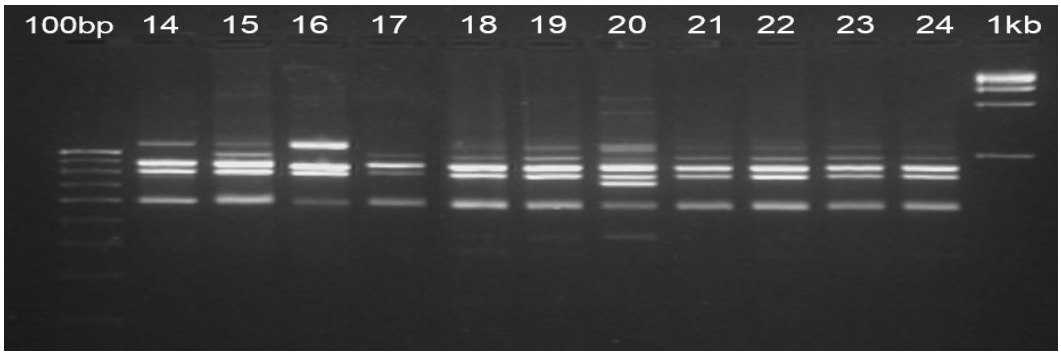
Table 8. Genetic characteristics of the ISSR markers used for analysing the greater yam accessions

Primer Code	Size Range (bp)	N_B	N_{PB}	P (%)	Hobs	PIC
UBC 808	250-2000	10	10	100 %	0.8841	0.8724
UBC 809	300-1500	12	12	100 %	0.8871	0.8731
UBC 810	400-1500	8	8	100 %	0.8618	0.8457
UBC 811	300-2000	11	11	100 %	0.8801	0.8701
UBC 817	750-2000	5	5	100 %	0.7389	0.6918
UBC 825	400-1500	8	8	100 %	0.869	0.8542
UBC 827	350-1800	11	11	100 %	0.8825	0.8756
UBC 807	300-1250	11	11	100 %	0.8811	0.88
UBC 864	500-2000	10	10	100 %	0.8752	0.8617
UBC 818	350-2000	11	10	90.9 %	0.8605	0.8501
UBC 836	250-900	9	9	100 %	0.8603	0.8442
$(GA)_9AC$	400-3000	9	9	100 %	0.8818	0.8818
$(GA)_9AT$	400-1800	12	11	91.7 %	0.8431	0.8357
UBC 14	750-2500	6	6	100 %	0.8116	0.7831
$(ACC)_6Y$	500-1500	11	10	90.9 %	0.8237	0.8134

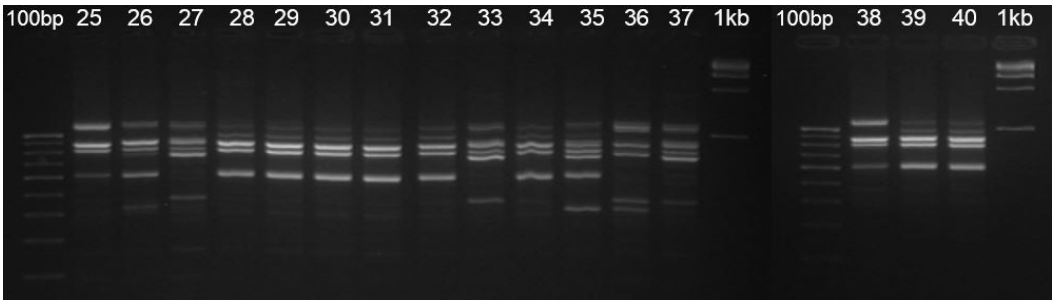
Molecular characterization using ISSR markers



1	2	3	4	5	6	7	8	9	10	11	12	13
Da 391	Da 240	Da 8	Da 215	Da 278	Da 289	Da 243	Da 303	Da 287	Da 222	Da 313	Da 312	Da 308

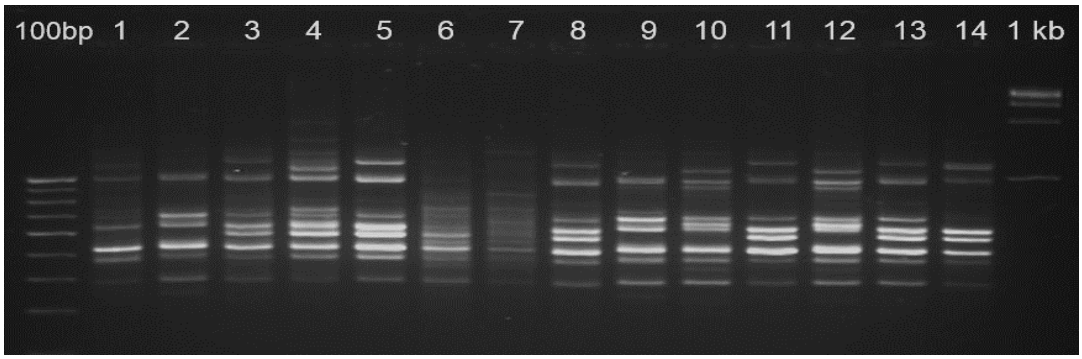


14	15	16	17	18	19	20	21	22	23	24
Da 122	Da 21	Da 28	Da 52	Da 65	Da 40	Da 68	Da 53	Da 33	Da 70	Da 27

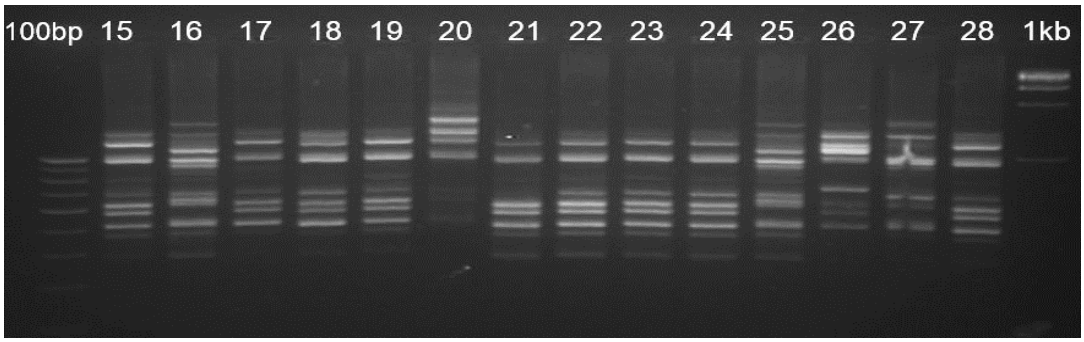


25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
Da 102	Da 114	Da 241	Da 73	Da 58	Da 120	Da 56	Da 84	Da 113	Da 88	Da 85	Da 118	Da 69	Da 99	Da 81	Da 390

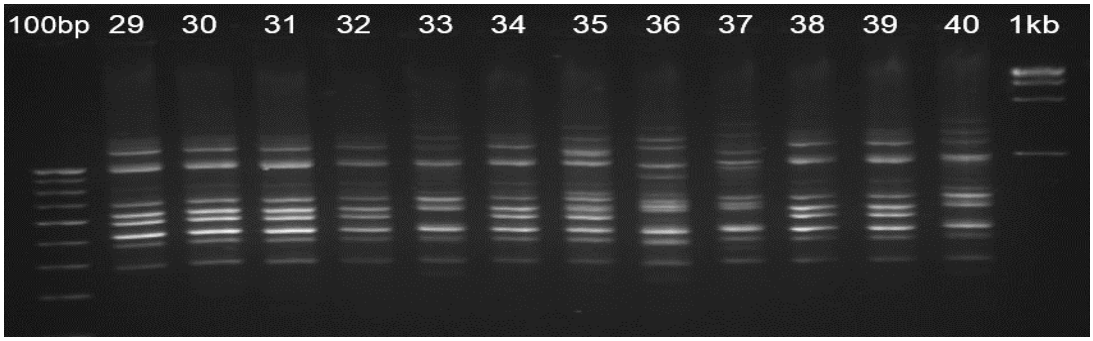
Plate 2. shows agarose gel (2%) profile of the ISSR primer UBC 810 for 40 accessions



1	2	3	4	5	6	7	8	9	10	11	12	13	14
Da 391	Da 240	Da 8	Da 215	Da 278	Da 289	Da 243	Da 303	Da 287	Da 222	Da 313	Da 312	Da 308	Da 122



15	16	17	18	19	20	21	22	23	24	25	26	27	28
Da 21	Da 28	Da 52	Da 65	Da 40	Da 68	Da 53	Da 33	Da 70	Da 27	Da 102	Da 114	Da 241	Da 73



29	30	31	32	33	34	35	36	37	38	39	40
Da 58	Da 120	Da 56	Da 84	Da 113	Da 88	Da 85	Da 118	Da 69	Da 99	Da 81	Da 390

Plate 3. shows agarose gel (2%) profile of the ISSR primer $(GA)_9AT$ for 40 accessions

4.1.5. SSR analysis of greater yam accessions

The amplicons obtained after the SSR analysis of the accessions using selected primers were initially resolved in 2% agarose gel (Plates 4 and 5). Band obtained in the product size as mentioned in Table 9.

Among the SSR markers studied, the number of the alleles per marker ranged from one to eight. All the SSR primers showed 100% polymorphism. The observed Heterozygosity values ranged from 0 (YM 5) to 0.8396 (Dab2C05). All other SSR primers showed Hobs value >0.7. The Polymorphism Information Content (PIC) ranged from 0 (YM 5) to 0.8191 (Dab2C05). In the present study an average of polymorphic alleles, Hobs value and PIC value obtained are six, 0.6904 and 0.6614 respectively.

4.1.6. Cluster analysis based on molecular data

Genetic similarity between different accessions for molecular markers (ISSR and SSR) was analysed based on Jaccard's similarity (J) coefficient using SIMQUAL programme of NTSYSpc v. 2.20 for 45 landraces of greater yam. Dendrogram based on ISSR markers showed the partition of the *D. alata* accessions into 3 different clusters at a similarity coefficient of 0.57 (Figure 1). Only one accession is present in the cluster I and III each with Da 340 and Da 331 showing maximum genetic divergence from other landraces. The cluster II formed 11 sub clusters depicting genetic diversity among the landraces grown in Kerala. Even though duplicates were not found, Da 58 and Da 56 recorded very high similarity.

SSR markers showed the partition of the *D. alata* accessions into ten clusters at a similarity coefficient of 0.57 (Figure 2) of which six clusters have one accession each (Da 340, Da 331, Da 209, Da 391, Da 122, Da 52). The cluster VII had the maximum number of accessions (24). The study based on SSR data revealed the similarity of Da 308 and Da 65 even though they are not duplicates.

4.1.6.1. Comparison of molecular analysis by ISSR and SSR markers

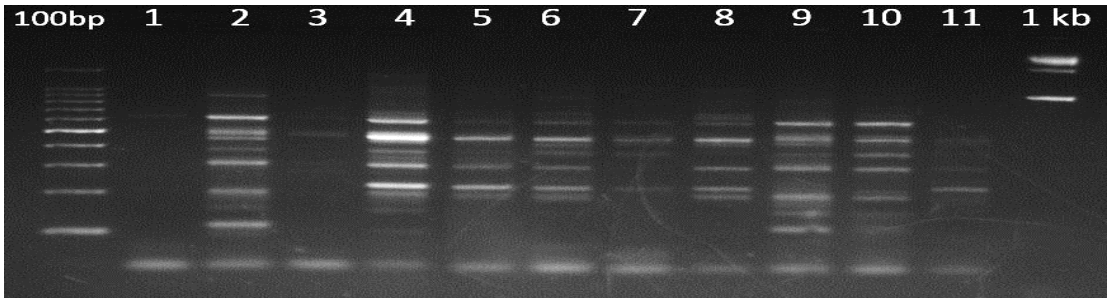
When ISSR markers were used for studying the diversity of greater yam accessions, number of polymorphic bands ranged from 6-12 while the range was 1-8 for SSR markers. Polymorphic Information content was also highest for ISSR markers (0.8818) as compared to SSR (0.8191) markers.

Based on ISSR markers greater yam accessions were grouped into three major clusters at a similarity coefficient 0.57. However, as per SSR markers, the accessions were grouped into ten clusters at a similarity coefficient of 0.57.

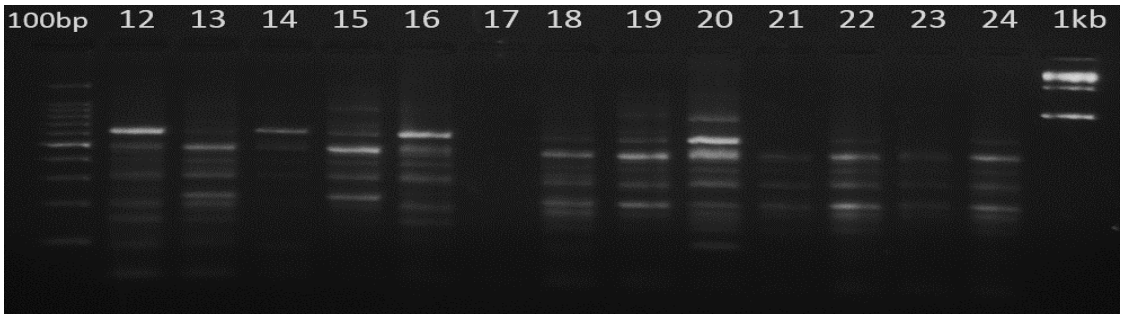
Two divergent clusters with one accession each formed in ISSR grouping whereas, six clusters with one accession each were formed in SSR grouping. Da 331 formed a unique divergent cluster in both grouping based ISSR as well as SSR markers.

This study showed that SSR markers are more relevant for elucidating genetic diversity of greater yam accessions.

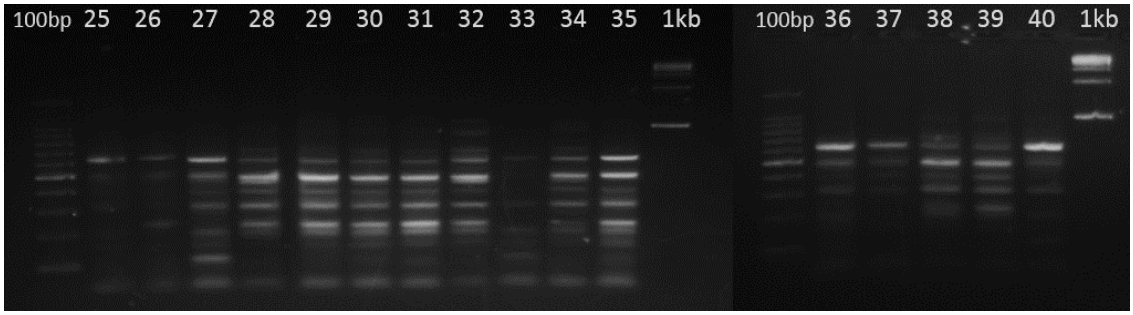
Molecular characterization using SSR markers



1	2	3	4	5	6	7	8	9	10	11
Da 391	Da 240	Da 8	Da 215	Da 278	Da 289	Da 243	Da 303	Da 287	Da 222	Da 313

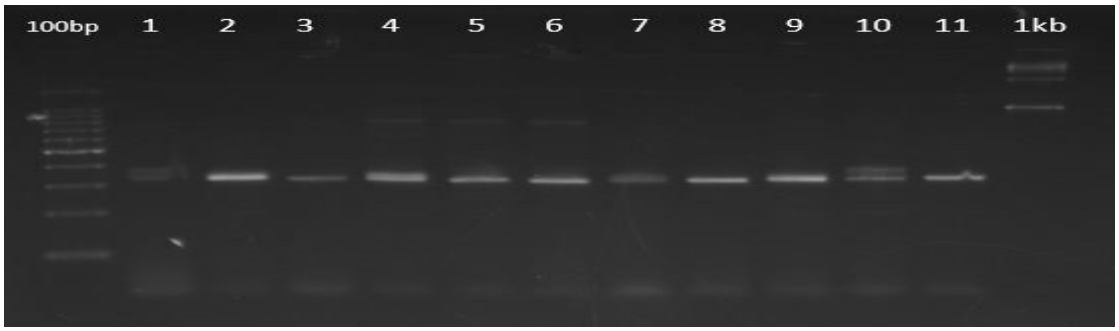


12	13	14	15	16	17	18	19	20	21	22	23	24
Da 312	Da 308	Da 122	Da 21	Da 28	Da 52	Da 65	Da 40	Da 68	Da 53	Da 33	Da 70	Da 27

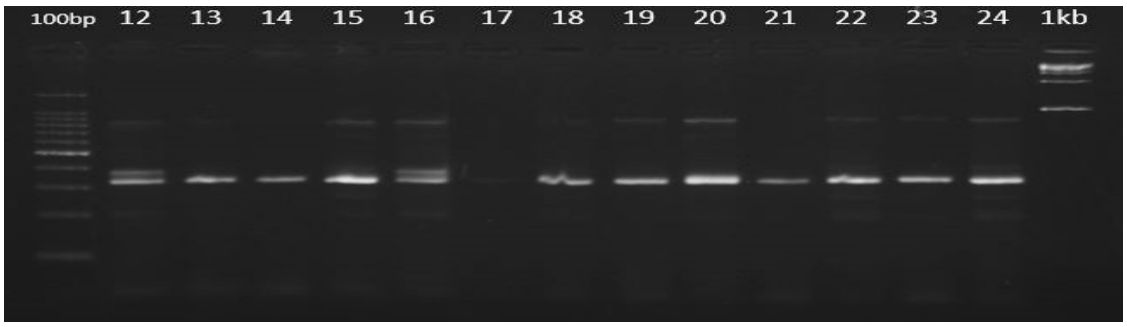


25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
Da 102	Da 114	Da 241	Da 73	Da 58	Da 120	Da 56	Da 84	Da 113	Da 88	Da 85	Da 118	Da 69	Da 99	Da 81	Da 390

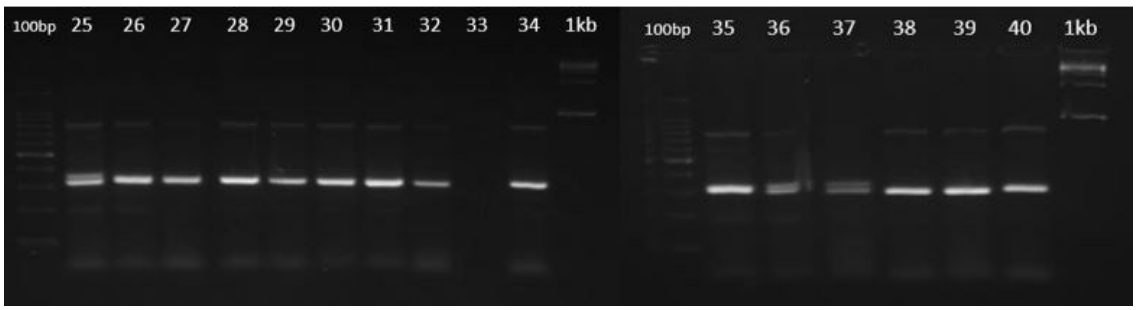
Plate. 4 shows agarose gel (2%) profile of the SSR primer YM 15 for 40 accessions



1	2	3	4	5	6	7	8	9	10	11
Da 391	Da 240	Da 8	Da 215	Da 278	Da 289	Da 243	Da 303	Da 287	Da 222	Da 313



12	13	14	15	16	17	18	19	20	21	22	23	24
Da 312	Da 308	Da 122	Da 21	Da 28	Da 52	Da 65	Da 40	Da 68	Da 53	Da 33	Da 70	Da 27



25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
Da 102	Da 114	Da 241	Da 73	Da 58	Da 120	Da 56	Da 84	Da 113	Da 88	Da 85	Da 118	Da 69	Da 99	Da 81	Da 390

Plate 5. shows agarose gel (2%) profile of the SSR primer Dab2D08 for 40 accessions

Table 9. Genetic characteristics of the SSR markers used for analysing the greater yam accessions

Primer Code	Size range (bp)	N_B	N_{PB}	P (%)	He	PIC
Dab2C05	100-1000	8	8	100 %	0.8396	0.8191
Dab2D06	300-1250	6	6	100 %	0.7937	0.7621
Dab2E07	150-650	6	6	100 %	0.8079	0.78
Da1A01	150-750	6	6	100 %	0.7152	0.6653
Dpr3F04	100-700	5	5	100 %	0.7288	0.6787
YM5	150-450	1	1	100 %	0	0
YM15	100-650	6	6	100 %	0.8193	0.7935
YM26	150-1500	6	6	100 %	0.8188	0.7929

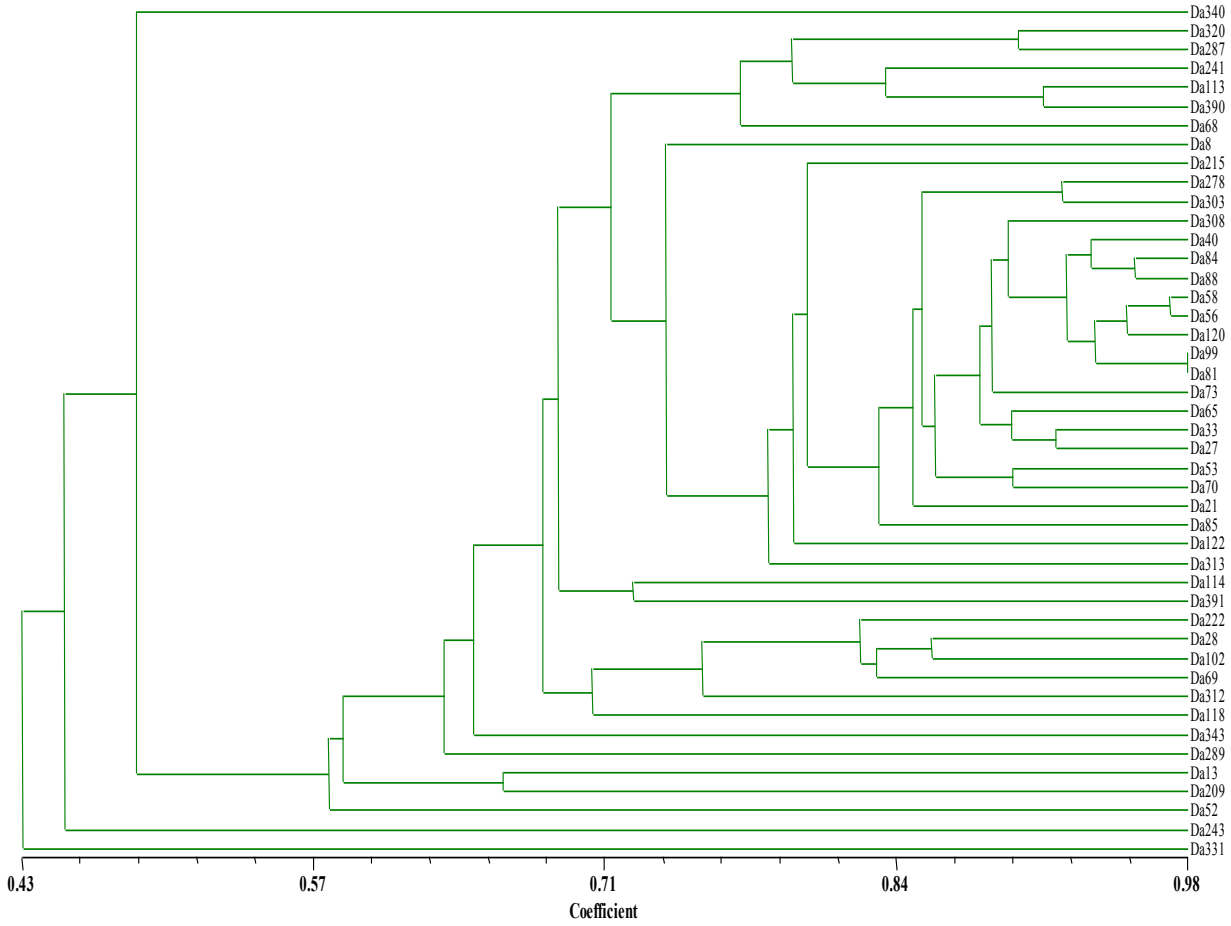


Fig 1. cluster diagram depicting genetic diversity based on ISSR markers

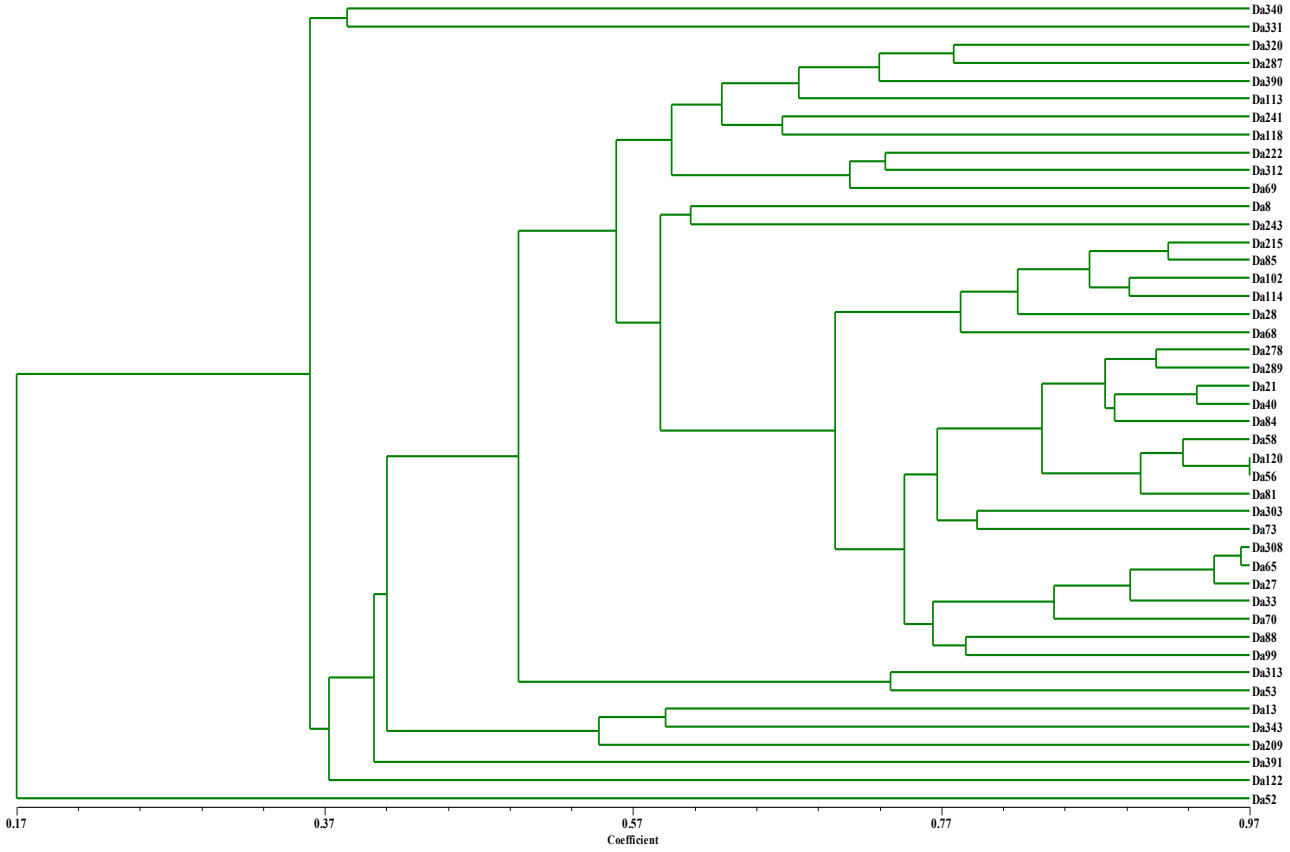


Figure 2. cluster diagram depicting genetic diversity based on SSR markers

4.2. Morphological Data Analysis

Morphologically analysed the 45 accessions of *D. alata* based on qualitative and quantitative data.

4.2.1. Variation in Qualitative Traits Assessed

Wide variation is observed on qualitative traits and it is given below

4.2.1.1. Young leaf colour

Plate 6. (C-H) shows the different types of young leaf colour observed among the 45 accessions of *D. alata*. There are six different colours observed namely, yellowish green, pale green, dark green, light brown, purplish green and purple colour. In all, 71.1%showed pale green colour, 13.3% had yellowish green, 6.7% showed dark green, 4.4% showed purplish green, 2.3% had light brown and 2.2% had purple colour.

4.2.1.2. Leaf shape

Plate 7. (A and B) depicts the main leaf shape characters observed among *D. alata* accessions. Five different shapes of leaves are observed. 51.0% observed as cordate, 26.7% observed as cordate long, 8.9% had cordate broad, 6.7% shows sagitate broad and 6.7% exhibit sagitate long leaves.

4.2.1.3.Anthracnose susceptibility

Anthracnose is the major disease caused to yams. Researches are going on for developing the anthracnose resistant varieties in greater yam. The results of the field screening of the greater yam landraces showed that 44.4% of accessions have intermediate susceptibility, 40% showed minimum susceptibility and 15.6% shows high anthracnose susceptibility.

4.2.1.4. Tuber shape

Tuber shape variation among the greater yam accessions studied is presented in Plate 8 that shows different types of shapes observed in *D. alata*. The *D. alata* accessions examined phenotypically based on their tuber shape exhibited wide

variation (six types, namely, round (11.1%), oval (11.1%), oval-oblong (31.1%), cylindrical (8.9%), conical (8.9%), spindle shape (13.3%), digitate (11.1%) and irregular (4.5%) shapes.

4.2.1.5.Tuber cortex colour

The tuber cortex colour showed high variability among the selected accessions and six different types of colours were observed. Among the accessions, 49.0% exhibit light purple, 22.2% shows light cream, 11.1% shows light yellow, 8.9% observed as dark purple, 4.4% shows purple colour and 4.4% exhibit light greenish yellow colour.

4.2.1.6. Tuber flesh colour

Plate 7 (D-I) shows variation observed in tuber flesh colour among 45 accessions of *D. alata* landraces. Majority of the accessions (86.7%) had off-white colour, 8.9% showed white colour, 2.2% with yellow colour and 2.2% exhibited purple coloured flesh.

4.2.1.7. Tuber skin texture

Identified important phenotypic variation between the accessions with respect to tuber texture. Four types of skin texture recorded- smooth, moderately smooth, warty and highly warty. Among the accessions 44.4% have warty skin, 33.3% have moderately smooth skin, 20 % have skin of highly warty and 2.3% exhibit smooth skin.

4.2.1.8. Cooking quality/ culinary quality

One important character taken for the study is cooking quality. There are four characters recorded under cooking quality/ culinary quality *viz.* softness, stickiness, colour and taste. From examining the cooking quality, 75.5% exhibited soft flesh, 48.9% had non-sticky flesh and 64.4% recorded non-coloured flesh. In case of taste of the tuber 57.8% recorded as good, 33.3% recorded as excellent, 6.7% have average taste and 2.2% exhibited poor quality.

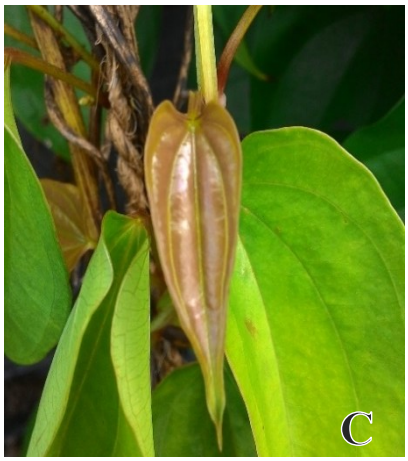


Plate 6. showing: greater yam field (A), areal tuber (B), variation in young leaf colours (C-H)

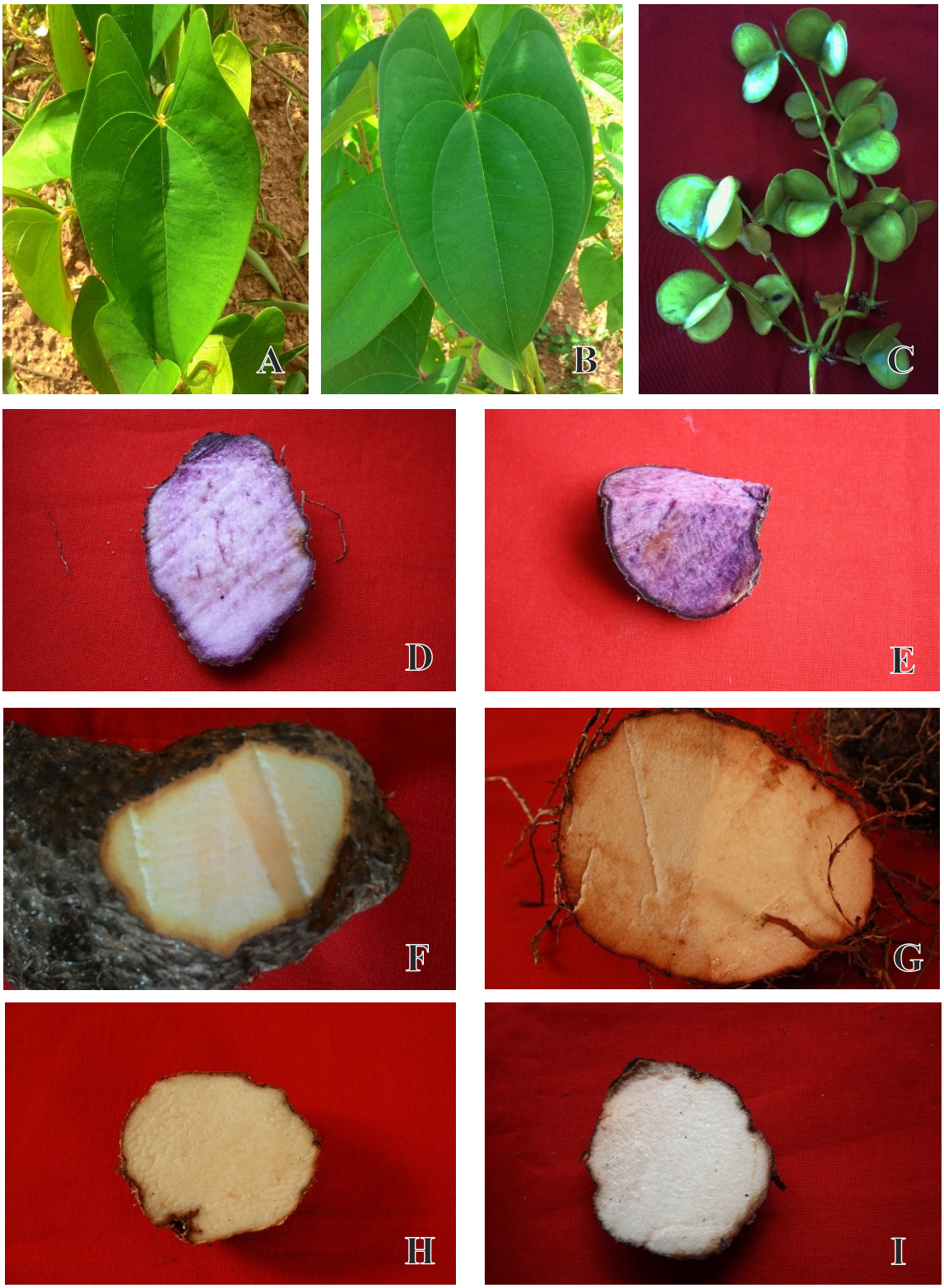


Plate 7. showing sagitate leaf (A), cordate leaf (B), seed (C) and variation in tuber flesh colour (D-I)

DA 340

DA 240

DA 8

DA 13

DA 343

DA 215

DA 278

DA 289

DA 243

DA 303

DA 287

DA 222

DA 313

DA 312

DA 308

DA 122

DA 209

DA 21

DA 28

DA 52

DA 65

DA 40

DA 68

DA 53

DA 33

DA 70

DA 27

DA 102

DA 114

DA 241

DA 73

DA 58

DA 120

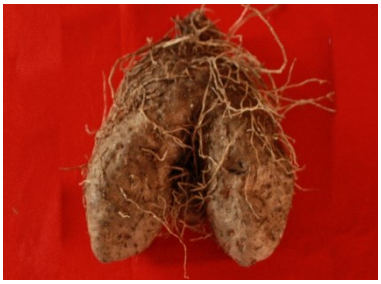
DA 56

DA 84

DA 113

DA 88

DA 85



DA 118

DA 69

DA 99

DA 331

Plate 8. showing tuber shape morphology of different accessions

Variation in Quantitative Characters Assessed

Among the 45 *D. alata* accessions, seven quantitative traits are recorded and wide range of variation observed (Table 10). The observed ranges for plant characters like mature stem diameter, leaf length, leaf breadth and petiole length are 2.1 cm (Da 343)-3.8 cm (Da 209), 10.5cm (Da 243)-22.0cm (Da 13), 6.3cm (Da 52)-13.3cm (Da 313) and 5.3cm (Da 53)-15.0cm (Da 222). The tuber characters measured have the range, 7.50cm (Da 53)-46.67cm (Da 390) for tuber length, 14.97cm (Da 391)-59.17cm (Da 340) for tuber girth and 0.15kg (Da 53)-6.88kg (Da 340) for tuber weight.

Among the 45 *D. alata* accessions, seven quantitative data is recorded and performed ANOVA (Analysis of Variance) and estimation of genetic parameters, genotypic and phenotypic correlation between the selected quantitative characters Among the agronomic traits evaluated, highest genotypic coefficient of variation (GCV) was recorded for length of tuber (36.51) followed by girth of tuber (31.15) while lowest GCV was recorded by leaf length (12.95) (Table 11). Among the traits studied, tuber characters recorded very high heritability as compared to shoot characters. The highest heritability was recorded by length of tuber followed by girth of tuber. Highest genetic gain (%) was recorded for girth of tuber (17.62) followed by tuber length (13.64).

The genotypic as well as phenotypic correlation among the traits were also studied (Table 12 and 13). Among the traits studied, highest genotypic correlation was recorded for girth of tuber with weight of tuber (0.7153). However phenotypic correlation of girth of tuber with weight of tuber (0.0680) was found to be negligible. Phenotypic correlation was found to be maximum among the leaf characters, leaf length with leaf breadth (0.5622) and leaf length with leaf breadth (0.4766). These characters also recorded significantly high genotypic correlation also. For length of tuber, both phenotypic (0.3512) and genotypic (0.3636) correlation with weight of tuber was found to be significant.

Table 10. Observations of quantitative data

Sample	Accession no.	Mature stem diameter (cm)	Leaf length (cm)	Leaf breadth (cm)	Petiole length (cm)	Tuber length (cm)	Tuber girth (cm)	Tuber weight (kg)
A1	Da340	3.25	13.23	9.03	11.83	20.02	59.17	6.88
A2	Da 240	2.75	14.23	8.03	8.23	29.33	24.10	1.10
A3	Da 8	2.13	18.50	10.13	9.57	20.10	22.37	0.98
A4	Da 13	2.40	22.00	13.30	10.33	13.70	25.33	0.60
A5	Da 343	2.10	17.00	9.57	7.43	25.33	30.57	1.28
A6	Da 215	3.43	13.03	7.53	7.00	15.00	31.57	0.63
A7	Da 278	3.33	13.10	8.43	9.33	20.00	54.73	2.03
A8	Da 289	2.43	14.47	8.27	9.43	22.37	31.63	1.30
A9	Da 243	2.73	10.50	7.03	6.37	12.67	32.73	1.05
A10	Da 303	2.30	14.50	8.40	9.60	14.33	29.93	0.92
A11	Da 287	2.10	12.00	8.77	10.23	27.73	22.83	1.05
A12	Da 222	2.77	16.50	10.47	15.00	27.60	25.03	1.23
A13	Da 313	2.37	12.00	13.27	8.23	13.30	22.07	0.35
A14	Da 312	2.83	14.30	9.30	9.17	21.77	23.07	0.65
A15	Da 308	2.57	16.50	7.63	8.53	32.17	23.10	0.74
A16	Da 122	2.67	13.10	6.37	6.73	19.23	47.90	1.41
A17	Da 209	3.80	14.37	8.50	6.57	17.17	17.63	0.29
A18	Da 21	2.53	12.27	6.17	5.37	17.20	45.60	1.91
A19	Da 28	2.67	14.33	9.83	12.50	21.57	27.43	0.94
A20	Da 52	2.40	11.97	6.33	6.43	12.47	20.17	0.36
A21	Da 65	2.50	14.57	8.43	6.23	9.23	22.97	0.30
A22	Da 40	2.27	16.33	9.20	8.53	13.93	24.30	0.36
A23	Da 68	3.07	13.87	7.77	8.57	17.37	17.87	0.36
A24	Da 53	2.33	14.37	8.33	5.33	7.50	22.63	0.15
A25	Da 33	2.57	12.63	7.23	7.53	15.27	36.27	1.33
A26	Da 70	2.90	14.97	7.97	7.50	16.23	28.80	0.68
A27	Da 27	2.93	15.40	8.03	8.00	16.27	34.77	1.20
A28	Da 102	3.10	13.73	7.63	7.83	21.40	31.00	1.09
A29	Da 114	2.73	16.40	9.47	8.17	24.27	22.00	0.78
A30	Da 241	3.57	13.37	9.97	7.83	25.33	22.33	0.58
A31	Da 73	2.83	14.83	8.27	5.93	15.30	33.93	0.69
A32	Da 58	3.60	16.00	8.70	6.43	12.50	36.10	0.69
A33	Da 120	3.00	13.90	7.67	5.67	16.23	27.33	0.68
A34	Da 56	3.53	15.60	8.60	9.60	16.17	29.83	0.74
A35	Da 84	3.50	15.90	8.93	8.87	13.43	24.13	0.45
A36	Da 113	3.17	14.97	9.27	8.27	26.77	19.20	0.66

Sample	Accesion no.	Mature stem diameter (cm)	Leaf length (cm)	Leaf breadth (cm)	Petiole length (cm)	Tuber length (cm)	Tuber girth (cm)	Tuber weight (kg)
A37	Da 88	3.43	14.33	7.20	7.20	17.70	24.67	0.62
A38	Da 85	3.33	14.60	7.67	7.77	19.60	34.67	1.15
A39	Da 118	2.73	13.70	7.90	6.93	16.77	27.13	0.40
A40	Da 69	2.90	16.00	8.70	9.60	12.87	32.93	0.54
A41	Da 99	3.23	16.50	10.30	8.67	14.10	35.33	0.75
A42	Da 81	2.73	15.53	8.00	9.77	11.63	28.27	0.28
A43	Da 390	3.37	17.50	10.87	8.17	46.67	34.00	3.25
A44	Da 331	2.50	10.57	9.27	9.17	20.33	20.33	0.51
A45	Da 391	2.57	16.67	10.00	10.50	14.63	14.97	0.78

Table 11. Evaluation of genetic parameters in greater yam

Character	GCV	PCV	Heritability	GA 5%	GA as % of mean
Mature stem diameter	13.854	19.1844	0.5215	0.5861	20.6098
Leaf length	12.9497	16.327	0.6291	3.1038	21.1582
Leaf breadth	13.8558	21.9894	0.397	1.5657	17.9853
Petiole length	19.6142	27.97	0.4916	2.367	28.33305
Length of tuber	36.5165	37.7897	0.9338	13.6422	72.6896
Girth of tuber	31.1495	32.8361	0.8999	17.6223	60.8721

Where, GCV= Genotypic coefficient of variation, PCV= Phenotypic coefficient of variation, GA= Genetic advance

Table 12. Genotypic correlation among agronomic traits in greater yam

Character	X1	X2	X3	X4	X5	X6	X7
X1	1						
X2	-0.0017	1					
X3	-0.0845	0.4912**	1				
X4	-0.1052	0.253	0.5062**	1			
X5	-0.0524	0.1489	0.2111	0.3324*	1		
X6	0.2261	-0.1963	-0.3349	-0.0286	0.0075	1	
X7	0.1663	-0.0699	0.0247	0.3364*	0.3636*	0.7153**	1

Where X1= Mature stem diameter, X2= leaf length, X3= leaf breadth, X4= petiole length, X5= tuber length, X6= tuber girth, X7= tuber weight

Table 13. Phenotypic correlation among agronomic traits in greater yam

Character	X1	X2	X3	X4	X5	X6	X7
X1	1						
X2	-0.0545	1					
X3	-0.1376	0.5622**	1				
X4	-0.0908	0.3517*	0.4766**	1			
X5	0.0617	0.1222	0.1404	0.2184	1		
X6	0.1792	-0.128	-0.1816	0.0067	-0.0114	1	
X7	0.1231	-0.0601	0.0092	0.2247	0.3512*	0.06804	1

Where X1= Mature stem diameter, X2= leaf length, X3= leaf breadth, X4= petiole length, X5= tuber length, X6= tuber girth, X7= tuber weight

4.2.2. Diversity assessment using morphological data

Based on Jaccard's index genetic similarity matrix observations recorded for the morphological characters of greater yam used for clustering with tree analysis concept. It revealed that the D. alata accessions exhibited greater degree of genetic variation for the 42 different morphological traits observed (figure 3). The similarity coefficient ranged 2.65 – 11.28. The 45 greater yam accessions were grouped into two major clusters with Cluster I having maximum number of accessions (29) and Cluster II with 16 accessions. The Cluster I and II again formed three sub clusters each at a similarity level 9.12. Da 312 and Da 102 shows highest similarity of 2.65. The major cluster divided into two sub clusters and Da 331 and Da 340 showed maximum divergence among the accessions and lies in the separate sub clusters. Among the accessions none were found to be completely similar. However maximum similarity was obtained between Da 56 and Da 84.

4.3. Biochemical analysis

The biochemical characters of tubers of *D. alata* landraces grown in different districts of Kerala are presented in Table 14. The dry matter per cent ranged from 27.53 (Da 56) to 48.24 (Da 69). The starch content, sugar content and protein content are determined on the dry weight basis. The values ranged from 36.0 (Da 40) -65.22 (Da 289) for starch, 2.73 (Da 278) – 9.62 (Da 70) for sugar and 5.86 (Da 390) – 14.51 (Da 331) for protein content on dry weight basis. Among the accessions evaluated Da 331 recorded the highest percentage of crude protein content (14.51) followed by Da 102 (13.52) and fourteen accessions recorded high crude protein above ten percent.

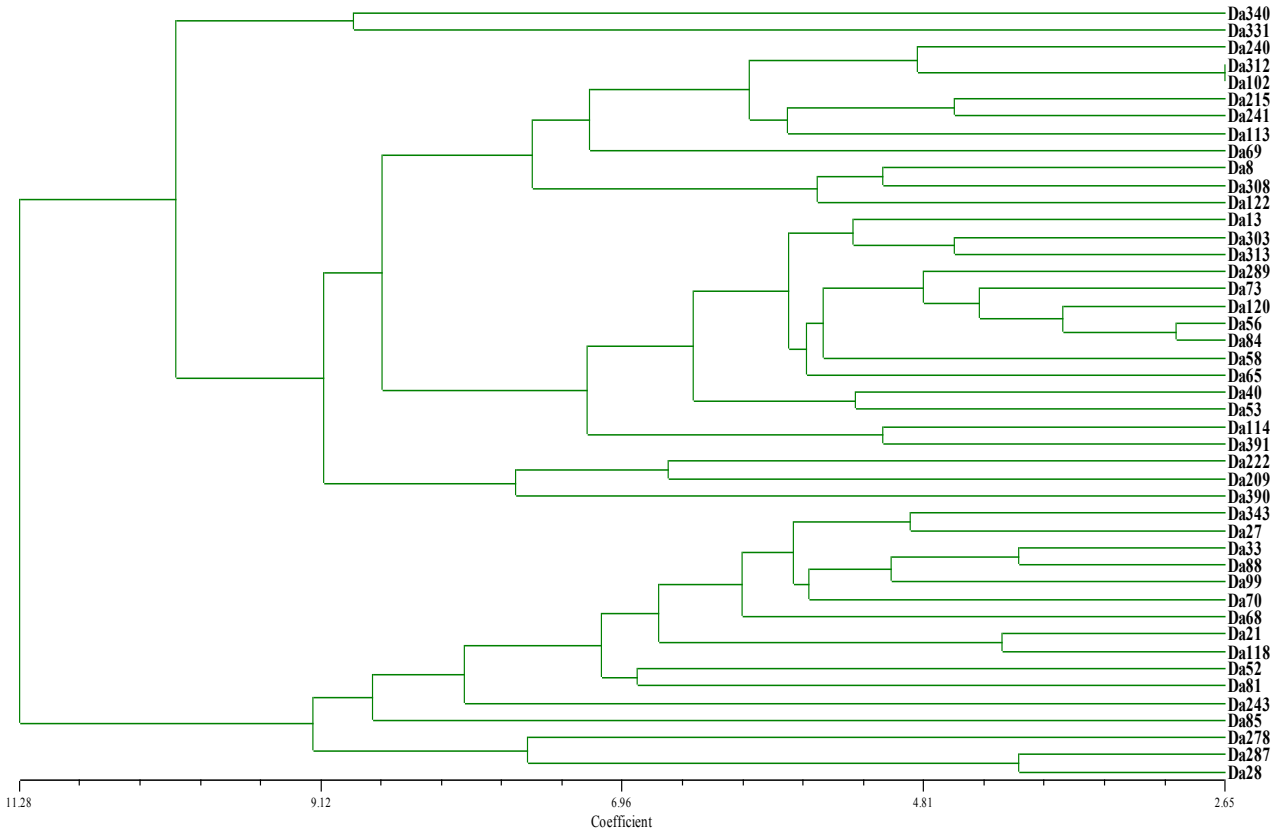


Figure 3. cluster diagram depicting genetic diversity based on morphological characters

Table 14. showing biochemical values for the *D. alata* accessions

Sample	Accession no.	Dry matter %	starch	sugar	Protein
A1	Da 340	31.48	57.69	7.58	8.65
A2	Da 240	37.11	55.56	4.85	7.84
A3	Da 8	33.01	56.96	5.81	9.19
A4	Da 13	31.43	60.00	8.33	8.11
A5	Da 343	34.70	46.88	5.26	11.09
A6	Da 215	42.25	62.50	5.26	9.37
A7	Da 278	34.51	60.81	2.73	9.64
A8	Da 289	33.51	65.22	4.59	9.46
A9	Da 243	31.09	58.44	7.14	11.72
A10	Da 303	34.70	52.94	6.54	8.74
A11	Da 287	38.45	60.00	7.94	10.45
A12	Da 222	31.30	62.50	3.13	9.01
A13	Da 313	41.20	60.00	4.35	10.63
A14	Da 312	32.76	64.29	3.33	10.82
A15	Da 308	34.72	60.00	9.10	7.931
A16	Da 122	30.03	50.56	8.55	8.11
A17	Da 209	28.86	57.69	7.94	8.83
A18	Da 21	31.86	58.44	2.81	7.93
A19	Da 28	36.50	62.50	8.06	7.48
A20	Da 52	33.50	59.21	5.56	9.55
A21	Da 65	33.76	60.00	3.89	8.92
A22	Da 40	39.19	36.00	9.35	10.45
A23	Da 68	37.33	60.81	6.33	7.66
A24	Da 53	31.48	45.92	8.06	11.72
A25	Da 33	32.20	50.00	7.81	8.47
A26	Da 70	29.32	45.00	9.62	13.07
A27	Da 27	34.19	59.21	5.75	9.01

Sample	Accession no.	Dry matter %	starch	sugar	Protein
A28	Da 102	35.23	48.91	6.58	13.52
A29	Da 114	30.09	51.72	4.07	9.91
A30	Da 241	39.32	55.56	3.73	7.03
A31	Da 73	33.10	58.44	7.14	8.47
A32	Da 58	32.95	57.69	5.32	10.09
A33	Da 120	33.53	50.56	8.20	10.72
A34	Da 56	27.53	56.25	9.43	8.83
A35	Da 84	31.42	43.27	6.54	8.11
A36	Da 113	43.10	58.44	5.75	9.37
A37	Da 88	30.80	54.88	6.02	9.46
A38	Da 85	35.84	63.38	5.32	8.56
A39	Da 118	37.55	53.57	3.16	10.82
A40	Da 69	48.24	48.39	6.62	10.72
A41	Da 99	33.26	57.69	6.85	9.28
A42	Da 81	36.70	54.88	5.24	9.37
A43	Da 390	38.86	64.29	7.30	5.86
A44	Da 331	36.07	54.88	3.91	14.51
A45	Da 391	33.2	58.47	4.2	7.6

4.5. **Principal component analysis (PCA)**

Forty two morphological traits were considered for PCA analysis with a Measure of Sample Adequacy greater than 0.5. The Eigen values and cumulative variance of the first fifteen components is given in Table 15 and variability is depicted in figure 4. The first six principal components of the analysis accounted for 50.12% of the total variance among accessions. The primary principal component (PC1) elucidated 13.58% of the overall variance, which was contributed mainly by young vein colour, colour of young leaves, leaf shape, petiole length, young leaf vein colour, tuber shape, tuber cortex colour and starch content. The distribution of accessions in the scatter plot revealed high divergence of Da 340, Da 331 and Da 390.

The principal component analysis was performed for molecular data as well. The Eigen values and cumulative variance of the first ten components for ISSR and SSR data is given in Table 16 and 17 respectively. The first six principal components of ISSR data accounted for 53.51% of the total variance among accessions and the PC1 (first principal component) explained 17.26% of the overall variation. The first six principal components of SSR data accounted for 59.38% of the entire variation among accessions and the primary principal component (PC1) showed 20.19% of the overall variation. The scatter plot depicting the variability among the accessions based on ISSR and SSR data is given in Figure 5 and 6. The scatter plot based on ISSR data revealed high divergence of Da 289, Da 331 and Da 243 while that based on SSR showed the divergence of Da 331, and Da 118. Table Eigen values and cumulative variance of the first ten PCs representing variability of greater yam accessions based on ISSR data.

Table 15. Eigen values and cumulative variance of the first fifteen PCs representing variability of greater yam accessions.

Eigenvalues of the Correlation Matrix				
	Eigenvalue	Difference	Proportion	Cumulative
1	5.70287400	1.56740673	0.1358	0.1358
2	4.13546728	0.65192007	0.0985	0.2342
3	3.48354721	0.63484085	0.0829	0.3172
4	2.84870636	0.33406474	0.0678	0.3850
5	2.51464162	0.14922580	0.0599	0.4449
6	2.36541582	0.10518255	0.0563	0.5012
7	2.26023327	0.30821789	0.0538	0.5550
8	1.95201538	0.03898562	0.0465	0.6015
9	1.91302976	0.44441406	0.0455	0.6470
10	1.46861570	0.10067026	0.0350	0.6820
11	1.36794543	0.05238317	0.0326	0.7146
12	1.31556227	0.08342311	0.0313	0.7459
13	1.23213916	0.10881764	0.0293	0.7752
14	1.12332152	0.16349285	0.0267	0.8020
15	0.95982867	0.06811207	0.0229	0.8248

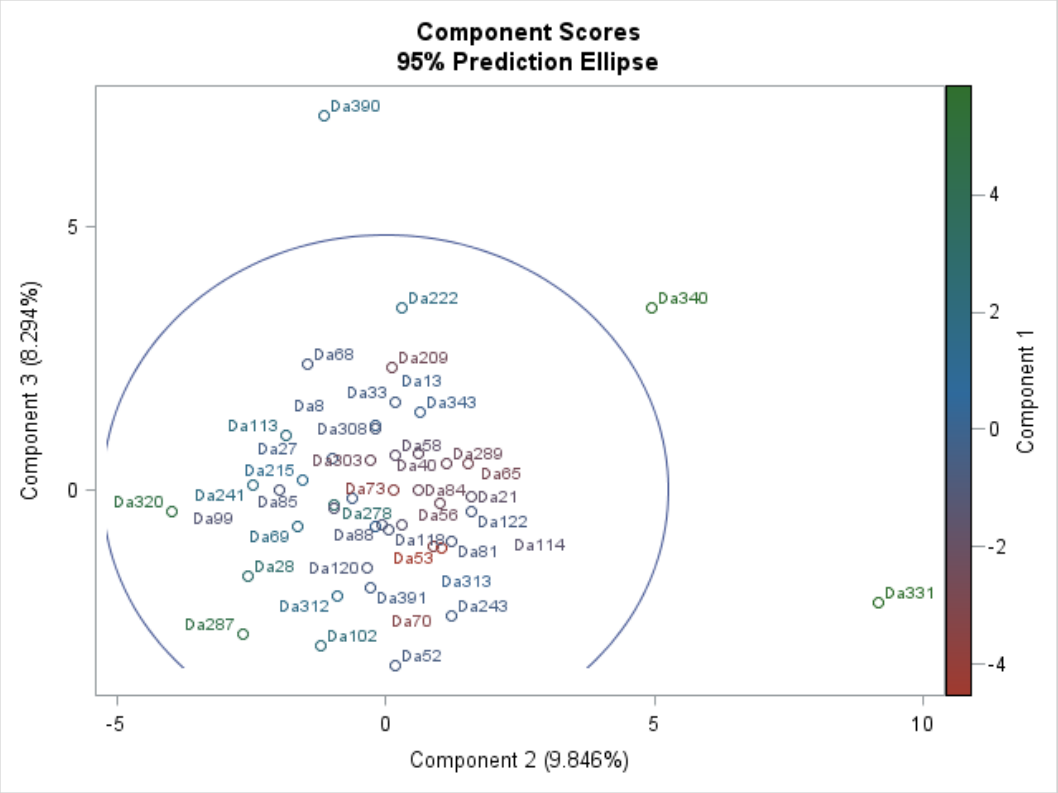


Figure 4. Scatter plot for the first three principle components (PCs) for morphological traits

Table 16. Eigen values and cumulative variance of the first ten PCs representing variability of greater yam accessions based on ISSR data.

Eigenvalues of the Correlation Matrix				
	Eigenvalue	Difference	Proportion	Cumulative
1	24.5161510	10.6923114	0.1726	0.1726
2	13.8238395	2.4886834	0.0974	0.2700
3	11.3351561	1.2718009	0.0798	0.3498
4	10.0633552	0.8224683	0.0709	0.4207
5	9.2408869	2.2302887	0.0651	0.4858
6	7.0105982	0.2407812	0.0494	0.5351
7	6.7698170	0.8444587	0.0477	0.5828
8	5.9253582	1.1485883	0.0417	0.6245
9	4.7767699	0.1883669	0.0336	0.6582
10	4.5884030	0.1110049	0.0323	0.6905

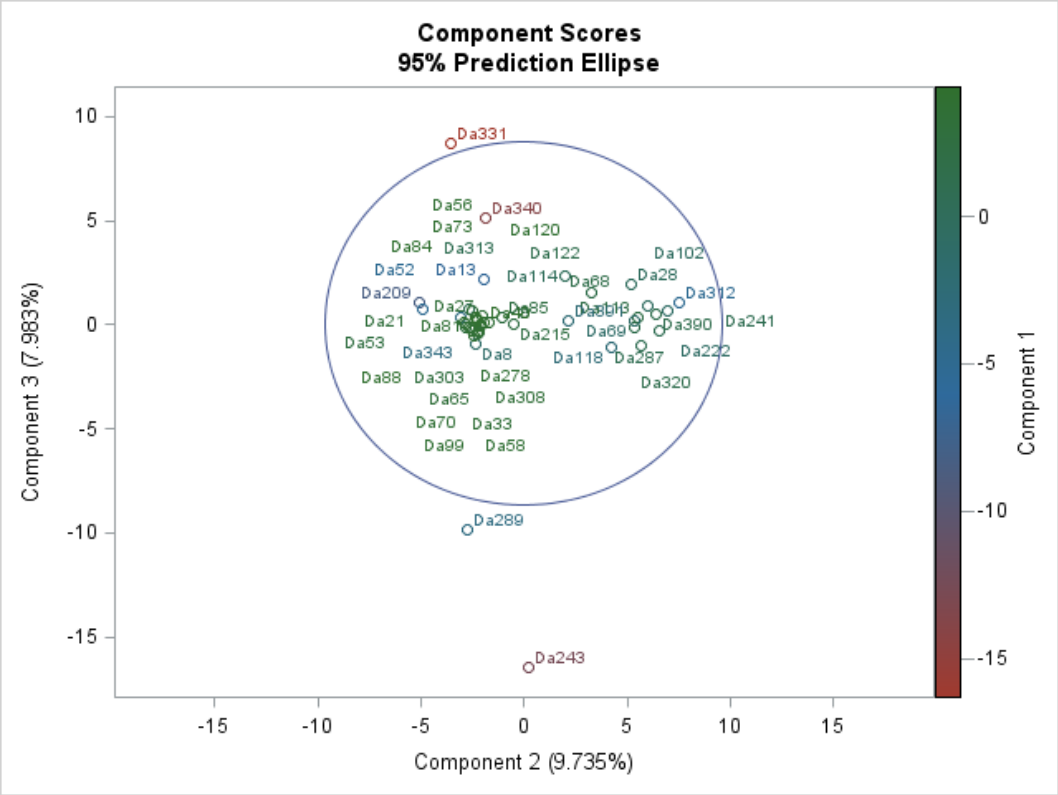


Figure 5. Scatter plot for the first three principle components (PCs) for ISSR

Table 17. Eigen values and cumulative variance of the first ten PCs representing variability of greater yam accessions based on SSR data.

Eigenvalues of the Correlation Matrix				
	Eigenvalue	Difference	Proportion	Cumulative
1	9.49126887	3.06261804	0.2019	0.2019
2	6.42865082	2.41246518	0.1368	0.3387
3	4.01618565	1.05777518	0.0855	0.4242
4	2.95841047	0.38600316	0.0629	0.4871
5	2.57240732	0.12876372	0.0547	0.5418
6	2.44364360	0.32330375	0.0520	0.5938
7	2.12033985	0.14211546	0.0451	0.6390
8	1.97822439	0.26278167	0.0421	0.6810
9	1.71544272	0.31904441	0.0365	0.7175
10	1.39639830	0.02032208	0.0297	0.7473
11	1.37607622	0.13282721	0.0293	0.7765

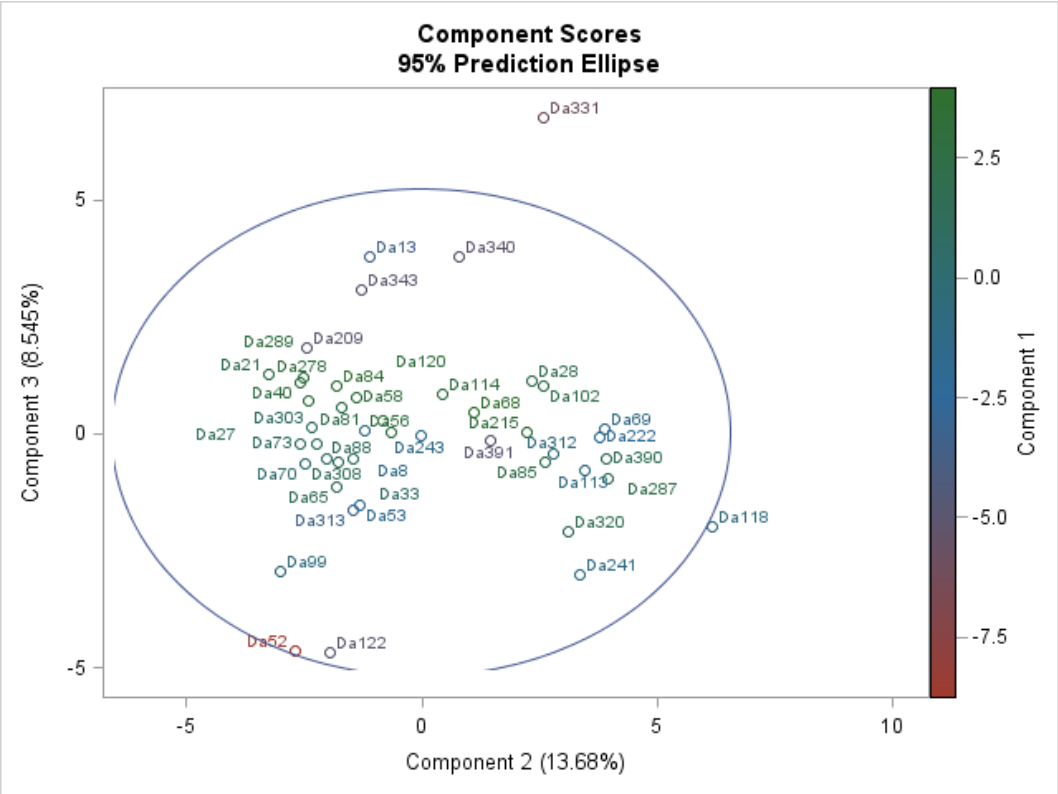


Figure 6. Scatter plot for the first three principle components (PCs) for SSR

DISCUSSION

5. DISCUSSION

Yam is a vital and nutritional security crop cultivating in many countries for its tubers. It is a source of income and also a part of socio-cultural events. They are crops which are grown for their thickened, modified roots or stems which develop into underground tubers. They have high amount of carbohydrates and are usually used as a cattle feed, dietary staple, raw material for the production of starch and alcohol or various processed food products industrially. They represent the second most important set of food crops in developing countries, following the cereals. It contains high amount of protein (2.4%) and vitamins (thiamine, riboflavin and ascorbic acid) and minerals like calcium, iron and phosphorus. It also has medicinal properties like the steroidal sapogenins which is used for producing cortisone and synthetic steroid hormones.

Greater yam (*Dioscorea alata*) is evolving as a vital tuber crop in India. Despite a growing interest in water yam, the published data on molecular classification and genetic diversity of this crop are very less. Precise information on genetic diversity is needed for the accomplishment of breeding programs, since genetic variance produces high heterotic effects. Hence, this research work attempts to combine morphological and molecular data for understand the extent of genetic variation present within the *D. alata* landraces collected from different places of Kerala.

5.1.Molecular analysis

Isolated DNA from 45 grater yam (*Dioscorea alata* L.) genotypes collected from different places of Kerala. Standardization of PCR conditions was undertaken for different types of molecular markers so as to obtain clear and consistent amplicons under reduced cycle duration.

In the present study, ISSR and SSR markers were selected based on previous reports in yams (Narina *et al.,* 2011 and Nascimento *et al.,* 2013). For preliminary screening of ISSR, 16 ISSR primers were used of which, 15 were selected based on their polymorphism and repeatability. In case of SSR 11 primers are screened and 9 primers were selected.

The ISSR markers are very useful in studying deviation in microsatellite region that are distributed particularly in the nuclear genome. Using these primers, 144 discernible DNA fragments were generated with 141 polymorphic ones. Total number of bands per ISSR primer ranged from 5 (UBC 817) to 12 (UBC 809 and $(GA)_9AT$). The present study revealed quite high polymorphism (98.2 %) in greater yam landraces. The high percentage of polymorphism for ISSR amplified product is common. Other workers obtained similar results in yam species: 95% (Wu *et al.,* 2014), 83 % (Zhou *et al.,* 2008). The high level of polymorphism of ISSR markers suggests that this technique is very useful and possibly powerful technique for the genetic diversity studies in yams.

In the present study, average heterozygosity and Polymorphism Information Content (PIC) observed are 0.86 and 0.85 respectively. In the study conducted by Velasco-Ramírez *et al.* (2014), they observed 0.45 and 0.49 for average heterozygosity and PIC value which is very less than the values obtained in this study. The higher PIC and observed heterozygosity values obtained in the present study for ISSR markers indicated the high variability of the population studied. It also indicated the usefulness of the ISSR marker identified and they can be used in elucidating genetic diversity studies among yams in future.

The cluster analysis done based on dissimilarity index of 45 accessions for ISSR. Based on that, the genotypes formed four clusters at 0.57 dissimilarity index. From the dendrogram analysis Da 99 and Da 81 had high similarity (0.98).Cluster 1, 2 and 4 have only one accession Da 331, Da 243 and Da 340 respectively. They were denoted as outlier with respect to other clusters. The cluster 3 contains large number of genotypes and it was again divided into sub clusters.

Simple sequence repeats (SSR) markers showed that genetic diversity is higher among accessions. In the present study, 47 bands identified in 45 accessions with a high percentage of polymorphism (100 %). Higher band number was obtained for the SSR primer Dab2C05 and lowest in SSR YM 5. Siqueira *et al.* (2014) reported 61 bands from the study using 90 accessions with high amount of polymorphism of

98.3% which is similar to the present study. Due to high polymorphism the SSR markers were able to clearly discriminate the *D. alata* landraces in Kerala.

Further, the allele number assessed the efficiency of each primer taken for the study by the calculation of polymorphic information content and observed heterozygosity. The values of observed heterozygosity ranged from 0 (YM 5) to 0.8396 (Dab2C05). The range of PIC is from 0 (YM 5) to 0.8191 (Dab2C05). In this present study, SSR primer produced an average of polymorphic alleles, *Hobs* value and PIC value obtained are six, 0.6904 and 0.6614 respectively. In the study of Otoo *et al.* (2015a), reported that mean PIC value was 0.91 which is more than the value recorded in the present study.

The cluster analysis done based on Euclidian distance of 45 accessions for SSR. Based on that, the genotypes formed 3 clusters at 0.37 dissimilarity index. From the SSR cluster analysis, Da 120 and Da 56 showed higher similarity (0.97).Cluster 1 have only one accession (Da 52) forming an outlier and cluster 3 have two genotypes Da 331 and Da 340. The cluster 2 comprised of large number of genotypes and it was then divided into sub clusters forming Da 122 as an outlier. The sub cluster is divided further to depict the genetic diversity.

The present genetic diversity study on greater yam landraces showed good variation among the accessions. This gives emphasis to the hypothesis that India especially Western Ghats could be the main center of origin of *D. alata.*

The cluster data from dendrogram was in agreement with data from cluster analysis done after PCA. The present findings was in conformity with the results obtained by Velasco-Ramírez *et al.* (2014).

5.2.Morphological analysis

The 45 *Dioscorea alata* accessions which are used for the study were genetically different and therefore based on their close associations or relationships clustered in groups. The descriptors for yams were efficient and helpful in differentiating the *Dioscorea alata* accessions into different clusters. There were no duplicate accessions identified based on the morphological classification and it can be

maintained as core collection. Morphological traits like tuber shape, tuber skin texture, tuber cortex colour, tuber flesh colour, leaf shape and young leaf colour were all found to be of great importance in distinguishing the accessions. Morphological characterization provided an inexpensive means of quickly evaluating the "water yam" accessions.

The use of qualitative traits in the classification of crops is very essential due to their relative stability over qualitative traits. Wide range of polymorphism was observed in some of the various qualitative characters used in the differentiation of the accessions namely, shape of tuber, colour of tuber flesh, tuber cortex colour, texture of tuber skin, young leaf colour and leaf shape. Similar observations have been reported in the studies done by Demuyakor *et al.* (2013) and Anokye *et al*. (2014).

Among the genotypes evaluated yellowish white/off-white emerged as the predominant character among the flesh colour of the accessions. It was followed by purple colour, white and yellow with lowest relative frequency. Islam *et al*. (2011), assessed 59 *Dioscorea alata* accessions from Bangladesh and observed white, yellow, off white, red, purple and yellow orange flesh colour. Similarly on the study conducted on *Dioscorea alata* landraces by Bressan *et al.* (2011), also observed that white, purple and yellow coloured flesh of tubers exist. In this study, the higher number of white, yellow and purple coloured tuber flesh observed were in agreement with Bressan *et al.* (2011), while red types were absent as reported by Islam *et al*. (2012), light purple tuber cortex colour were in the majority among the accessions studied followed by light cream, light yellow dark purple, purple colour and greenish colour. Regarding the tuber skin texture, majority of the germplasm had warty skin while others have moderately smooth and smooth skin. Regarding the tuber shape, it showed a large range of variation morphologically for the accessions. The observed tuber shapes are oval-oblong, spindle shape, round, oval, cylindrical, conical and irregular shapes. In the diversity pattern study of greater yam in Brazil (Siqueria *et al.,* 2014) three tuber shapes, namely oblong, irregular

and round were found. But in this study more than three shapes found indicating more variability for this crop in India.

For other qualitative characters, wide variation was observed in terms of the shape of the leaves. Most of the materials had cordate leaf shape ranging between cordate long and cordate broad. Very few accessions had sagitate leaf shape also. The present study have similar observation in the study of morphological characterization of *D. alata* in Ghana (Anokye *et al.,* 2014).

The results obtained from the PCA showed that the characters that most contributed to the species variability were related to shape of tuber, flesh colour and colour of tuber cortex. Further morphological data clustering with tree analysis concept and tree construction using Jaccard's similarity matrix approach gave three main clusters. In the cluster formation tuber flesh colour contributed maximum polymorphism. This is identified that purple coloured flesh colour having accessions (Da 340 and Da 331) were divergent from other landraces. Similar observations found in the study of genetic diversity analysis in greater yam native to India (Sheela *et al.,* 2016). Mahalakshmi *et al.* (2007), also stated the use of morphological traits for obtaining a core collection for *D. alata* by assessing a total of 772 accessions. From the results, 45 accessions can be used as part of the core collection using both qualitative and quantitative characters.

5.3.Biochemical analysis

D. alata cultivars are used as a staple food in many communities of tropical world. Lebot *et al*. (2006), studied the physico-chemical properties of 48 *D. alata* accessions from Vanuatu regions. The result revealed that the dry matter, starch and protein varied from 14.81-31.42 percent, 63.6-78.6 percent and 8.8-17.0 percent respectively among these accessions.

Baah *et al.* (2009), studied the nutritional and biochemical characterization of *D. alata* tubers. In this study they investigated the chemical and nutritional content of 16 *D. alata* varieties. The dry matter content ranged from 22.3 to 33.8 % on a fresh

weight basis. The ranges of crude protein, sugar and starch were 4.3-8.7, 3.6-11.0 and 60.3-74.4 percent on dry weight basis, respectively.

In the study on Biochemical quantification of crude protein, starch, fat, ash , crude fibre and dry matter content in different collection of greater yam found in Orissa , Behera *et al.* (2009), reported that the dry matter, starch, protein were varied from 24.91-33.33%, 78.36-82.51% and 7.31-9.67% on dry weight basis, respectively.

In the present study on 45 landraces in Kerala, The dry matter per cent ranged from 27.53 (Da 56) to 48.24 (Da 69). The starch content, sugar content and protein content are studied on the dry weight basis. The values ranged from 36.0 (Da 40) - 65.22 (Da 289) for starch, 2.73 (Da 278) – 9.62 (Da 70) for sugar and 5.86 (Da 390) – 14.51 (Da 331) for protein content on dry weight basis. The values for the studied nutritional characters is higher than the reported studies. This indicated that the landraces of *D. alata* grown in Kerala have better nutritional quality than the landraces from other parts of the world.

SUMMARY

6. SUMMARY

Yam plant is a vine cultivated for its big, edible underground tubers. More than 600 Dioscorea species exist globally. Yam is primarily consumed for its carbohydrates, as well as for protein, vitamins and minerals. It also has medicinal properties like the steroidal sapogenins, used in the production of cortisone and synthetic steroid hormones. More than hundred cultivars of *Dioscorea* exists; however, only a few of them worth of commercial importance.

Greater yam is one of the oldest cultivated yam species with the largest distribution in world. They are one of the important tropical crops which require hot, humid climates and may stop to grow when the temperature fall down below 68ºF. Greater yam is known for its high nutritional content, with crude protein content of 7.4%, vitamin C content ranging from 13.0 to 24.7 mg 100 g-1, and starch content of 75-84%.

In the present study, DNA was isolated from 45 greater yam (D. alata L.) genotypes. Standardization of PCR conditions was undertaken for different types of molecular markers so as to obtain clear and consistent amplicons under reduced cycle duration. Changing annealing temperature for certain SSR primers gave better results. Molecular markers viz. ISSR and SSR primers were selected based on the previous studies in yams. For preliminary screening of ISSR, 16 ISSR primers were used of which, only 15 were selected based on their polymorphism and repeatability. For preliminary screening of SSR, 11 SSR were used of which, only 9 were selected based on their polymorphism and repeatability.

The amplicons obtained after ISSR analysis of accessions using the selected primers were initially resolved in two percent agarose gel. Genetic properties of the fifteen ISSR markers which are used for analysing the greater yam landraces is given in Table. Total number of bands per ISSR primer ranged from 5 (UBC 817) to 12 (UBC 809 and (GA)9AT). The polymorphism of the ISSR primers studied ranged from 90.9% to 100%. (ACC)6Y and UBC 818 recorded the lowest polymorphism. In the present study, the ISSR primer produced an average of 9

polymorphic alleles with mean Hobs and values of PIC 0.8619 and 0.8472 respectively.

The amplicons obtained after the SSR analysis of the accessions using selected primers were initially resolved in 2% agarose gel. Among the studied SSR markers, the total allele number per marker ranged from one to eight. All the SSR primers showed 100% polymorphism. In the present study an average of polymorphic alleles, Hobs value and PIC value obtained are six, 0.6904 and 0.6614 respectively.

Dendrogram based on ISSR markers showed the partition of the D. alata accessions into three different clusters at a similarity coefficient of 0.57.In the analysis Da 340 and Da 331 showed maximum genetic divergence from other landraces. Even though duplicates were not found, Da 58 and Da 56 recorded very high similarity. SSR markers showed the partition of the D. alata accessions into ten clusters at a similarity coefficient of 0.57 of which six clusters have one accession each (Da 340, Da 331, Da 209, Da 391, Da 122, Da 52). The study based on SSR data revealed the similarity of Da 308 and Da 65 even though they are not duplicates.

The 45 accessions of greater yam is morphologically analysed using the descriptors of yam. According to the morphological data, there were no duplicate accessions identified based on the morphological classification and it can be maintained as core collection. Morphological traits like shape of tuber, tuber flesh colour, colour of tuber cortex, texture of tuber skin, young leaf colour and leaf shape were all found to be of great importance in distinguishing the accessions. Morphological characterization provided an inexpensive means of quickly evaluating the “water yam” accessions.

Among the 45 *D. alata* accessions, seven quantitative traits are recorded and wide range of variation observed. The seven quantitative data is recorded and performed ANOVA (Analysis of Variance) and estimation of genetic parameters, genotypic and phenotypic correlation between the selected quantitative characters.

Among the agronomic traits evaluated, highest genotypic coefficient of variation (GCV) was recorded for length of tuber (36.51) followed by girth of tuber (31.15) while lowest GCV was recorded by leaf length (12.95). Among the traits studied, tuber characters recorded very high heritability related to shoot characters. The higher value of heritability was noted by tuber length followed by girth of tuber. Highest genetic gain (%) was recorded for girth of tuber (17.62) followed by tuber length (13.64).

The cluster dendrogram based on morphological data revealed that the D. alata accessions exhibited greater degree of genetic variation for the 42 different morphological traits observed. Among the accessions none were found to be completely similar. However maximum similarity was obtained between Da 56 and Da 84.

The biochemical characters of tubers of D. alata landraces grown in different districts of Kerala are determined. The dry matter per cent ranged from 27.53 (Da 56) to 48.24 (Da 69). The starch content, sugar content and protein content are determined on the dry weight basis. The values ranged from 36.0 (Da 40) -65.22 (Da 289) for starch, 2.73 (Da 278) – 9.62 (Da 70) for sugar and 5.86 (Da 390) – 14.51 (Da 331) for protein content on dry weight basis. Among the accessions evaluated Da 331 recorded the highest percentage of crude protein content (14.51) followed by Da 102 (13.52) and fourteen accessions recorded high crude protein above ten percent.

Forty two traits were selected for PCA analysis of yam accessions morphologically with a Measure of Sample Adequacy greater than 0.5 and the first six principal components of data accounted for 50.12% of the overall variation among accessions. The principal component 1 (PC1) elucidated 13.58% of the overall variation, which was contributed majorly by young vein colour, colour of young leaves, leaf shape, petiole length, young leaf vein colour, tuber shape, tuber cortex colour and starch content. The distribution of accessions in the scatter plot revealed high divergence of Da340, Da331 and Da 390.

The present study revealed good variability among accessions using molecular and morphological analysis. There were no duplicates found among the accessions and these can be maintained as core collection and used for future diversity studies. Da 331 is found to be highly divergent among the accessions from the SSR, ISSR, morphological and biochemical analysis and it can be used for future breeding purposes.

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APPENDICES

APPENDIX I

CTAB Extraction Buffer

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

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.

APPENDIX IV

Wash solution

Ammonium acetate	15mM
Ethanol	70%

APPENDIX V

Chloroform: Isoamyl alcohol

Chloroform	24ml

Isoamyl alcohol	1ml

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

APPENDIX VI

Potassium ferricyanide (1 %)

Exactly 1.0 g potassium ferricyanide (AnalaR) dissolved in 100 ml of distilled water and store for further use in a brown bottle.

APPENDIX VII

Sodium hydroxide (2.5N)

Dissolve 10.0 g sodium hydroxide pellets in 100 ml distilled water.

APPENDIX VIII

Hydrochloric acid (2.0 N)

Dilute concentrated hydrochloric acid six times by adding 250 ml distilled water to 50ml Con. HCl.

APPENDIX IX

80% ethanol

100% ethanol	80 ml
Distilled water	20 ml

APPENDIX X

Sodium hydroxide (40%)

NaOH	400g

Dissolve 400g Sodium hydroxide in 1 litre distilled water

APPENDIX XI

Boric acid (4%)

Boric acid	40g

Dissolve 400g boric acid in 5 to 6 litres of very hot distilled water. Mix and add more hot distilled water up to 9 litres

ABSTRACT

Genetic diversity analysis of greater yam (*Dioscorea alata* L.) landraces in Kerala

ATHIRA JYOTHY

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Abstract of the Thesis

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DEPARTMENT OF PLANT BIOTECHNOLOGY

COLLEGE OF AGRICULTURE

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KERALA, INDIA

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ABSTRACT

Yams belonging to Dioscoreaceae family are important climate resilient food security crops and widely cultivated in India, one of its center of origin, and in Africa. In India, greater yam (*Dioscorea alata* L.) is one of the major cultivated yam species and it provides a very good source of dietary carbohydrates in regions of tropic and subtropics. This research work attempts molecular, morphological and biochemical characterization of 45 landraces collected from different districts of Kerala for greater understanding of the distribution and extent of genetic variation present within the *Dioscorea alata* germplasm.

In the present study, 45 accessions conserved in the National repository of tuber crops germplasm at ICAR-CTCRI, Sreekariyam were characterized based on 25 qualitative and 13 quantitative traits including the major yield components and three biochemical characteristics. There were no duplicate accessions identified based on the morphological classification and it can be maintained as core collection.

The biochemical study on dry matter, starch, sugar and crude protein content were carried out to determine the nutritional quality of the accessions studied. The dry matter percentage ranged from 27.53 to 48.24 percent. The percentage starch content of the accessions ranged from 36.0 to 65.2 and 13.2 to 26.4 on dry and fresh weight basis respectively. Sugar content ranged from 2.4 to 9.6 percent on dry weight basis. Crude protein content on dry weight basis varied from 5.86 to 14.51 percent and Da331 recorded the maximum crude protein content.

The genetic diversity on molecular basis was determined using nine SSR and 15 ISSR markers. All the SSR primers and ISSR primers studied showed very high polymorphism. The ISSR primers on an average produced 9 polymorphic alleles with mean observed heterozygosity (*Hobs*) and values of polymorphism information content (PIC) 0.8619 and 0.8472 respectively. The SSR primers on an average produced six polymorphic alleles. The *Hobs* and PIC values obtained were 0.6904 and 0.6614 respectively. Highest number of bands was obtained for the SSR primer Dab2C05 and lowest in SSR YM 5.

Clustering based on ISSR marker analysis grouped the genotypes in to four clusters. Da 99 and Da 81 recorded high similarity (0.98). The Clusters 1, 2 and 4 have only one accession each *i.e.* Da 331, Da 243 and Da 340 respectively. Clustering and Principal Component Analysis (PCA) of the data validated the variation among the *Dioscorea alata* accessions. The distribution of accessions in the scatter plot revealed high divergence of Da340, Da331 and Da 390. Based on SSR marker analysis, the genotypes formed 3 clusters at 0.37 dissimilarity index. and Da 120 and Da 56 showed higher similarity (0.97).Cluster 1 have only one accession (Da 52) forming an outlier and cluster 3 have two genotypes Da 331 and Da 340.

The highly polymorphic ISSR and SSR primers identified can be used for further genetic diversity characterization in future. The results from the present study serves to facilitate the development of better varieties by plant breeding and marker assisted breeding programs.