GENETIC DIVERSITY ANALYSIS OF WILD YAMS OF WESTERN GHATS

by

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(2011-09-113)

THESIS

Submitted in partial fulfilment of the
Requirement for the degree of

MASTER OF SCIENCE (INTEGRATED) IN BIOTECHNOLOGY

Faculty of Agriculture

Kerala Agricultural University, Thrissur

B. Sc-M. Sc. (Integrated) Biotechnology

DEPARTMENT OF PLANT BIOTECHNOLOGY

COLLEGE OF AGRICULTURE

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KERALA, INDIA

2016

CERTIFICATE

Certified that this thesis entitled **"Genetic diversity analysis of wild yams of Western Ghats"** is a record of research work done independently by Irfa Anwar (2011-09-113) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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ACKNOWLEDGEMENT

It gives me immense pleasure and satisfaction to look over the past and remember everyone who have helped and supported me along this long but fulfilling travel.....
I joyously take this opportunity to express my heartfelt gratitude to:

Dr K. Rajmohan, former Course Director, who spearheaded the endeavour of carrying out the project at a national institute of immense reputation and has been a great mentor right from the beginning of this course till the end.

Dr James George, Director of CTCRI, whose whole–hearted benevolence and encouragement, gave me the opportunity to execute my thesis at CTCRI.

Dr Sheela M. N., my beloved Major Advisor who gave me the intellectual freedom to explore the work on my own and at the same time the guidance to recover whenever my steps faltered. In addition to supporting my participation at various conferences, her valuable advice at professional and personal fronts was a constant encouragement.

Dr B.R Reghunath, DEAN, Professor and Head of Department of Plant Biotechnology for motivating me to be excellent and hardworking, extending meaningful suggestions and scrutinizing the thesis chapters impeccably.

Dr Swapna Alex, my dearest teacher and Course director, who constantly assisted and motivated me to aim high and confront every obstacle and challenge in life with ease.

Dr Vijayaraghava Kumar for his immense support and help in using different statistical softwares and data analysis.

Dr Asha K. I. for helping me using her experience and immense knowledge in morphological identification and guiding me and correcting my write-ups to make it excellent and well-written.

Dr Sreekumar J., for allowing me to utilise the informatics lab- internet facility for Review of literature.

Dr A. Asha Devi for her wholehearted cheering, guidance and help at any time of emergency.

Dr C. Krishnaradhika N.and Dr Vishalakshi for encouragement and support all throughout the work.

Dr C. Mohan for being a good teacher and for providing spiritual motivation at times of stress.

Dr Lekha Sreekantan, my beloved and most compassionate teacher for motivating, cheering, guiding and the one who teached me to dream about the future and achieve the goals in flying colours.

Dr Soni K. B., Dr Deepa S. Nair and all other faculty members at College of Agriculture, Vellayani for manifesting their love and concern.

Dr T. Mahesh Kumar, Dr Padmaja, Dr J. T. Sherief and Mrs Sujatha for their earnest support, assistance and the required facility at times of need in their respective divisions.

Mr PrakashKrishnan B. S., who helped with the technical aspects of my work including handling of various instruments, standardisation of programmes and providing me with valuable informations , thus enriching my practical knowledge.

Mr Abhilash P.V and Mr Shershah, Mrs Aswathy and Ms Vidya P for their active and timely support and guidance.

Mr Vishnu of Diognostic Lab, who were always cheerful in offering assistance to use gel documentation facility.

Leen Abraham and members of Transgenic lab for being Co-operative in extending lab facility.

To my beloved, Mr Shefin srajudeen, who's support, care and encouragement played a great role throughout this year for me, who teached me to handle hard situations with atmost confidence and courage and to dream about my future and to accomplish my project to its fullest.

To my senior Mr Ambu Vijayan for wholehearted support, care, concern, guidance and active interventions throughout the work in tackling difficult situations.

To all staff of Division Of Crop Improvement for their whole- hearted help and manpower at times of planting especially Mr Ajeesh T, Ms Aritha, Mr Viswamparan Nair and Mr Babu.

All my dear colleagues of IBC 2011- especially Keerthana P. V., Lakshmipriya Athira Jyothy, Deepthy Chandran, Jayakrishnanan, Geethu S Nair, Saranya, Aswini, Geethu Krishna and Krishnaraj S. for their unlimited help, warm encouragement and lively companionship throughout these years.

My mom and dad, for all their love, care, encouragement and support, without which I would have never reached where I am.

Finally I humbly thank the Almighty for showering his blessings and bestowing the wisdom, perseverance and physical ability to accomplish this work.

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LIST OF ABBREVATIONS

◦C	Degree Celsius
%	Percentage
µg	Microgram
µl	Microlitre
µM	Micromolar
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
mM	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 HCl	Tris (Hydroxyl Methyl) aminomethane hydrochloride
U	Enzyme unit

UV	Ultraviolet
V	Volt
v / v	volume/ volume
w / v	weight/ volume

INTRODUCTION

1. INTRODUCTION

The English term 'Yam' is believed to be originated from tribal African word "*niam*" meaning "to sample" or "to taste" (Coursey *et al.,* 1967). Yams belong to Dioscoreacea family. These are monocotyledonous, perennial, herbaceous vine constituting an important part of the forest flora forming edible aerial and underground tubers.

Approximately 850 *Dioscoreae* species are known all around the world. Yams, the edible starchy tubers, are of cultural, economic and nutritional importance in the tropical and subtropical regions of the world (Bhandari and Kawabata*,* 2005). In fact they are one of the principal sources of food and nutrient energy for many people in the tropics. Yam has been suggested to have nutritional superiority when compared with other tropical root crops (Baquar and Oke, 1976). They are reported as good sources of essential dietary nutrients since they are the rich source of fiber, starch, proteins vitamins and minerals (Sheikh *et al.,* 2013).

The wild yams are the major food crop among the tribal community. They depend on the yams not only during the period of drought and food scarcity but also form a part of their regular intake. Wild yams are considered as a "life saving" crop among many tribal community. The tribes consume various species of *Dioscorea* like *Dioscorea pentaphylla, Dioscorea oppositifolia, Dioscorea wallichii, Dioscorea pubera, Dioscorea wightii, Dioscorea bulbifera, Dioscorea hispida* etc. Among them *Dioscorea pentaphylla* and *Dioscorea oppositifolia* are considered safe and taste good to eat but tubers of *Dioscorea hamiltonnii* tastes bitter and considered to be toxic and therefore requires thorough processing before consumption (Narayanan *et al.,* 2011).

The bitterness and toxicity in these wild yams are caused due to the presence of high level of saponin, the saponin is actually a steroidal saponin, which is a secondary metabolite produced by yams namely diosgenin. The consumption of these tubers without processing causes vomiting and diarrhoea. When large amount are ingested it will cause itching sensation among children (Webster *et al.,* 1984).

The diosgenin have many pharmaceutical properties and extracts from the tubers of *Dioscorea villosa* have been traditionally used for the treatment of diabetes, nephritis, asthma, cardiovascular diseases and hypercholesterolemia (Tada *et al.,* 2009). Cases are also reported from the Munda tribe of Jharkhand that processed tubers of *Dioscorea bulbifera* are consumed by Munda tribes for relieving from gastric disorders and constipation (Singh and Kumar, 2015).

Wild yams and domesticated cultivars occur throughout the tropical and subtropical world. In India, they are grown practically in all the States but the major yams producing States are Kerala, West Bengal, Bihar, Orissa, Assam, Gujarat and Maharashtra. The genetic variability available in different parts of Kerala, Assam, Nagaland, Maharashtra and Goa have been collected and maintained at National Repository for tuber crops germplasm , ICAR-CTCRI. Since wild yams form one of the major food source for the tribal population of Kerala, it is imperative to study the genetic diversity of the wild yams seen in Western ghats and their nutritional quality. DNA fingerprinting will also helps to protect the genetic wealth from biopyracy.

This research work attempts to combine morphological and molecular data for greater understanding of the distribution and extent of genetic variation existing within the wild yam accessions collected from western ghats of India. Being one of the major food source of the tribals, nutritional evaluation of the wild yams is highly essential. The exploitation of the genetic diversity so determined serves to facilitate the development of better varieties by plant breeding and crop improvement programmes.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1 ROOT AND TUBER CROPS

Tuber crops are the third important food crops after cereals and legumes and are either a staple or subsidiary food for about one-fifth of the world population. They contribute about 6% of the world's dietary calories and are important sources of animal feed and raw materials for industrial products. In terms of annual volume of production, cassava, potato, and sweet potato rank among the top 10 food crops produced in developing countries. Yams, taro and tannia are the other important root and tuber crops produced globally. Taro (arvi), elephant foot yam (jimikand), and yams are grown as vegetable crops in homestead or in semi-commercial scale throughout India.

The tuber crops are rich in minerals, vitamins, antioxidants and dietary fibre. They may play an important role in mitigating hidden hunger through diet diversification. Most of the tuber crops have higher biological efficiency as food producers with high dry matter production per unit area per unit time. The tuber crops have proved to be life sustaining crops in times of natural calamities and famine. Most of these crops are bestowed with resilience to global warming and climate change and potential for better return under adverse soil and weather condition. They also have the potential of reversing extensive soil degradation primarily caused by intensive cultivation of grain crops.

Tuber crops are also used in industrial scale for the production of bio-ethanol and also for biopolymer production to contract the adverse effect of plastics in the environment. Thus with increasing world population the demand for carbohydrates also will increase and being rich source of carbohydrates, roots and tuber crops can play an essential role in ensuring food security especially to low income population.

2.2 ORIGIN OF YAMS

Within the tropics, species of *Dioscorea* are to be found almost everywhere where the rainfall is sufficient for their growth. They occur in both Old and New Worlds in the wild state, while even the Australian continent, whose flora is in many ways unique, has a few indigenous species. Different members of the genus are adapted to such diverse ecologies as rain forest and savannah; few are found in on coastal plains, and on mountains at altitudes of several thousand metres. Virtually the only tropical areas where no *Dioscorea* are to be found are swamps, the near desert districts where the rainfall is less than about 80cm per annum, and on mountains of altitude sufficiently great for heavy frost to occur.

The origin of Asiatic food yams in South-East Asia probably Burma or Siam is generally accepted. Vavilov (1950) has suggested that *D. alata* and *D. esculenta* are derived from the Indian centre of origin, Burma and Assam, while the former of these had a subsidiary distribution from the Indo-Malayan centre rather farther south east, from which centre *D. hispida, D. pentaphylla* and *D. bulbifera* also came into cultivation. The two more temperate species of the section Enantiophyllum, *D. opposita* and *D. japonica* has Chinese centre of origin.

The possibility of independent origins of yam cultivation in Africa and America had been proposed by Watt as early as 1890. Many closely related species are also known in the wild state in West Africa, including *D. praehensilis*, a possible ancestor of the cultivated *D. rotundata*. The great antiquity of yam cultivation in West Africa is also indicated by the number and importance of cultural and superstitious practices associated with the cultivation of the crop, and by African tradition.

The available evidence, however, clearly indicates four distinct centres of origin for the edible yams. First of all, a site somewhere in the Indo-Chinese peninsula, where *D. alata, D. pentaphylla,* and probably *D. esculenta, D. bulbifera* and some species were cultivated. Secondly, a centre in southern china, where the more temperate adapted species *D. opposita* and *D. japonica* and possibly *D. esculenta* originated. Thirdly, a location on the fringe of the West African forest

belt either within the savannah, or possibly in the Dahomey gap, where savannah conditions penetrate south to the sea where *D. cayenensis, D. rotundata* and *D. dumentorum* were originated. Lastly, *D.trifida* and probably some other less important species of *Dioscorea* have centre of origin in the Caribbean area: again, there may be a subsidiary centre in South America.

2.3 INDIGENOUS TRADITIONAL KNOWLEDGE

2.3.1 Kattunaikka, Paniya and Kuruma tribal community, Wayanad Kerala, India

Wayanad district is with a hilly terrain on the southern Western Ghats, which conserves a very impressive ethnic diversity of ten different tribal groups. The main three tribal communities are Kattunaikka, Paniya and Kuruma. These tribal communities conserve knowledge of 165 edible plants and various other indigenous traditional knowledge of various crops and edible species found only in the wild and play a crucial role in the food security of tribal and rural families. Tuber crops play an important role in the diets of these tribal communities. For instance, various wild species of *Dioscorea*, *Colocasia* and *Amaranthus* which are the source of vitamins and nutrients, supplement the food needs of a multitude of families who live near to forests (Roy *et al*., 1998).

For certain communities consumption of wild tubers is included in their regular dietary intake and considered as “life saving” crops at the period of acute famine and scarcity (Narayanan *et al.,* 2011). The Paniya community possesses knowledge regarding 136 taxa of wild edible plants, with Kattunaikkas coming next with knowledge of 97 taxa. Amongst these tribal communities, the Kurumas are at the bottom of the knowledge-ladder with knowledge of 42 taxa of wild edible plants.

About 24 varieties of wild tubers are consumed based on characteristics like edibility, taste, colour, size, direction of growth, fiber content, cooking properties and occasionally the pattern of underground tuber proliferation. The important wild yams include *Dioscorea hamiltonii, Dioscorea oppositifolia, Dioscorea wallichii,*

Dioscorea pubera, Dioscorea hispida, Dioscorea bulbifera, Dioscorea wightii, Dioscorea tomentosa and Dioscorea pentaphylla.

Dioscorea hispida tastes bitter and considered toxic and hence thoroughly processed before consumption. *Dioscorea tomentosa* is not consumed due to high mucilaginous content. *Dioscorea wallichii* is rich in fibre content. *Dioscorea oppositifolia* and *Dioscorea pentaphylla* tastes good and were preferred for consumption since they are rich in starch and pulp and are popular among tribal communities.

2.3.2 Munda tribes of District Kundi, Jharkhand, India

Munda tribe is the third leading tribal community out of total 30 tribal communities of the State Jharkhand and is the dominating tribal community of Khunti district. The Munda people are living in remote places in close vicinity to nature. Although the above ground parts like, leaves, stems etc. dries up, the munda people have unique traditional knowledge of searching the tubers even in absence of above ground parts and utilizing them as food.

Munda people use, about nine wild edible yams or *Dioscorea* species like *Dioscorea alata, Dioscorea bulbifera, Dioscorea hispida, Dioscorea quartiniana, Dioscorea glabra, Dioscorea pubera* and an unidentified *Dioscorea sp*. The tribes consume underground as well as aerial tubers. Most of the yams can be eaten after roasting them in low heat and peeling off the skin, but some species of yams *viz., D. bulbifera and D. dumetorum* needs to be processed due to the presence of toxic substances.

D. dumetorum posses hallucination properties and tribal people mix small portion of the tuber in their local alcoholic drink. The wild yams also have ethanomedical properties. The extracts of wild yams and even the cooked form is used for ailments like tuber of *Dioscorea alata* relieves from constipation and of *Dioscorea bulbifera* is given for gastric disorders and constipation. The utilization of the natural resource for food and lifestyle is the sole reason behind the maintenance of good health of the Munda tribal community in Jharkhand (Singh and Kumar, 2015).

2.4 NUTRITION CONTENT

Yam is a food source for more than 100 million people and is an important income source for small farmers in many developing countries (Lebot, 2009). Yam is primarily eaten for its carbohydrates (Tetchi *et al.,* 2007), as well as protein, potassium, sodium, magnesium, copper and zinc (Baah *et al.,* 2009). Yams are also known for phytochemical contents like flavanoids, cholesterol, alkaloids, terpenoids, cardiac glycosides, saponin, and steroids (Sheikh *et al.,* 2013) and also rich in vitamins like ascorbic acid, niacin, riboflavin and thiamine. The bitterness and toxicity is due to the presence of saponin and alkaloids. The studies on phytochemical, mineral and vitamins of different species of *Dioscorea* showed that *Dioscorea dumentornm* is having high phytochemical as well as mineral content.

2.5 MORPHOLOGY

The members of genus *Dioscorea*, are plants consisting of a rhizome or tuber, from which stems and roots emerge annually during the growing season. All members of genus *Dioscorea* are dioecious, male flowers have more inflorescence than female flowers. The female flowers are born in small numbers in axillary spikes, and the male inflorescence formed in large number in panicles. The flowers are small, but insignificant in colour, generally being white, creamish or greenish shades or sometimes brown and are entomophilous.

Fruits are formed in dry, dehiscent, trilocular capsules, 1-3cm long. The seeds are small, light, flattened and winged to aid dispersal by wind. Leaves are simple or composite, cordate leaves carried on comparatively long petioles and in some species like *Dioscorea trifida, Dioscorea dumentorum and Dioscorea pentaphylla* leaves are lobed. Several species of *Dioscorea* bear stipules at the bases of the petioles, which are well developed in some forms of *D. rotundata*, in *D. sansibarensis* and in some other species, but in most they are only rudimentary, while in *D. alata* and *D. bulbifera* they are borne on occasional plants.

The phyllotaxy is usually described as either opposite or alternate, while often the arrangements is alternate on the lower parts of stems and opposite on younger growth.

Stem of all the *Dioscorea,* except for the few dwarf species, are unable to support their own weight for any great height and climb by twining. In most genera the direction of twining is constant, but in *Dioscorea* it is a characteristic of each section, all the members of some sections like *D. hispida, D.esculenta, D. bulbifera* and *D. tomentosa* twining to the left and all those like *D. rotundata, D. wallichi* and *D. pubera* , some others to the right. In some species both leaf and stem will be covered with hair or spines, the form and size of which are useful for the purpose of identification.

Rooting systems of yams are comparatively weak. The development of roots commence at the beginning of the growing season, with the emergence of several thick roots from the "head" or rhizomatous end of the tubers, from which area the stems also arise. These grow rapidly, and ensure that the developing plant is firmly held in the ground. These roots are normally unbranched, and may extend to considerable distances. In a number of species, including such economic plants as *D. esculenta* and some of the Enantiophylla, some roots, especially those which are near the surface of the ground, are armed with spines.

Rhizome in the true sense, *i.e.* a horizontal swollen perennial stem, is a primitive feature in the *Dioscoreaceae.* Tuber of the *Dioscorea,* including those which form the edible portion of the food yams, are stem tubers. Yams form usually only a single tuber, at most two or three which may be individually very large. Weight of *Dioscorea alata* range over 50kg and being 2-3 meters in length while tubers of inferior forms of same species may be only 1kg in weight. The form may vary between globular, flattened or greatly elongated while large tubers may be much branched or lobed.

D. esculenta tubers are small and spindle shaped, while in *D. dumentorum* and in *D. hispida* tubers are fused together in irregular cluster. Plants produce bulbils in the axil of leaves. These are organs, which are especially adapted for vegetative propagation under natural conditions, have the appearance and

morphology of condensed stems. In *D. bulbifera,* the main underground tuber is considerably reduced and aerial tubers or bulbils are the main storage organ of the plant (Coursey, 1967).

2.6 STUDIES ON PHARMACEUTICAL PROPERTIES OF YAM

Yam tubers, belonging to the monocotyledonous *Dioscorea* genus are a great interest in the pharmaceutical industry due to the presence of a secondary metabolite, a steroidal saponin, named as diosgenin. It's used as a starting material for the production of contraceptives, steroids etc.

2.6.1 In the treatment of menopausal syndrome

Rhizome of *D. opposita* (shanyao) has been included in the Pharmacopoeia of the People's Republic of China as a Chinese herbal medicine. It is far the most commonly used *Dioscorea* species for improving female health and for regulating menstruation. The study mainly deals with identification of yam tuber extract having the ability to prevent menopausal syndrome, thus to elevate serum estrogen level. The DOI protein isolated from the tuber extract of *D. opposita* was reported to be potent estrogen – stimulating activity.

BLAST analysis of the N-terminal sequence of DOI in NCBI showed a high E- value (10^{-3}) that indicated it was a novel protein. The results showed DOI was a member of the chitinase-like super family. BLAST analysis of the partial amino acid sequence of DOI revealed that it has high homology with the 27.9-kDa chitinase from *D. japonica* and the 31.4-kDa chitinase from *D. oppositifolia.*

Thus with the extraction of a novel protein DOI from *Dioscorea opposita* of molecular weight 33.5kDa have the efficiency to prevent the decline in immune function during menopause, by triggering the action of natural killer cells, T lymphocytes and B lymphocytes which would have been otherwise declined due to general aging in women. DOI also is a potent therapeutic agent for menopausal osteoporosis. In addition DOI also decreased the viability of MCF – 7 breast cancer cells and ovarian cancer cells in the dose dependent manner thus decreased the side effects of hormone replacement therapy (Wong *et al.,* 2015).

2.6.2 In the treatment of Doxorubicin – Induced cardiotoxicity

Doxorubicin (DOX) belongs to the family of anthracyclines, and is one of the most effective anti cancer drug. But surveys showed that 10% of patients treated with DOX develop cardiomyopathy after the cessation of treatment (Fu *et al.,* 1990). The increased cardiac oxidative stress and lipid peroxidation leading to myocardial apoptosis (Singal and Iliskovic*,* 1998). Thus, apoptosis plays a role in the development of heart failure *via* a loss of cardiomyocyte.

Diosgenin have favourable effects on anti inflammatory (Yamada *et al.,* 1997), anti-oxidant activities (Hsu *et al.,* 2006), (Son *et al.,* 2007). Previous studies showed that food rich in diosgenin possesses the protective effect on myocardial injury in rats due to apoptosis and necrosis (Vasanthi *et al.,* 2010). Another study showed that diosgenin has a beneficial role against aortic remodelling induced by oxidative stress in diabetic state and decreased lipid peroxidation in aorta (Pari *et al.,* 2012).

Diosgenin treatment against DOX induced cardiotoxicity in mice showed the increase in the production of Caspase-3 active component, which helps in decreasing the DOX induced cardiotoxicity. Thus diosgenin act as a heart protective agent by altering GSH, by reducing reactive oxygen species (ROS) against DOX (Chen *et al.,* 2015).

2.6.3 In treatment against Skin aging

Skin aging is mainly due to programmed aging that occurs with time and also caused by environmental factors such as exposure to U. V rays etc. The remedy against skin aging was natural or synthetic estrogen because decline in endogenous estrogen level is also a reason for cutaneous changes which leads to skin aging.

The application of synthetic estrogen suppresses skin aging in aged skin but prolonged use is associated with the risk of breast cancer. The diosgenin was reported to have anti inflammatory and E2 mimicking effects paved the way for opting diosgenin as a potential drug against skin aging. Thus diosgenin induces keratinocytes proliferation and skin thickness by increasing BrdU (helps in cell division) uptake in the cells.

Diosgenin also has anti cancer properties and suppresses growth and induce apoptosis of leukemia, osteosarcoma, and human colon carcinoma and erythroleukemia cells. Action of diosgenin is *via* cell cycle arrest and activation of tumour suppressor genes like p53, release of apoptosis inducing factor and modulation of caspase -3-pathway.

Yams, particularly D. *dumentornm and D. cayenensis* have been well respected by the herbalist community for generations due to their potency in enhancing fertility in males and this is also due to the presence of steroidal sapogenins such as diosgenin (Okwu and Ndu, 2006).

Table No 1. *Dioscorea* species as commercial sources of steroid precursors

Species	Distribution	Parts used	Principal constituent (on dry weight basis)
D. deltoidea	North - western Himalayas from Kashmir to Nepal and China at 1000-3300m.	Rhizomes	Diosgenin (2-5%)
D. prazeri	Wetter parts of northern Bihar, Nepal, Sikkim, Bhutan, Abor hills and Naga hills upto 1800m	Rhizomes	Diosgenin (2-5%)
D. floribunda	Grown in parts of Karnataka (especially Bangalore and Coorg), Assam and Goa	Rhizomes	Diosgenin (3-3.5%)
D. composita	Grown in Jammu	Rhizomes	Diosgenin (2-4%)
(Csir, 1985).			

D. floribunda is rich in diosgenin and is cultivated in America for pharmaceutical purpose in the manufacture of cortisone (Coursey, 1967).

2.7 MOLECULAR MARKER STUDIES IN YAMS

Only a few studies have been carried out on genetic relationships between yam cultivars using molecular markers *viz.* restriction fragment length polymorphism (RFLP) markers (Terauchi *et al.,* 1992), random amplified polymorphic DNA (RAPD) markers (Dansi *et al.,* 2000b) and amplified fragment length polymorphism (AFLP) markers (Mignouna *et al.,* 2003; Tostain *et al.,* 2003). Of all the techniques, microsatellites are one of the most informative markers with desirable genetic attributes including hypervariability, multiallelic nature, codominant inheritance, reproducibility, relative abundance, and extensive genome coverage (Kalia *et al*. 2011).

Tostain *et al.* (2007) studied the genetic diversity of 146 accessions from Benin was analysed using 10 polymorphic simple sequence repeat (SSR) nuclear markers and agromorphological traits. An average of 8.4 alleles per locus was detected. The mean heterozygosity was 0.57 and the mean polymorphism information content (PIC) for polymorphic markers was 0.51. 23% of cultivars were found to have an identical genotype for the 10 markers. Significant heterozygosity was present in *D. rotundata*, at a comparable level with that in wild Japanese yam *D. tokoro* (Terauchi and Konuma, 1994). Three microsatellite markers, Dab2D06, Dab2D08 and Dab2E07, which have shown a large diversity among studied cultivars, are particularly useful for the study of genetic diversity of yams. SSR markers proved to be powerful tools for fingerprinting each cultivar and analysing their genetic relationships.

Siqueira *et al.* (2014) studied on 12 microsatellite primers to generate DNA profiles of 72 local varieties and 17 commercial accessions of water yam collected from four different regions in Brazil. Also, four morphological traits were evaluated on individual plants under field conditions. The traits included: tuber shape, tuber size, tuber skin colour and tuber flesh colour in accordance with IPGRI descriptors. The number of bands or amplification products per locus, the number of polymorphic bands and percent of polymorphism were evaluated visually. The morphological characterization showed considerable diversity. High polymorphism was found with 100 % polymorphism observed for 11 primers and a discriminating power value of 0.92, on average. The highest Shannon index value (H 0 = 0.41) was for the accessions from Southeast region, among the local accessions from different regions in Brazil.

Wellington *et al.* (2013) studied on genetic diversity of *D. trifida* maintained by traditional farmers in Brazil. Fifty three accessions were characterized using eight Simple Sequence Repeats (SSR) and 16 Inter Simple Sequence Repeats (ISSR) markers. Genetic diversity analyses were based on POPGENE Software, version 1.3 (Yeh *et al.,*. 1997). This study showed that it is possible to use both ISSR and SSR techniques for characterizing and discriminating morphologically distinct or similar yam accessions. The eight

SSR primer pairs were highly polymorphic and informative among the 53 *D. trifida* accessions analyzed in this study. The heterologous primers Da1A01 and Dab2C05 showed 100 % polymorphism, and high discrimination power among accessions, equal to 0.97 and 0.91, respectively. The primers specific for *D. trifida*, developed by Hochu *et al.* (2006), besides providing good resolution of bands in the gel electrophoresis, showed high polymorphism, with an average of 93.8 %, and a high number of bands per primer (7 bands, on average). UBC 7, UBC 898, JOHN and MANNY ISSR primers were highly polymorphic with a high number of bands (more than 10 bands) per primer as well as high discrimination power among accessions. The level of polymorphism was high, 95 % for SSR and 75.8 % for ISSR.

The Jaccard coefficient among 53 accessions of *D. trifida* ranged from 0.40 to 0.96, with a variation of 56 % similarity for SSR marker and from 0.66 to 0.97, with a variation of 31 % for ISSR. Mantel test results revealed that data obtained with the SSR and ISSR markers are correlated ($r = 0.5$; $p = 0.0002$), demonstrating similar relationships between data from both marker classes.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The study entitled "Genetic diversity analysis of Wild yams of Western Ghats" was carried out at the Division of Crop Improvement, 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

In order to study the genetic variability among the wild yam species, the molecular characterization using molecular markers *viz.* ISSR SSR and morphological markers were carried out among the following wild yam genotypes (Table 2.).The plant materials used for the study comprises of 24 accessions of *Dioscorea* collected from germplasm maintained in the field gene bank at ICAR-CTCRI, Thiruvananthapuram.

3.1.1 **Maintenance of accessions**

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

Table.2.Accessions of wild yam species used for the study

Sl .No	Species Name	Acc code No.	Sample Code No.
1	*D. alata*	Da 1	CTDa - 1
2	*D. alata 340*	Da 2	CTDa - 340
3	*D. belophyla*	Dbe1	CTDbe-1
4	*D. bulbifera*	Dbu 1	CTDbu-2
5	*D. bulbifera*	Dbu2	CTDbu-1
6	*D. esculenta- CTDE 178*	De 1	CTDe-1
7	*D. floribunda*	Df 1	CTDf-1
8	*D. floribunda*	Df 2	CTDf-2
9	*D. glabra*	Dg 1	CTDg-2
10	*D. hispida*	Dh 1	CTDh-1
11	*D. hispida*	Dh2	CTDh-2
12	*D. opposita*	Do 1	CTDo-2
13	*D. oppositifolia*	Dol 1	CTDol-1
14	*D. oppositifolia*	Dol2	CTDol-2
15	*D. oppositifolia*	Dol 3	CTDol-1
16	*D. pentaphyla*	Dpe 1	CTDp-1
17	*D. rotundata 657*	Dr 1	CTDr-1
18	*D. tomentosa*	Dt 1	CTDt-1
19	*D. tomentosa*	Dt2	CTDt-2
20	*D. vexans*	Dv 1	CTDv-1
21	*D. wallichii*	Dw 1	CTDw -1
22	*D. wallichii*	Dw2	CTDw -2
23	*D. wallichii*	Dw3	CTDw -3
24	*D. wightii*	Dwi4	CTDwi -1
25	*D. pubera*	Dpu1	CTDpu -1
26	*D. hamiltonnii*	Dha1	CTDha -1
27	*D. intermedia*	Di1	CTDi -1
28	*D. spicata*	Ds1	CTDs -1

3.2. Molecular marker analysis

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.

Table 3. List of ISSR primers used for molecular characterization

Sl. No.	Primer name	Sequence (5' ⟷ 3')	T_A (° C)
1	UBC 808	AGAGAGAGAGAGAGC	56° C
2	UBC 809	AGAGAGAGAGAGAGACG	56° C
3	UBC 810	GAGAGAGAGAGAGAGAT	56° C
4	UBC 811	GAGAGAGAGAGAGAGAC	56° C
5	UBC 817	CACACACACACACACAA	56° C
6	UBC 825	ACACACACACACACACT	56° C
7	UBC 827	ACACACACACACACACG	56° C
8	UBC 848	CACACACACACACACARG	56° C
9	UBC 864	ATGATGATGATGATGATG	56° C
10	UBC 818	CACACACACACACACAG	56° C
11	UBC 836	AGAGAGAGAGAGAGAGYA	56° C
12	$(GA)_9$ AC	GAGAGAGAGAGAGAGAGAAC	56° C
13	$(GA)_9$ AT	GAGAGAGAGAGAGAGAGAAT	56° C
14	UBC 14	AGAGGGTTCTCTTG	56° C
15	$(ACC)_6$ 7	ACCAAACAACAACAACAAY	56° C

Table 4. List of SSR primers used for molecular characterization

Sl. No.	Primer name	Sequence (5' ⟷ 3')	Sequence (3' ⟷ 5')	T_A (°C)
1	Dab2C05	CCCATGCTTGTAGTTGT	TGCTCACCTCTTTACTTG	51(° C)
2	Dab2D06	TGTAAGATGCCCACATT	TCTCAGGCTTCAGGG	51(° C)
3	Dab2E07	TTGAACCTTGACTTTGGT	GAGTTCCTGTCCTTGGT	51(° C)
4	Da1AO1	TATAATCGGCCAGAGG	TGTTGGAAGCATAGAGAA	51(° C)
5	Dpr3F04	AGACTCTTGCTCATGT	GCCTTGTTACTTTATTC	51(° C)
6	YM 5	AATGAAGAAACGGGTGAGGAAGT	CAGCCCAGTAGTTAGCCCATCT	58(° C)
7	YM 15	TACGGCCTCACTCCAAACACTA	AAAATGGCCACGTCTAATCCT	58(° C)
8	YM 26	AATTCGTGACATCGGTTTCTCC	ACTCCCTGCCCACTCTGCT	58(° C)
9	MT 13	TAACAAACAAAAAATGAAAC	TAACAGTGATTGAGCTAGGA	59(° C)
10	MT 10	TCGTGTCCATCTTGCTGCGT	GAAAAGCGGAGATGAAGAGCA	59(° C)

3.2.2 Glass wares and materials in Molecular Biology Lab

Eppendorf tubes, pestle and mortar, micropipette tips for 1 ml, 200 µl and 10 µl, PCR tubes were autoclaved and used. Eppendorf tube stand, PCR tube holders, micropipettes, ice bags, polythene covers, labels, wipes, bottles, spatula, 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 transilluminator, 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 wild yam accessions were collected from germplasm of CTCRI, Thiruvananthapuram. Leaves were collected in plastic bags and transferred to lab in an ice box.

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 ground 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 Eppendorf tubes and 10µl Proteinase K (10mg/ml) was added. The tube was incubated in 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 eppendorf 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 12,000g 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 12,000g 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 was removed 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.

3.2.4.2 Using DNeasy® Plant Mini Kit

Young leaves collected were weighed out about 100-200mg. grind it in pestle and mortar using liquid added 400 µl Buffer AP1 and 4 µl RNase A. Vortexed and incubated for 10 min at 65°C. Inverted the tube 2–3 times during incubation. Mixed and incubated for 5 min on ice. Centrifuged the lysate 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. Discard 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 microcentrifuge tube. Added 100 c Buffer AE for elution. 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 -20^0C 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 quantified using Nanodrop spectrophotometer. It helped to assess its yield and purity of isolated DNA. TE buffer 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 micromolar concentrations by adding a volume of nuclease free water equal to ten times the number of nanomoles of DNA present in the tube and stored as main stock. Working stock of 10 mico 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 PCR mixture (Table No 5&6) and PCR programme (figure 1 &2) used for amplification is given below.

Table 5. ISSR reaction mixture

Components	Stock concentration	Required concentration	Volume for one reaction (15µl)
Takara 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	4 µl
dH_2O	---	---	2.6 µl
Total			15 µl

PCR conditions

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

Lid – 105^0C

94^0C – 5 min (initial denaturation)

94^0C – 1min (denaturation)

56^0C – 1 min (annealing)

72^0C – 1 min 30sec (extention)

} Cycles x 35

72^0C – 10 mins (final extension)

4^0C – hold

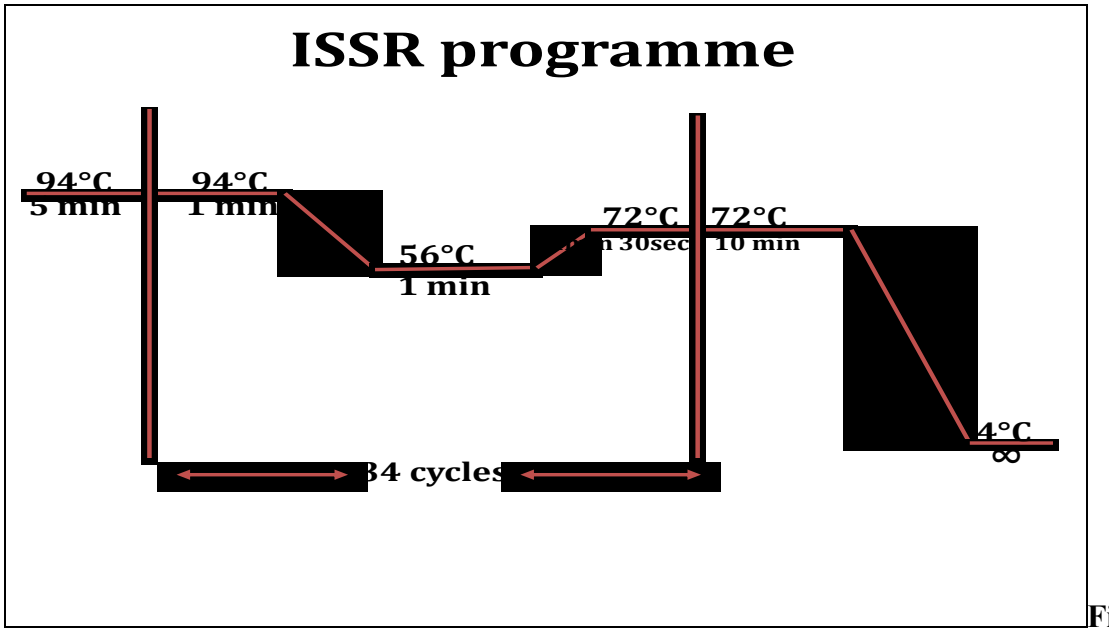


Figure 1. ISSR PCR programme

The amplified products were separated on 2.5% agarose gel along with 1Kb and 100bp ladders to identify molecular weight of obtained bands and for polymorphism studies.

Table 6. SSR reaction mixture

Components	Stock concentration	Required concentration	Volume for one reaction (15µl)
Takara 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	0.45 µl
DNA	10ng/µl	50ng	5 µl
dH_2O	---	---	1.05 µl
Total			15 µl

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 (extention)

Cycles x 35

72^0C – 8 mins (final extension)

4^0C – hold

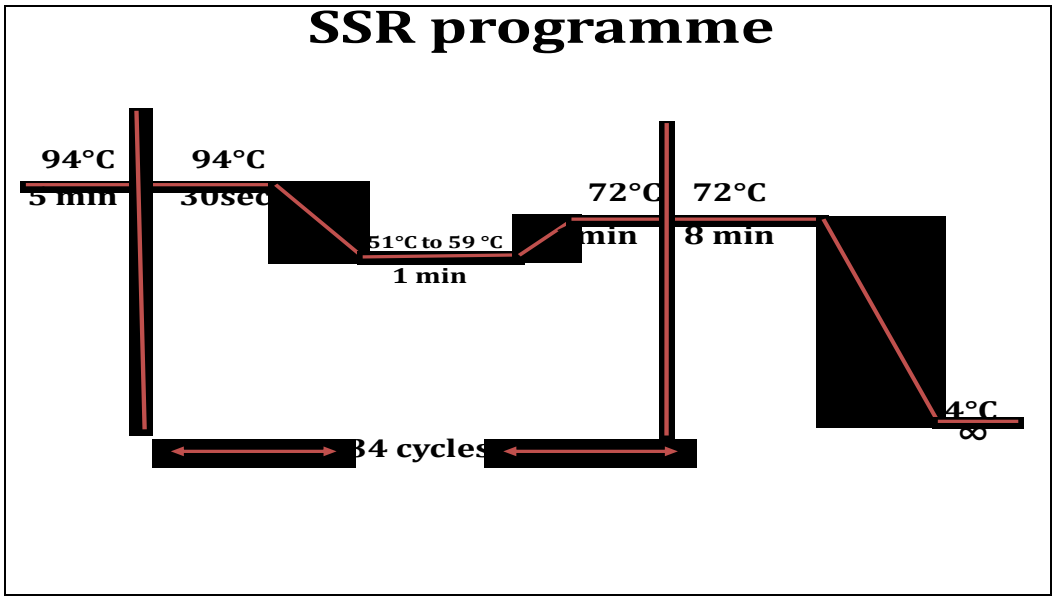


Figure 2. SSR PCR programme

Annealing temperature changed to 58^0C for primers YM 5, YM 15, YM 26 for better results. The amplified products were separated on 2.5% 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 the easiest and most popular way of separating and analyzing DNA. Here DNA molecules are separated on the basis of charge by applying an electric field to the electrophoretic apparatus. Shorter molecules migrate more easily and move faster than longer molecules through the pores of the gel and this process is called sieving. Increasing the agarose concentration will also increase sieving effect. The gel might be used to look at the DNA in order to quantify it or to isolate a particular band. The DNA can be visualized in the gel by the addition of ethidium bromide the intercalating agent along with agarose solution when exposed on UV light.

Weighed 3.0g 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 cooled for 20 mins. Filled the horizontal electrophoretic tank with 1X TBE buffer. After that the solidified gel is transferred to the electrophoretic apparatus and removed the combs. Loaded 10 µl samples and also added 2 µl 100 bp and 1 µl 1 Kb ladders for reference. Run the gel at 100V for 1 to1.5 hour. Visualized the band under UV transilluminator and documented the image on alpha imager. The images finally scored to detect polymorphism.

3.2.10 Morphological analysis

The plant and tuber morphology was done using descriptors of yam (IPGRI/ IITA, 1997). There are 33 descriptors are used for characterization. RHS colour chart is used for determining the colour characters. The respective colour codes are given for each characters.

List of qualitative characters used for morphological analysis

1. Twining direction
2. Mature vine colour
3. Young vine colour
4. Presence of hair/ spine on the vine
5. Presence of wings and its colour
6. Leaf shape
7. Leaf types
8. Leaf arrangements
9. No of leaf lobes
10. Leaf tip shape
11. Leaf margins
12. Leaf pubescence
13. Petiole colour
14. Infloresence type
15. Flower colour at maturity
16. Fruit formation
17. Tuber skin colour
18. Tuber cortex colour
19. Tuber flesh colour
20. Tuber shape

List of quantitative characters used for morphological analysis

1. No of tuber per plant
2. Weight of tuber per plant
3. Length of tuber
4. Girth of tuber

List of Morphological characters scored using Royal herbarium society (RHS) - Colour chart

1. Tuber Skin colour
2. Tuber Flesh colour
3. Tuber Cortex colour

3.2.11 Biochemical characterization

The tuber samples were used for analyzing the biochemical properties and nutritional qualities. The harvested tuber are collected and cleaned for analyzing dry matter, starch, sugar, fat, fibre and protein contents.

3.2.11.1 Dry matter analysis

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

$$\text{Dry matter percentage} = \frac{\text{Dry weight}}{\text{Fresh weight}} \times 100$$

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

3.2.11.2 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.

Treatment with 80% ethanol extracts the sugars present in the tuber sample. The extract was filtered and the filtrate was used for the analysis of sugars. The residue was acid hydrolyzed for the analysis of starch. The starch was completely hydrolyzed by treatment of with 2N Hydrochloric acid (HCl), while the non-reducing sugars were converted to reducing ones, using Con. HCl. Analysis of both of the components was done in a similar manner, based on the number of reducing groups.

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

The extracted sugars were separated from the residue, by filtration through Whatman No.1 filter paper. The filtrate were collected separately for sugar estimation. Residue on the filter paper was washed with two lots (10 ml each) of distilled water to remove the adhering sugar particles and the filtrate added to the original filtrate. The residue was transferred back into the conical flask using 20.0 ml of 2N HCl. The starch in the residue was then hydrolyzed by leaving the flasks on a hot plate at 100^0 C for 30 minutes. The hydrolysates were cooled to room

temperature and their volume increased to 100 ml using distilled water. This supernatant was then directly used for titration for starch.

The alcoholic sugar filtrate was treated with 1.0 ml concentrated HCl and heated for 30 minutes on a hot plate at 100^0 C. The volume of the sugar extract was raised to 50 ml and used for titration.

Titrimetric Assay

Pipetted out 10 ml of Potassium ferricyanide into 100 ml Erlenmeyer flasks. 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 was indicated by change of colour from blue-green to violet. A few more drops were added carefully, to reach the end point, which was 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.

Calculations

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

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

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 the following formula is used:

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

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

3.2.11.3 Protein analysis

Protein is the most essential nutrient present in many food crops including tuber crops. The major element present in proteins is nitrogen, which generally constitutes 16% of the total make up. Determination of the nitrogen content is the easiest way to compute the crude protein content in food crops. The crude protein content is obtained by multiplying the total nitrogen content with a factor of 6.25.

The nitrogen in the protein present in fresh tubers, leaves, flour samples, processed food products etc. is converted to ammonium sulphate during digestion with sulphuric acid. The ammonium sulphate is then converted to ammonia using 40% NaOH which is absorbed into boric acid solution and titrated against standard HCl. The nitrogen content is determined using Semi automated Kjeltec apparatus.

In the present study, Kjehdahl method was used to determine crude protein content. This method involved stages of digestion, distillation and titration.

Procedure

200 mg of well grinded tuber sample weighed and transferred it to the digestion tube. Digestion mixture (Na/K sulphate + $CuSO_4$) and 10 ml H_2SO_4were added to the sample. The tube was then kept in the digestion rack for 2 h at 420^0C. The sample was then cooled and 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.

Calculation

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

3.2.11.4 Estimation of fat

The total fat (ether extractives) present in food crops contributes considerably to the energy content. Approximately, 1.0g of fat gives an energy of 9.0Kcal. Extraction of fat using organic solvents and quantification by gravimetry is the easiest way to determine the fat content of tuber crops, which are generally low in fat. Processed food products from the tuber crops require more number of extractions to completely separate the fat, while fresh tubers, dry chip flour, leaves

etc. require only two extractions with alcohol: ether(3:1) and a single extraction with chloroform: ethanol(1:1).

Reagents

1. Alcohol : ether mixture (3:1)

Mixed 3 parts of ethyl alcohol (absolute) with 1part of diethyl ether(AR). Stored in a tightly capped bottle.

2. Chloroform: methanol mixture (1:1)

Mixed 1 part of chloroform (AR) with 1part of methanol (AR) and stored in a tightly capped bottle.

Procedure

Weighed 2.0g powdered sample into wide mouthed boiling tube(30 ml). Added 20ml alcohol: diethyl ether mixture (3:1) to this and stirred well. Keep in thermostatic water bath for 2 hours at 55°C. Centrifuged at 3000rpm for 10min and decanted the clear supernatant to a weighed Petri dish. Added another lot of alcohol: diethyl ether mixture, and extracted for two hours. Centrifuged and decanted to the same plate. Added 20ml of chloroform: methanol (1:1) to the residue. Extracted at 50°C for one hour. Centrifuged and decanted to the same Petri dish and dried the Petri dish in oven at 60°C. Took the weight of the dish.

The quantity of fat was calculated based on the following formula:

Weight of fat = (weight of petridish + extract) - weight of empty petridish

$$\% \text{ Crude fat (ether extractives)} = \frac{\text{weight of the fat} \times 100}{2.0}$$

3.2.11.5 Determination of crude fibre

Crude fibre in plants is the fraction resistant to digestion with acid and alkali and this determines broadly the nutritional quality of a food crop. The procedure is less cumbersome than the dietary fibre determination and involves less costly reagents.

Reagents

1. Sulphuric acid – 1.25%. Weighed 1.25g concentrated H_2S0_4 in a small beaker and increased with distilled water to 100ml

2. Sodium hydroxide – 1.25% weigh 1.25g Na0H and distilled water and increased to 10ml.

3. Alcohol 100%

Procedure

The sample was oven-dried at105°C. Then 2g of the powdered dried sample, was placed in a beaker and 200ml of boiling 1.25% $H_2S0_4$was added. Placed the beaker on a hot plate and boiled for 30min, occasionally rotating the beaker. Cooled and filtered by suction through a Buchner funnel. Rinsed the beaker with two 50ml portions of boiling water. Transferred the residue carefully to a beaker and added 200ml, 1.25% NaOH. Boiled for 30min. Cooled and filtered as above and washed twice with 50ml boiling water. Finally, washed with 25ml 95% alcohol. Oven dried the residue for 2 h at 130°C. Cooled in a desiccator and weighed.

$$\% \text{ Crude fibre in the ground sample} = \frac{(\text{Loss in weight in ignitition}) \times 10}{\text{Weight of sample}}$$

3.3. Statistical Analysis

3.3. 1 Statistical Analysis of Molecular data

Clear and reproducible bands were only selected for scoring. Binary Scoring was carried out by assigning "1" for presence of bands and "0" for absence of band. A binary matrix of presence / absence was obtained from gels for each marker. The data matrix created in excel format was used as the input for cluster analysis. Estimation of genetic diversity parameters results in an overview of genetic variability and could be used as a criterion for comparing both marker efficiency and groups from different studies.

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

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

wheren_i is the number of alleles per locus and K is the number of loci.

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

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

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

Heterozygosity per locus was calculated according the formula:

$$He = 1-p^2-q^2$$

Where, $p^2 = fi$

Average heterozygosity per marker was calculated based on:

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

3.3.2 Cluster Analysis

A binary matrix of presence/absence was obtained from gels foreach marker. Jaccard's similarity coefficient was calculated for use in clustering analysis by Unweighted Pair-group Method with Arithmetic Average (UPGMA).Genetic similarity between different accessions was estimated based on jaccard's similarity (J) coefficient using SIMQUAL programme of NTSYSpc v. 2.20 (Rohlf, 2005). Jaccard's similarity coefficients of different accessions were used to construct UPGMA dendrogram for morphological qualitative, ISSR and SSR markers using SAHN programme of NTSYSpc v. 2.20 (Rohlf, 2005). Dendrogram grouping the 45 accessions based on ISSR, SSR 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 analysed for analysis of variance (ANOVA) and LSD test was performed to identify genotypes that were significantly different from each other.

Correlation and principal component analysis (PCA) was done using PRINQUAL procedure of SAS 9.3 software (SAS, 2011)

Genetic parameters were estimated to identify genetic variability among accessions and determine genetic and environmental effects 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).

$\sigma 2\ p = \sigma 2\ g + \sigma 2\ e,$

$\sigma 2\ g = (Msg - Mse)/r$

Where: σ2 p = Phenotypic variance, σ2 g = Genotypic variance, σ2 e= Error variance (error mean square); r = number of replications .

Genotypic coefficient of variation (GCV) and phenotypic coefficient of variation (PCV) were estimated as:

GCV = √(σ2 g/X)*100

PCV = √(σ2 p/X)*100

Where: X= grand mean

Broad sense heritability was estimated as:

H^2 = σ2 g/σ2 p

Where: H^2 = broad sense heritability

Genetic advance (GA) expected under selection assuming a selection intensity of 5% was estimated as:

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

Where GA = expected genetic advance ; *K* = Selection differential (2.06 at 5% selection intensity) σA= Phenotypic standard deviation

Genetic advance as a percentage of mean (GAM) was also estimated using the formula:

GAM = (GA/X)* 100

RESULT

4. RESULTS

The results of the study entitled "Genetic Diversity of Wild Yams on Western Ghats" was carried out at the Division of Crop Improvement, ICAR – Central Tuber Crops Research Institute, Sreekariyam, and Thiruvananthapuram during 2015 – 2016 are presented in this chapter.

4.1. VARIATION IN QUALITATIVE TRAITS ASSESSED

4.1.1 Tuber shape

Tuber shapes of different wild yam species were presented in plate no 3.Tuber shape varied from round to cylindrical. The different shapes include ovate, round, ovate – oblong, cylindrical, long cylindrical and irregular. Within the species different accessions varied in shape (shown in plate no 3). Two accessions of *D. hispida* tubers (plate no.1) varied in tuber shape produicing irregular and round tubers. Similarly *D. wallichi* also have accessions showing ovate and long cylindrical tubers. *D. oppositifolia* are long cylindrical in shape generally, where as tubers of *D. pubera* and *D. bulbifera* are short and round or ovate in shape.

Tuber skin texture of *D. floribunda* was very rough and dry whereas the tubers of *D. oppositifolia* are smooth and fresh. Grainy texture was observed in *D. bulbifera.*

Tubers of *D. hispida*, *D. alata* and *D. floribunda* were long and profusely hairy where as tubers of *D. tomentosa, D. bulbifera, D. wallichi, D. esculenta* were moderately hairy.

The tuber flesh colour of *D. floribunda* and *D. bulbifera* (plate no 4) were dark yellow in colour and another accession of *D. floribunda* have yellow with purple flesh colour. White tuber flesh colour was more frequent among accessions. Thus tuber flesh color of the accessions varied from white to red purple according to RHS colour chart. Tuber weight/plant ranged from 0.01kg *(D. oppositifolia)* to 5.66kg *(D. hispida)* (Table 7). Tuber length of the accessions ranged from 2.57 cm to 79.67cm and tuber girth varied from 1.50 cm to 37.83 cm.

Plate no 1. Different accessions of *D.*

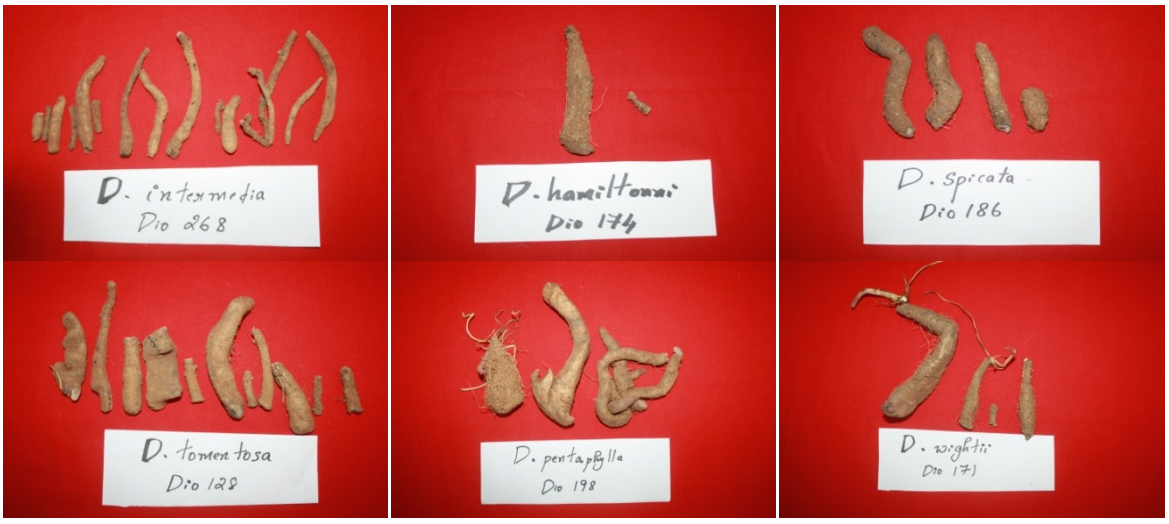


Plate no 2. Different species of wild yams collected from NBPGRI

Plate no 3. Tubers of different wild yam

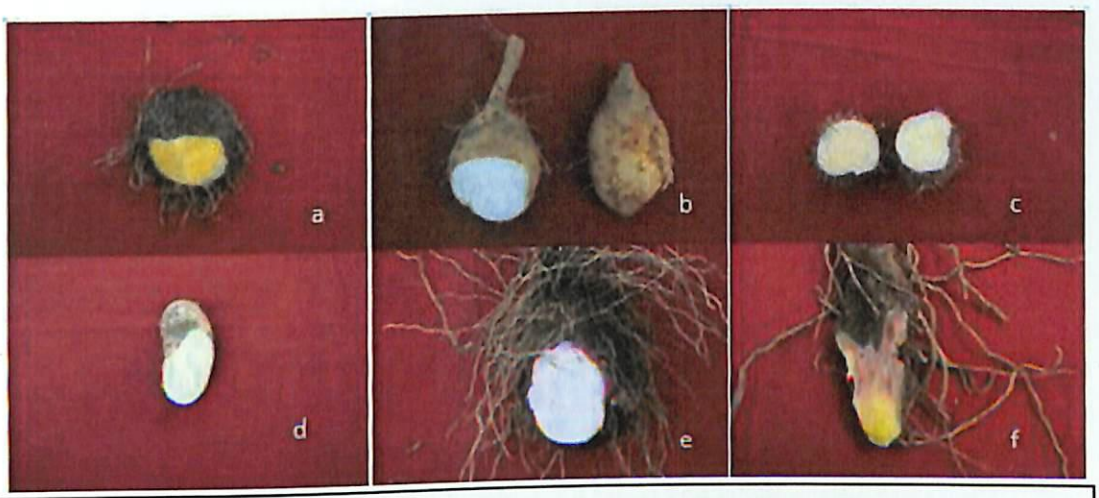


Plate no 4. Tuber flesh colour – *a)D. belophylla, b) D. wallichi, c)D. bulbifera d) D. hispida e)D. alata f) D. floribunda*

4.1.2 Aerial shoot parts analysis:

The accessions were observed to have two leaf types namely simple and compound. The more frequent leaf type was simple, in exception to some accessions namely *D. pentaphylla, D. hispida and D. tomentosa* with compound leaves. *D. pentaphylla* showed three to five leafed conditions whereas *D. tomentosa* have simple leaf type at young stage and compound leaves were formed at the stage of maturity (Plate no 5 and plate no 6).

Leaves of D. *floribunda* (Plate no 6) were simple in type and observed to be very unique with white patches on the leaf. The leaves of *D. wallichi* and *D. opposita* were found to be shiny.

The young leaf colour varied from light green to purple in colour (Plate no 7), wherein light green young leaves were more frequently seen in species like *D. pentaphylla, D. opposita, D. esculenta, D. hispida* etc and the young purple leaves were observed in *D. wallichi.*

The leaf shape varied from (Plate no 8) ovate to sagitate long. Leaf pubescence was observed in *D. tomentosa and D. pentaphylla.* Leaf lobes were frequently observed as no lateral leaf lobes apart from (lasiophytam) the compound leaved species like *D. hispida, D. tomentosa,* and *D. pentaphylla* having very deep lobes.

Aerial shoot parts of wild yams:

Plate no 5. Compound leafs of a) *D. hispida* b) *D. pentaphylla* c) *D.*

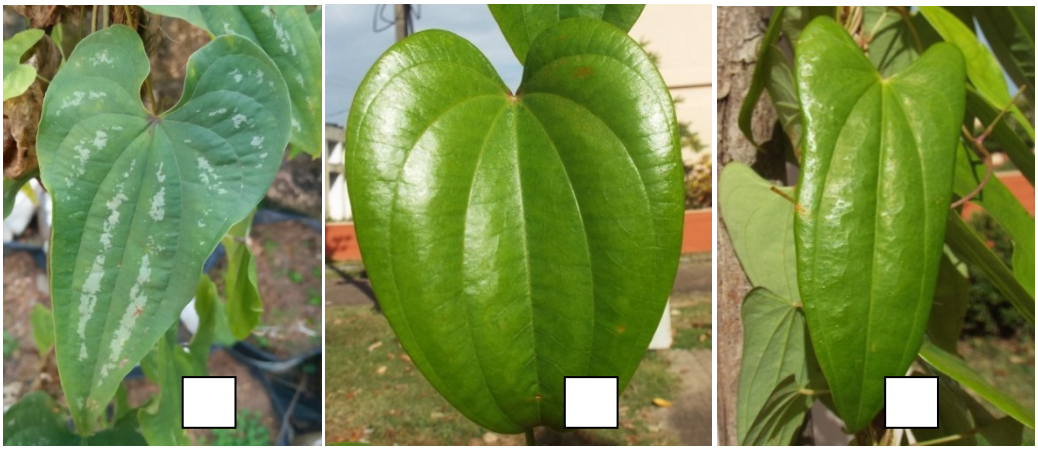


Plate no 6. Simple leaves of a) *D. floribunda* b) *D. wallichi* c) *D. opposita*

Aerial shoot parts of wild yams:

Plate no 7. Young leaf colour of a*) D. opposita* b) *D. wallichi* c) *D. pentaphylla* d) *D.*

Plate no 8. Leaf shapes of species a) *D. esculenta* (sagitate long) b) *D. bulbifera (sagitate long)*

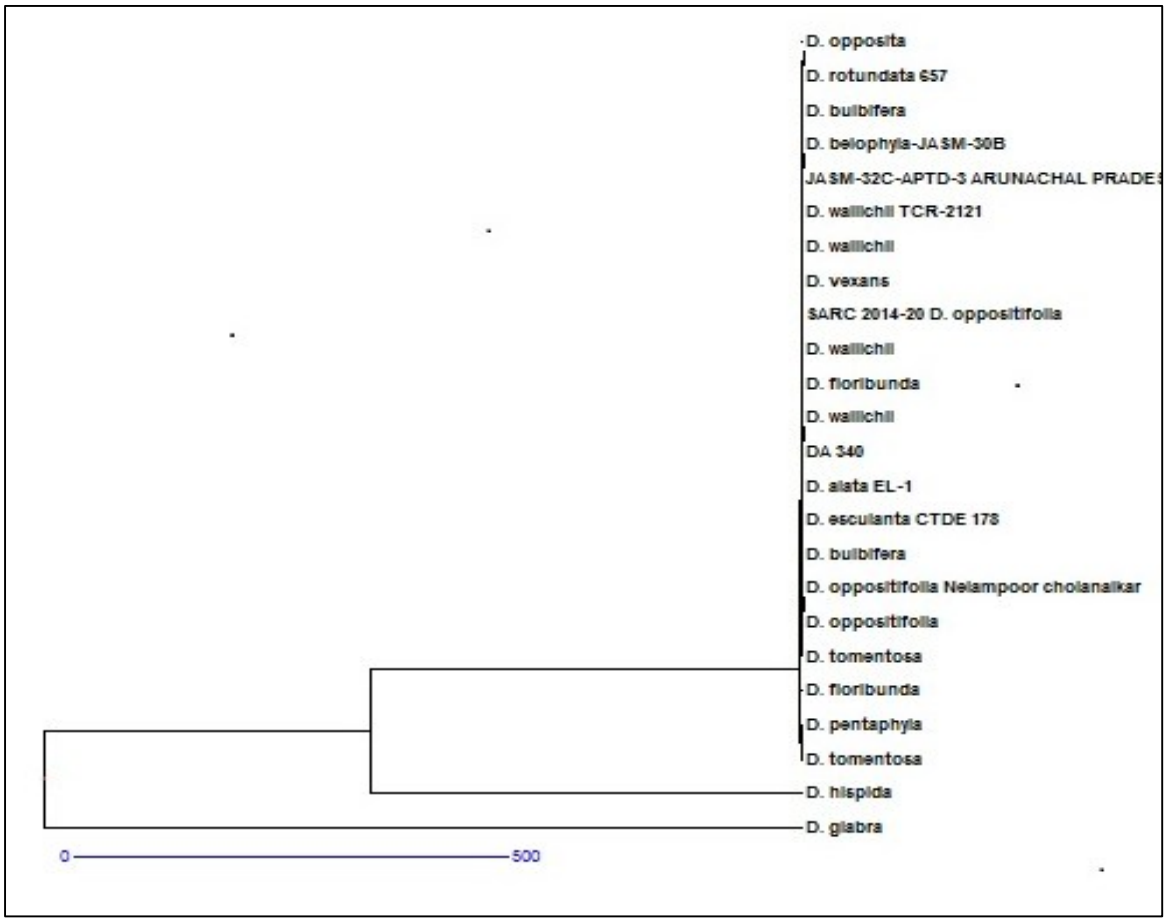
Table 7. Analysis of Morphological (Quantitative) Data

No.	Species Name	Accession code	Weight of tuber (Kg)	Length of tuber(cm)	Girth of tuber(cm)
1	*D. alata EL-1*	Da-1	0.02	2.67	3.33
2	*D. alata 340*	Da-340	2.80	36.67	4.30
3	*D. belophyla-JASM-30B*	Dbe-1	0.04	5.33	7.83
4	*D. bulbifera*	Db-1	0.88	12.33	21.17
5	*D. bulbifera*	Db-2	0.15	6.67	13.33
6	*D. esculenta CTDE 178*	De-1	4.90	14.66	13.83
7	*D. floribunda*	Df-1	0.17	20.00	9.17
8	*D. floribunda*	Df-2	5.66	43.30	28.83
9	*D. hispida*	Dh-1	2.86	8.66	18.00
10	*D. hispida*	Dh-2	3.48	12.00	37.83
11	*D. oppositifolia NC*	Dol-1	0.146	3.17	4.67
12	*D. oppositifolia SARC 2014-20*	Dol-2	0.044	30.00	1.50
13	*D. oppositifolia*	Dol-3	0.01	6.60	4.50
14	*D. pentaphylla*	Dp-1	0.02	3.17	12.50
15	*D. rotundata 657*	Dr-1	1.39	28.00	9.77
16	*D. tomentosa*	Dt-1	0.87	79.60	4.17
17	*D. tomentosa*	Dt-2	0.17	29.00	5.50
18	*D. vexans*	Dv-1	0.24	6.67	9.67
19	*D. wallichii*	Dw-1	0.06	22.00	3.50
20	*D. wallichii*	Dw-2	0.38	52.00	4.33
21	*D. wallichii*	Dw-3	0.19	27.17	19.00
22	*D. wallichii*	Dw-4	0.12	13.00	3.00
23	*D. wallichi*	Dw-5	0.51	2.57	3.67
	Mean		**1.01**	**20.80**	**10.01**
	SD		**1.62**	**17.90**	**9.01**
	CV (%)		**159.92**	**86.06**	**89.94**

4.1.3 Cluster analysis based on Aerial shoot parts

Cluster analysis was done based on Euclidean distance for 23 accessions. Dendrogram (figure 3) showed the clustering of accessions based on the morphological characters of aerial shoot parts. About 18 morphological characters were studied for the analysis. Two clusters (C1 and C2) were formed wherein Cluster C1 clearly showed that *D. glabra* is a complete outlier. *D. glabra* is observed as a divergent from all other accessions studied.C2 is sub clustered in to C2A and C2B, wherein C2A is an oulier *(D. hispida)* and found to be divergent from all other accessions in cluster C2B. C2b is the largest cluster observed in the dendrogram with 22 accessions

Figure 3 The clustering of the accessions based on morphological data



4.2.1 PCR STANDARDIZATION

The good quality DNA shown in figure 4 was obtained using manual method and poor quality DNA shown in figure 5 was obtained using using DNeasy Plant Mini Kit. Standardization of PCR conditions was attempted for ISSR markers to obtain clear and consistent amplicons under reduced cycle duration. Changing annealing temperature for ISSR primers gave better results. Usage of Takara emerald master mix reduced preparation time for PCR.

PRIMER SCREENING

For preliminary screening of ISSR, 15 ISSR primers were used and all the primers were selected based on their polymorphism and repeatability. Figure 6, 7&8 showed that gel profiles of ISSR used at annealing temperature of 56°C. From 15 ISSR primers chosen for the study UBC 808 is the efficient primer for wild yams for future studies. UBC 808 gave maximum number of polymorphic allele (24)

For preliminary screening of SSR, 10 ISSR primers were used and 9 primers were selected based on their polymorphism and repeatability. Figure 10, 11&12 showed the gel profile of the SSR primers used at annealing temperature of 51°C, 58°C & 59°C. MT 10 does not showed any amplification, hence done a gradient analysis and optimized annealing temperature at 59°C.

4.2 MOLECULAR ANALYSIS OF DATA

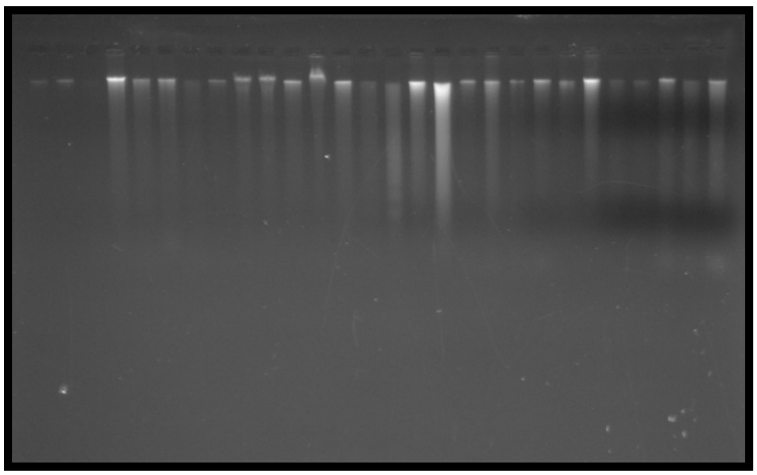


Figure 4. Shows low purity DNA with sheared bands obtained using DNeasy Plant

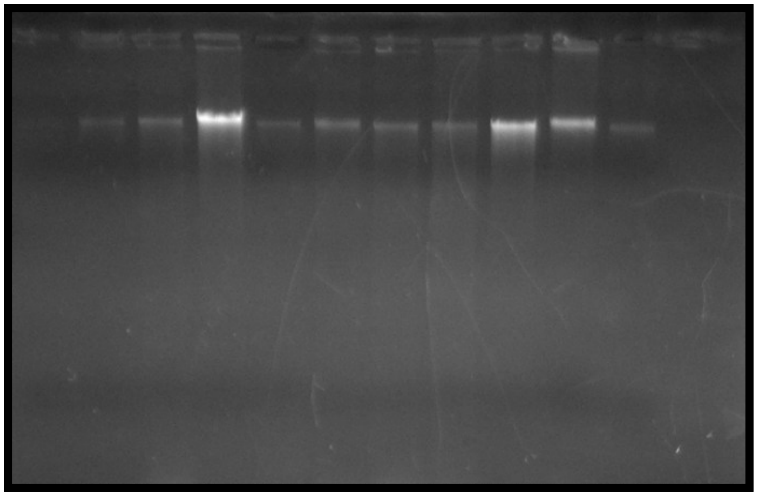


Figure 5. Shows good quality DNA obtained using Manual method (Raj *et al., 2014*).

4.2.2 Molecular Data Analysis

ISSR analysis of wild yams

The amplicons obtained after ISSR analysis of 24 accessions using the selected primers were initially resolved in 2.5% percent agarose gel (Plates 14 to 16). Genetic characteristics of the fifteen ISSR markers used to analyze the wild yams accessions is given in the Table 9. Total number of bands per ISSR primer ranged from 13(UBC 848) to 24(UBC 808). The primer UBC 808 recorded the highest number of bands (24) followed by$(GA)_9$ AC that recorded 22 bands, the primers UBC 810 and UBC 827 with 21 bands . The primers *viz.* $(ACC)_6$ Y, UBC 836, UBC 818, UBC 864 recorded 20 bands . The bands obtained from all the 15 ISSR primers were found to be polymorphic. Bands obtained in the product size from 100bp to 2Kb.

Cluster analysis on ISSR primers

Based on ISSR data, the genotypes formed two major clusters at 0.49 dissimilarity index. *D. hamiltonni* is 49% similar to all other genotypes in cluster 2. *D. esculenta* was found to be highly divergent than all other genotypes, formed as an outlier. Cluster 2 is biggest with 22 accessions and they were grouped in to several sub clusters. *D. pubera* and *D. wightii* showed 85% similarity whereas *D. pentaphylla* showed 82% similarity to *D. pubera* and *D. wightii. D. alata* and *D. oppositifolia* have 78% similarity. The clustering pattern based on ISSR markers is given in figure 9. The comparison of the cultivated yams with wild yams showed that greater yam (*D. alata*) is closer to *D. oppositttifolia* while *D. rotundata* is closer to *D. opposita.* The aerial potato *D. bulbiferra* formed separate cluster.

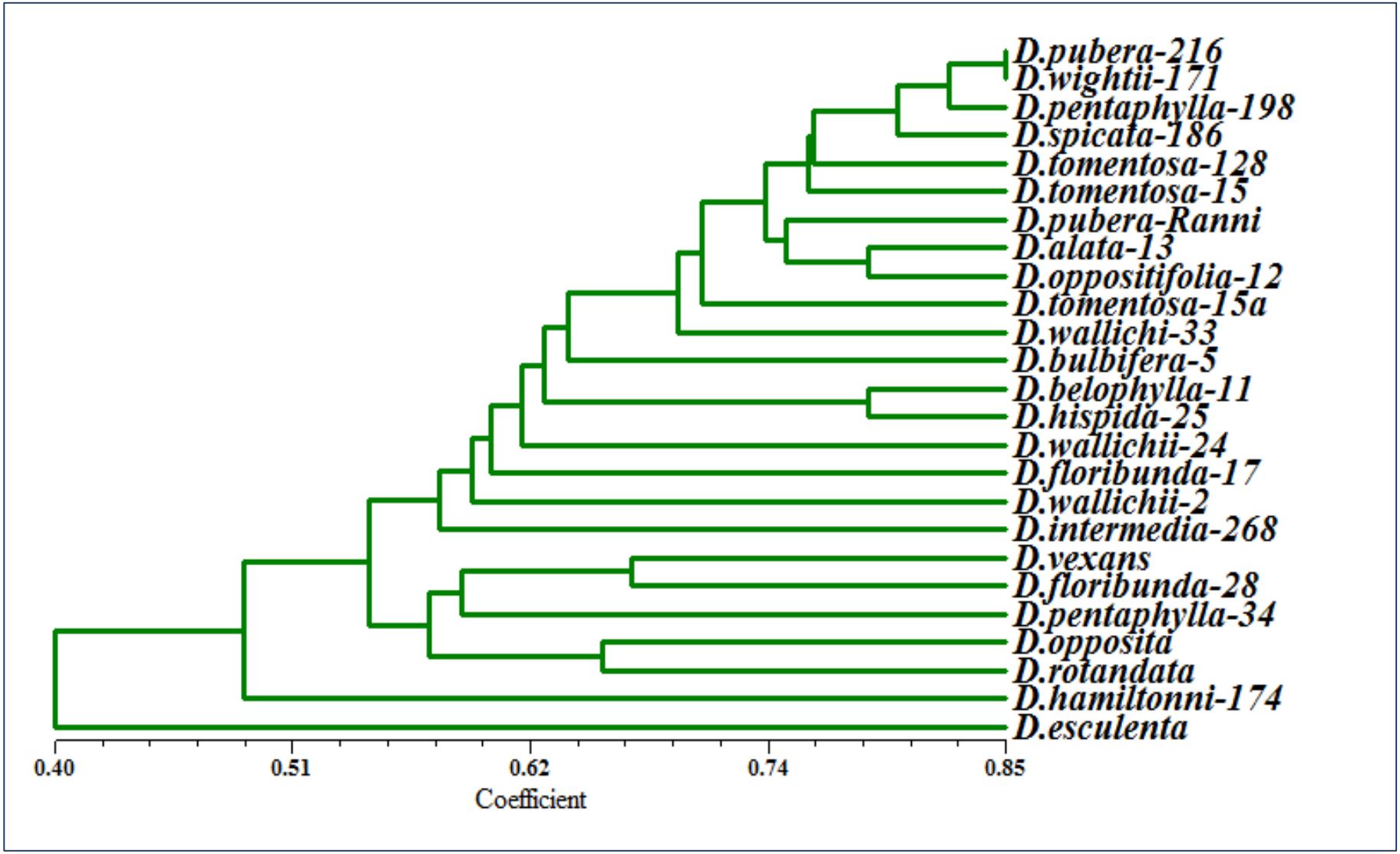
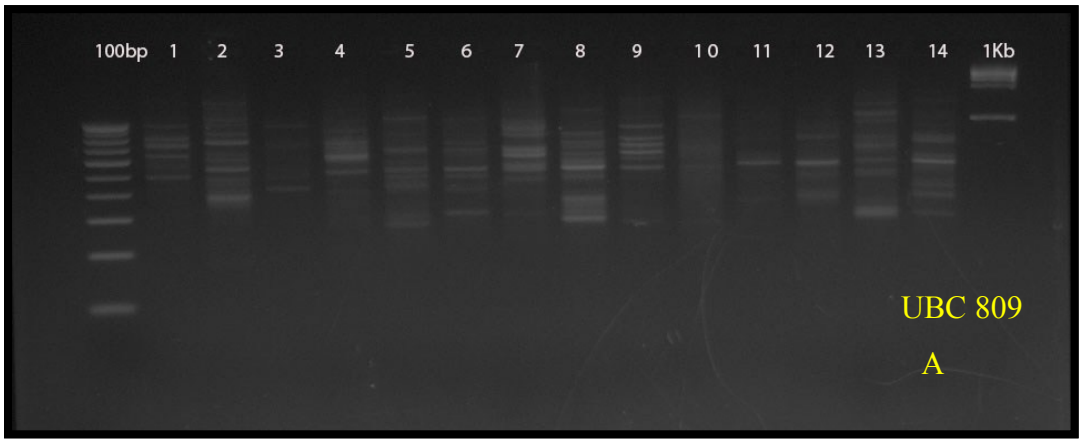
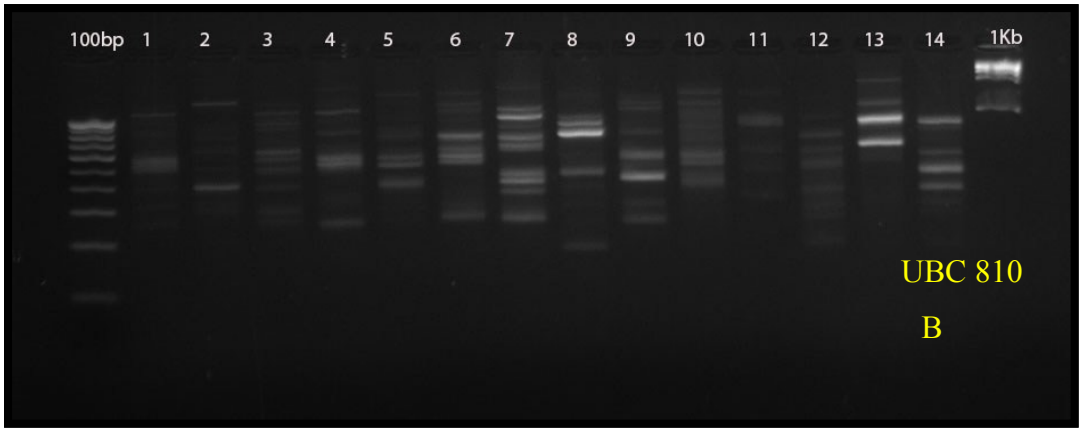


Figure 9. CLUSTER DENDROGRAM BASED ON ISSR

4.2.2.1 MOLECUAR CHARACTERIZATION USING ISSR PRIMERS

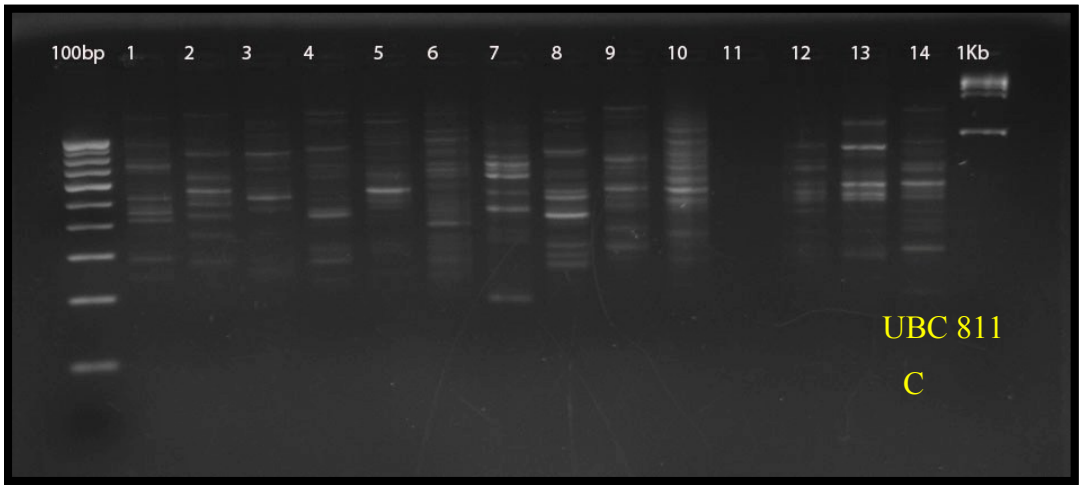


	1	2	3	4	5	6	7	8	9	10	11	12	13	14
UBC 809	**Dpu1**	**Dw1**	**Dp1**	**Dwi1**	**Dt1**	**Dt2**	**Dw2**	**Dpu2**	**Ds1**	**Dt2**	**Di1**	**Dw3**	**Da1**	**Dol1**

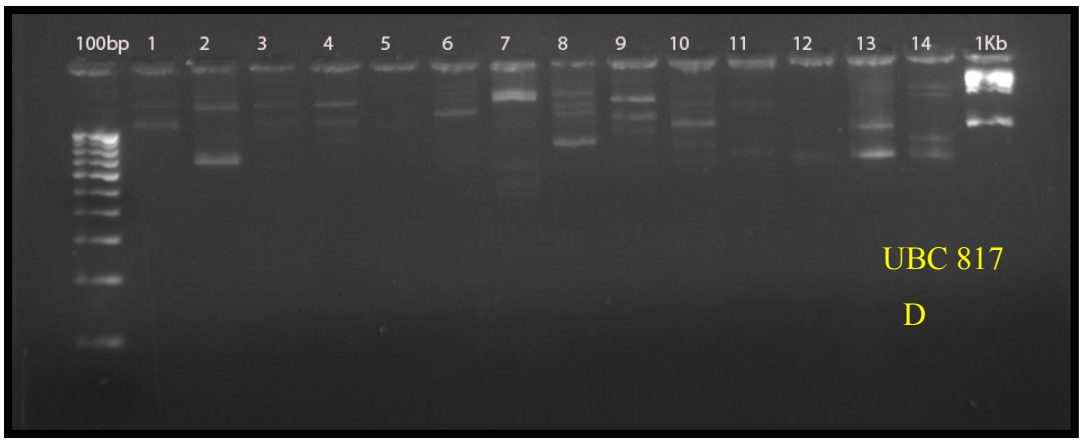


	1	2	3	4	5	6	7	8	9	10	11	12	13	14
UBC 810	**Dpu1**	**Dw1**	**Dp1**	**Dwi1**	**Dt1**	**Dt2**	**Dw2**	**Dpu2**	**Ds1**	**Dt2**	**Di1**	**Dw3**	**Da1**	**Dol1**

Figure 6. (A and B) shows the gel (2.5%) profile of the ISSR primers UBC 809 and UBC 810

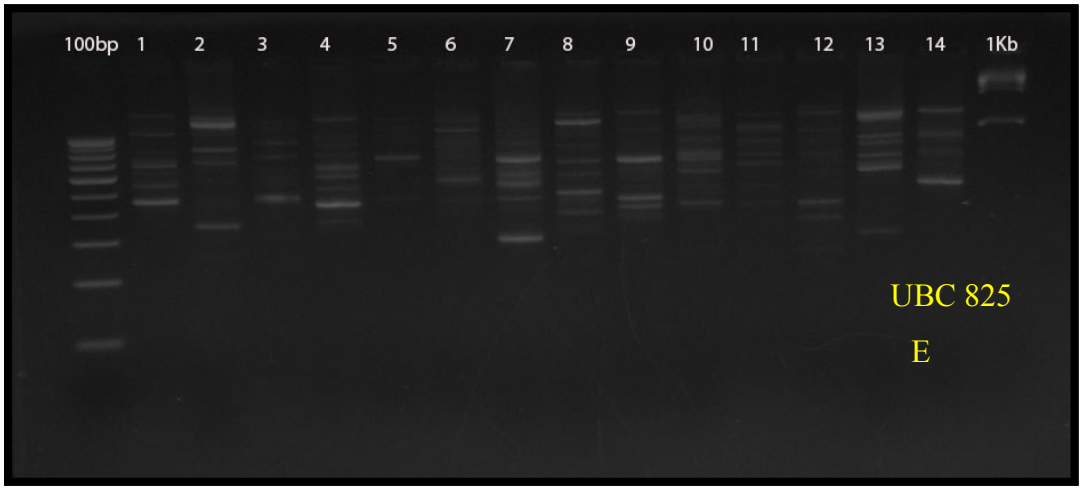


	1	2	3	4	5	6	7	8	9	10	11	12	13	14
UBC 811	**Dpu1**	**Dw1**	**Dp1**	**Dwi1**	**Dt1**	**Dt2**	**Dw2**	**Dpu2**	**Ds1**	**Dt2**	**Di1**	**Dw3**	**Da1**	**Dol1**

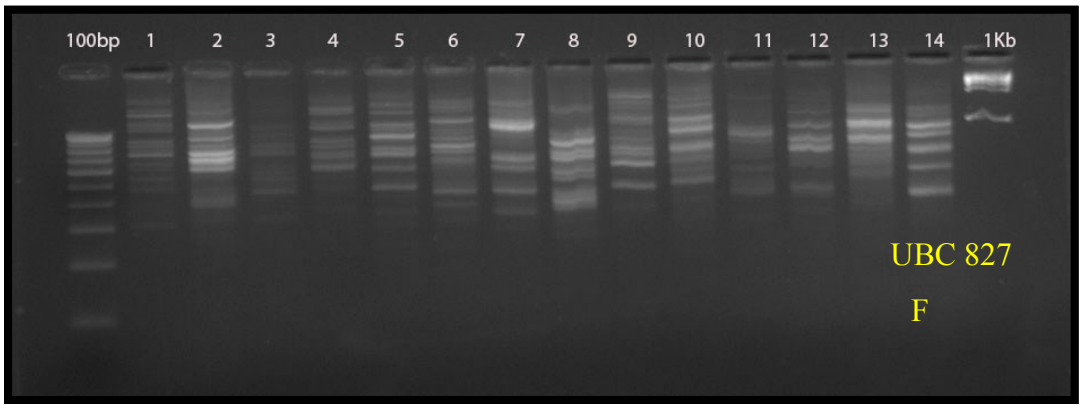


	1	2	3	4	5	6	7	8	9	10	11	12	13	14
UBC 817	**Dpu1**	**Dw1**	**Dp1**	**Dwi1**	**Dt1**	**Dt2**	**Dw2**	**Dpu2**	**Ds1**	**Dt2**	**Di1**	**Dw3**	**Da1**	**Dol1**

Figure 7. (C and D) shows the gel (2.5%) profile of the ISSR primers UBC 811 and UBC 817



	1	2	3	4	5	6	7	8	9	10	11	12	13	14
UBC 825	**Dpu1**	**Dw1**	**Dp1**	**Dwi1**	**Dt1**	**Dt2**	**Dw2**	**Dpu2**	**Ds1**	**Dt2**	**Di1**	**Dw3**	**Da1**	**Dol1**



	1	2	3	4	5	6	7	8	9	10	11	12	13	14
UBC 827	**Dpu1**	**Dw1**	**Dp1**	**Dwi1**	**Dt1**	**Dt2**	**Dw2**	**Dpu2**	**Ds1**	**Dt2**	**Di1**	**Dw3**	**Da1**	**Dol1**

Figure 8. (E and F) shows the gel (2.5%) profile of the ISSR primers UBC

Table 8. Genetic characteristics of the ISSR markers used to analyze the accessions of wild yams

S.No	PRIMER	N_B	N_{PB}	P%
1	UBC 808	24	24	100%
2	UBC 809	18	18	100%
3	UBC 810	21	21	100%
4	UBC 811	22	22	100%
5	UBC 817	17	17	100%
6	UBC 825	20	20	100%
7	UBC 827	21	21	100%
8	UBC 848	13	13	100%
9	UBC 864	20	20	100%
10	UBC 818	20	20	100%
11	UBC 836	20	20	100%
12	$(GA)_9$ AC	22	22	100%
13	$(GA)_9$ AT	19	19	100%
14	UBC 14	16	16	100%
15	$(ACC)_6$ y	20	20	100%

4.2.3 MOLECULAR DATA ANALYSIS OF SSR PRIMERS

Among nine SSR primers used for molecular characterisation, Dab2D06 was the good primer for diversity study of wild yams and it recorded maximum number of polymorphic alleles (19) followed by YM26 with 17 alleles. The SSR markers *viz.* Dab2E07, Da1AO1 and Dpr3FO4 also recorded higher number of polymorphic alleles. The lowest number of alleles was recorded for the loci, MT10. All other eight SSR markers revealed good polymorphism (> 10 alleles/ loci).

Cluster analysis of SSR primers

In the present study, the SSR markers gave unique results. Based on SSR markers the genotypes were grouped into two clusters (C1 and C2). C2 is the smallest cluster with six genotypes, wherein *D. wightii* and *D. spicata* shows the highest similarity with 91%. *D. rotundata* was divergent of all genotypes and forms a complete outlier.

In C1 *D. pentaphylla* and *D. floribunda* were found to be similar with approximately 87%. *D. alata* and *D. oppositifolia* grouped in on sub cluster. In SSR and ISSR *D. alata* showed greater similarity to *D. oppositifolia. D. esculenta* (ISSR) and *D. hamiltonni* (SSR*)* are divergents identified and they formed a complete outlier in the cluster dendrogram as shown in figure 13.

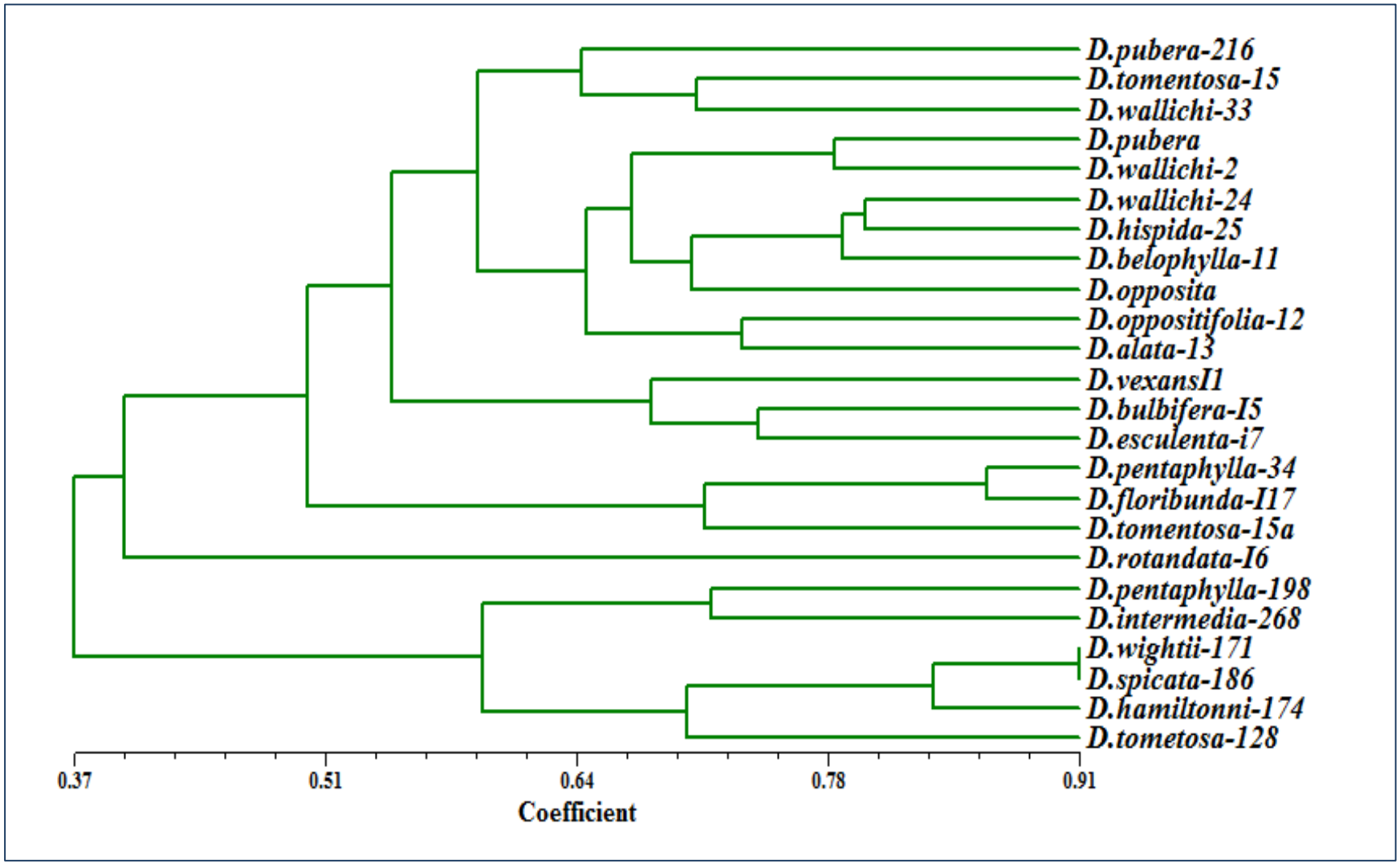
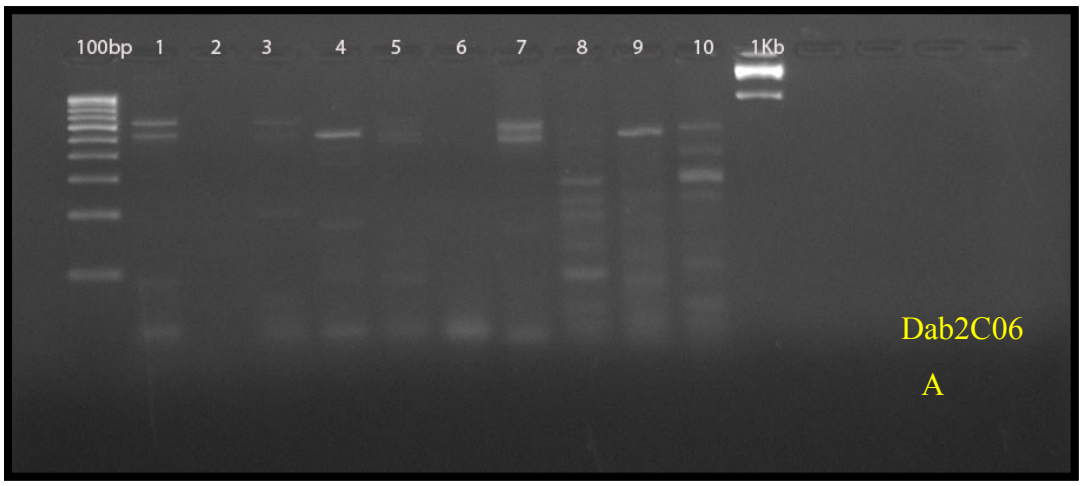
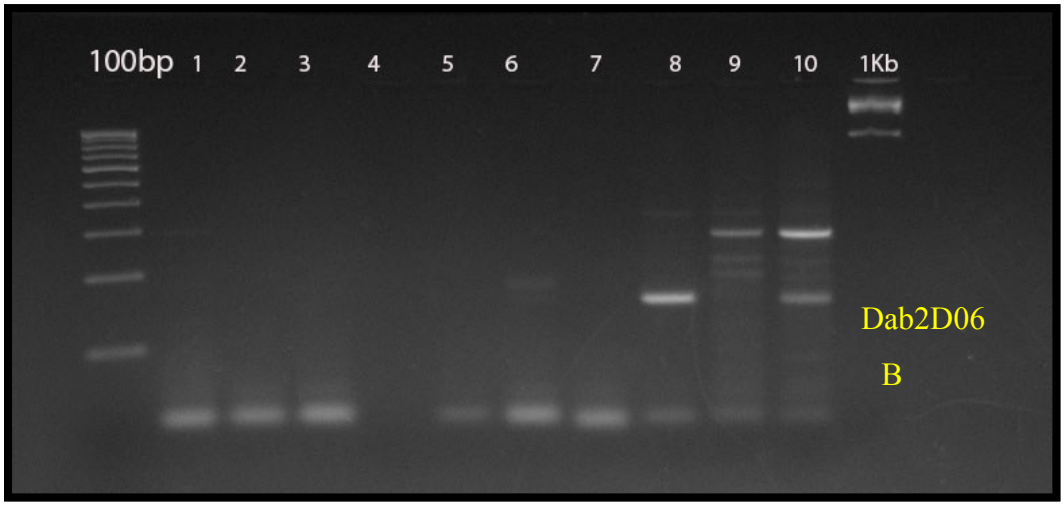


Figure 13. CLUSTER DENDROGRAM BASED ON SSR

4.2.3.1 MOLECULAR DATA ANALYSIS OF SSR PRIMERS

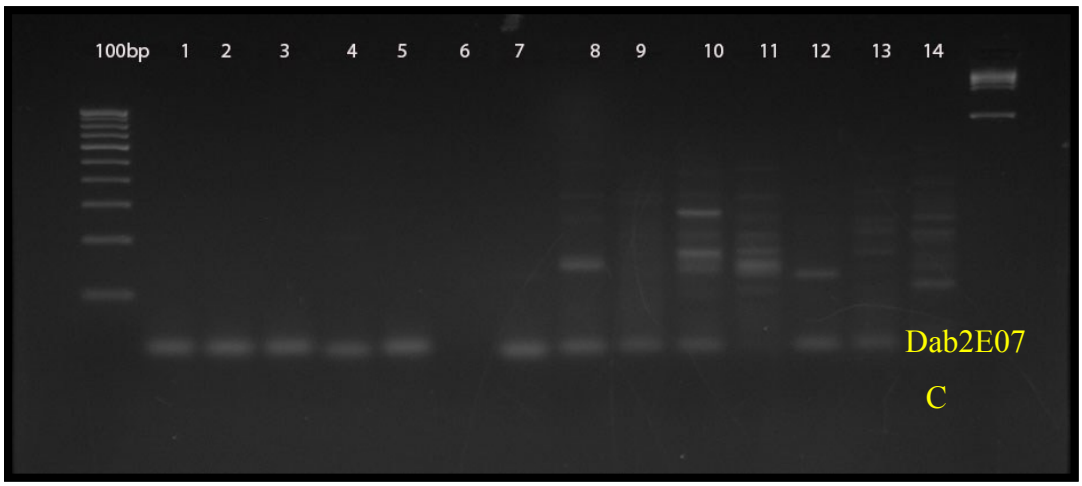


Dab2 C06	1	2	3	4	5	6	7	8	9	10
	Dpu 1	Dp 1	Dwi 1	Dt 1	Ds 1	Di 1	Dh 1	Dol 1	Dt 2	Da 1

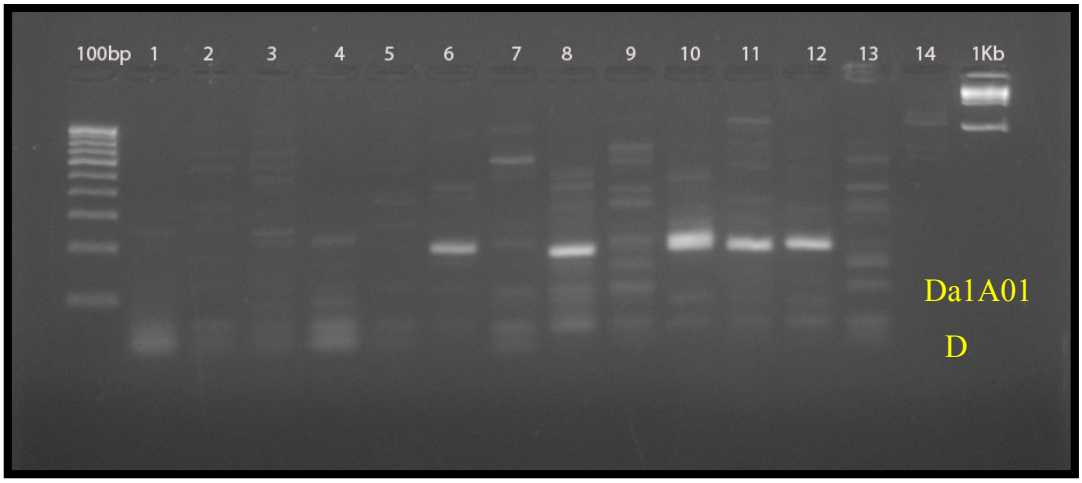


Dab2 D06	1	2	3	4	5	6	7	8	9	10
	Dpu 1	Dp 1	Dwi 1	Dt 1	Ds 1	Di 1	Dh 1	Dol 1	Dt 2	Da 1

Figure 10 (A and B) shows the gel (2.5%) profile of the SSR primers Dab2C05 and Dab2D06

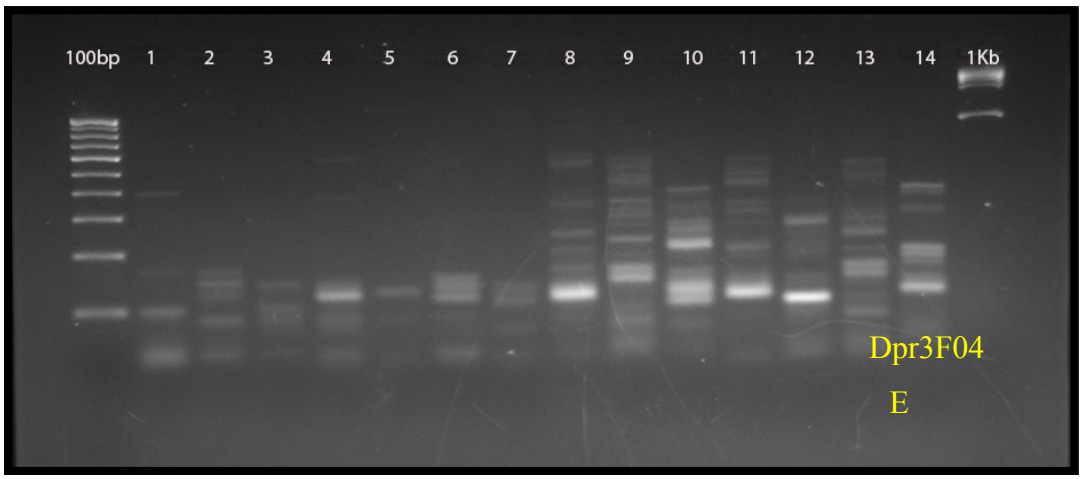


Dab2 E07	1	2	3	4	5	6	7	8	9	10	11	12	13	14
	Dpu 1	Dp 1	Dwi 1	Dt 1	Ds 1	Di 1	Dh 1	Dol 1	Dt 2	Da 1	Dpu 2	Dw 1	Dt 3	Dw 2

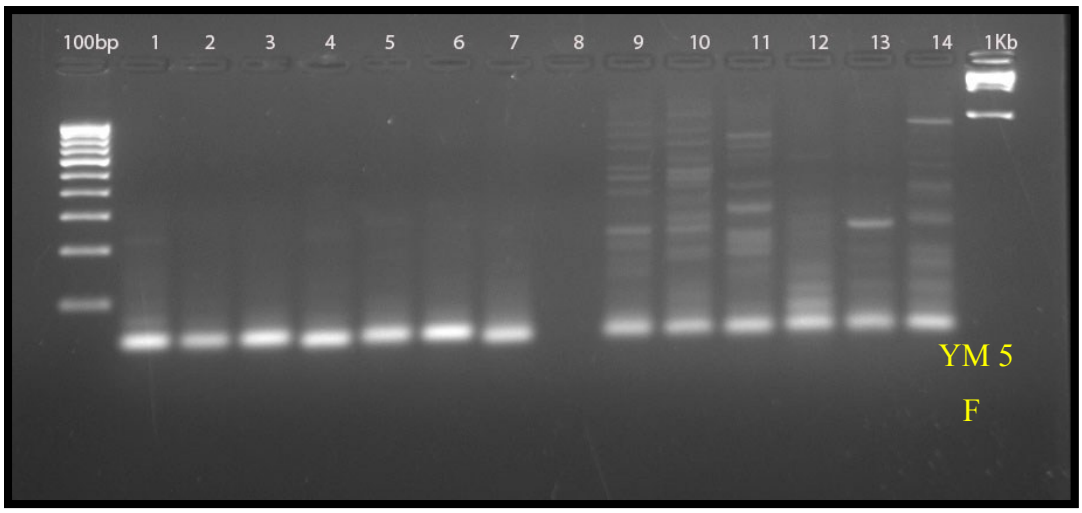


Da1 A01	1	2	3	4	5	6	7	8	9	10	11	12	13	14
	Dpu 1	Dp 1	Dwi 1	Dt 1	Ds 1	Di 1	Dh 1	Dol 1	Dt 2	Da 1	Dpu 2	Dw 1	Dt 3	Dw 2

Figure 11. (C and D) shows the gel (2.5%) profile of the SSR primers Dab2E07 and Da1A01



Dpr3 F04	1	2	3	4	5	6	7	8	9	10	11	12	13	14
	Dpu 1	Dp 1	Dwi 1	Dt 1	Ds 1	Di 1	Dh 1	Dol 1	Dt 2	Da 1	Dpu 2	Dw 1	Dt 3	Dw 2



YM 5	1	2	3	4	5	6	7	8	9	10	11	12	13	14
	Dpu 1	Dp 1	Dwi 1	Dt 1	Ds 1	Di 1	Dh 1	Dol 1	Dt 2	Da 1	Dpu 2	Dw 1	Dt 3	Dw 2

Figure 12. (E and F) shows the gel (2.5%) profile of the SSR primers Dpr3F04 and YM 5

Table 9. Genetic characteristics of the SSR markers used to analyze the accessions of wild yams

S.No	PRIMER	N_B	N_{PB}	P%
1	Dab2C05	13	13	100%
2	Dab2D06	19	19	100%
3	Dab2E07	15	15	100%
4	Da1AO1	15	15	100%
5	Dpr3F04	15	15	100%
6	YM 5	14	14	100%
7	YM 15	13	13	100%
8	YM 26	17	17	100%
9	MT 10	2	2	100%

4.3 Biochemical evaluation of Wild Yams

Biochemical analysis were done for Drymatter(%), Starch(%), Sugar(%), Crude protein(%) , Crude fibre(%) and Fat%. The variation for chemical traits is given in Table 10 and also depicted in Figure 14 to figure 19.

From the analysis it was observed that drymatter content is highest for *D. floribunda* with 48.93% followed by *D. wallichi* 44.71% and lowest value is observed in *D. oppositifolia* with 24.02% and in *D. vexans* with 24.21% .Starch content was observed to be highest in *D. belophylla* followed by *D. oppositifolia* and the lowest was recorded in *D. tomentosa*. Crude protein content was highest in *D. floribunda* with 14% followed by *D. hispida* with 12.46% and lowest value was recorded in *D. wallichi* (4.81%). Fiber content was highest in *D. vexans* with 1.29% and lowest value was recorded in *D. bulbifera* with 0.06%.

Fat content was observed to be highest in *D. floribunda* with 16.45% followed by *D. hispida* with 12.88% and the lowest value was recorded in *D. belophylla* with 11.77%. Sugar content was observed to be highest in *D. vexans* with 4.35%, followed by *D. oppositifolia* and lowest value was recorded *D. bulbifera* with 1.43%.

The nutritional content of wild yams is rich in starch, sugar, fibre, fat and protein. In Biochemical Analysis, it was observed that *D. belophylla* have higher sugar value than *D. alata*. *D. floribunda and D. bulbifera* have higher protein value than *D. alata. D. belophylla* have higher starch value than *D. alata.*

From the genetic correlations estimated (Table 11) the fibre and sugar contents showed negative correlation to drymatter content whereas sugar, protein and fibre showed negative correlations to starch.

Evaluation of genetic parameters in wild yams for biochemical traits is given in Table 13. The highest genotypic covariance (GCV) was estimated in fat (fresh weight basis) of 133.9, phenotypic covariance (PCV) was also estimated to high in fat (fw basis).

From the study it is clear that tubers of wild yams are rich source of nutrients than the cultivated variety *D. alata* and after processing it can be administered for human consumption as nutritional supplements.

Table 10. Biochemical data analysis (based on dryweight %)

SL. NO	ACCESSION CODE	DRY MATTER %	STARCH %	SUGAR%	CRUDE PROTEIN %	CRUDE FIBER%	FAT%
1	CTDa - 340	28.02	58.13	2.08	11.51	0.12	1.85
2	CTDbe- 1	26.79	150.00	1.56	6.65	0.00	1.77
3	CTDb-1	27.40	104.06	1.82	9.08	0.06	1.81
4	CTDb-2	28.07	33.33	1.43	6.56	0.63	2.55
5	CTDe-1	27.74	68.70	1.63	7.82	0.34	2.18
6	CTDf-1	48.93	40.91	3.57	14.00	0.81	16.45
7	CTDf-2	38.33	54.80	2.60	10.91	0.58	9.31
8	CTDh-1	43.63	47.86	3.09	12.46	0.69	12.88
9	CTDh-2	40.98	51.33	2.84	11.68	0.63	11.10
10	CTDo-1	24.02	56.25	1.67	4.81	0.37	3.20
11	CTDo-2	33.61	128.57	4.17	6.83	0.12	4.20
12	CTDr-2	28.82	92.41	2.92	5.82	0.25	3.70
13	CTDt-1	31.21	110.49	3.54	6.32	0.18	3.95
14	CTDt-2	26.19	25.71	2.50	6.30	0.23	3.35
15	CTDv-1	24.21	50.00	4.35	6.83	1.29	6.55
16	CTDtw-1	25.20	37.86	3.42	6.56	0.76	4.95
17	CTDtw-2	41.89	60.00	3.13	5.25	0.45	3.65
18	CTDtw-3	33.54	48.93	3.27	5.91	0.61	4.30
19	CTDtw-4	39.05	40.91	2.00	6.13	0.41	2.30
20	CTDtw-5	44.71	60.00	2.78	7.00	0.24	2.75
21	CTDu -1	35.61	56.25	1.67	6.48	0.30	2.45
	Mean	**33.24**	**65.55**	**2.67**	**7.85**	**0.43**	**5.01**
	SD	**7.58**	**32.70**	**0.88**	**2.64**	**0.31**	**4.04**
	CV(%)	**22.82**	**49.88**	**32.94**	**33.58**	**71.55**	**80.61**
	CD (5%)	**1.8201**	**1.6231**	**0.5877**	**4.0901**	**0.0754**	**1.3314**

Figure 14. Variation for Drymatter content among wild yams

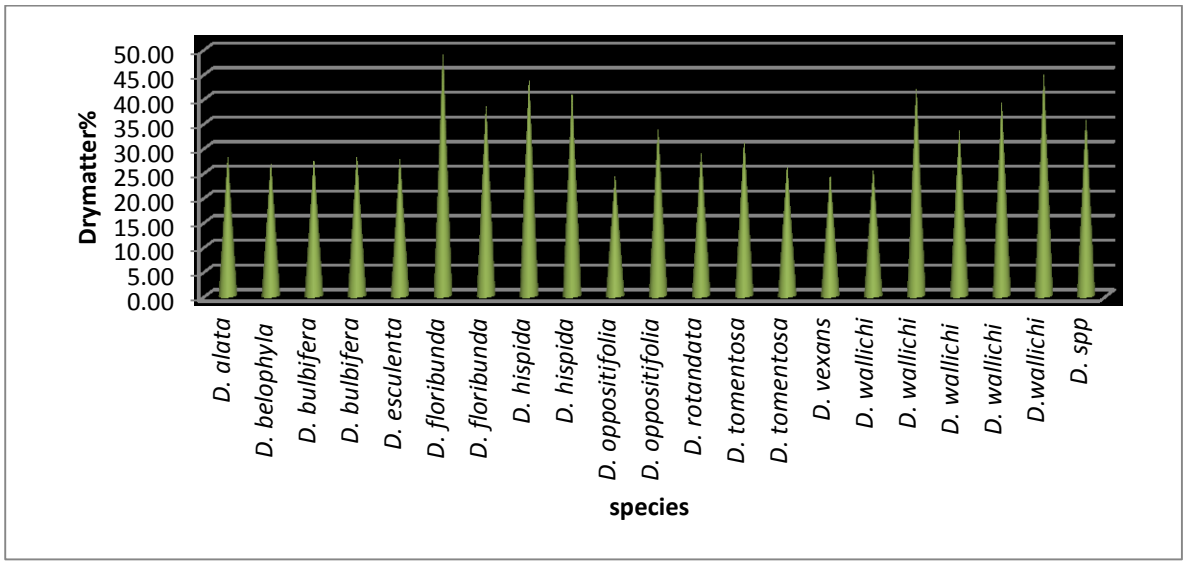


Figure 15. Variation for starch content among wild yams

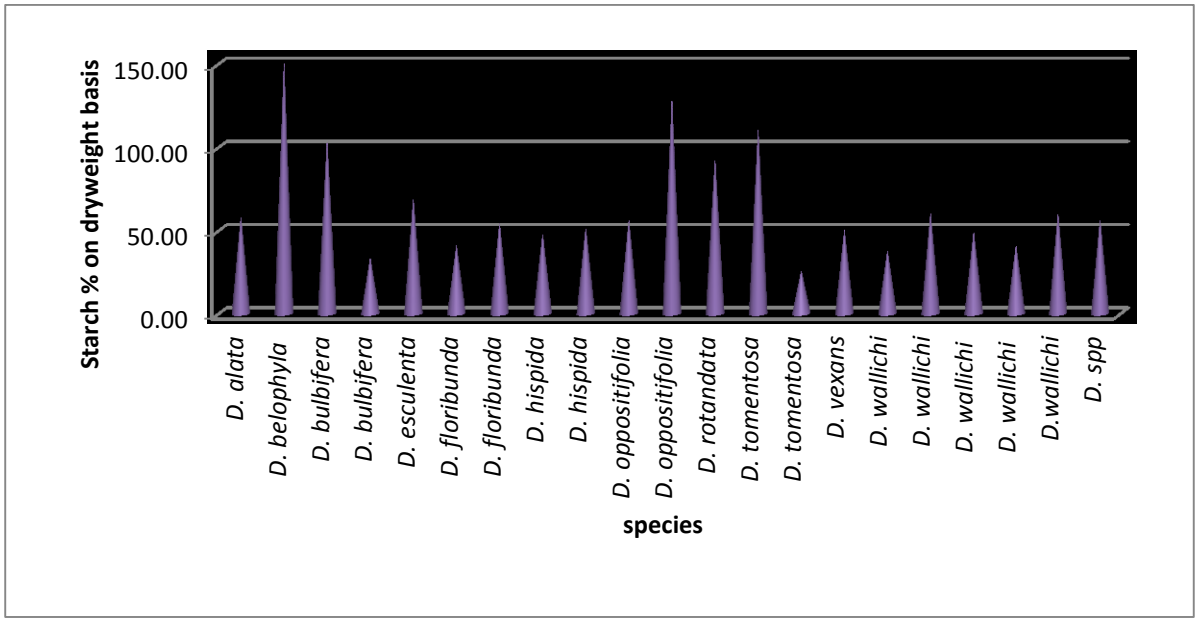


Figure 16. Variation for Sugar content among wild yams

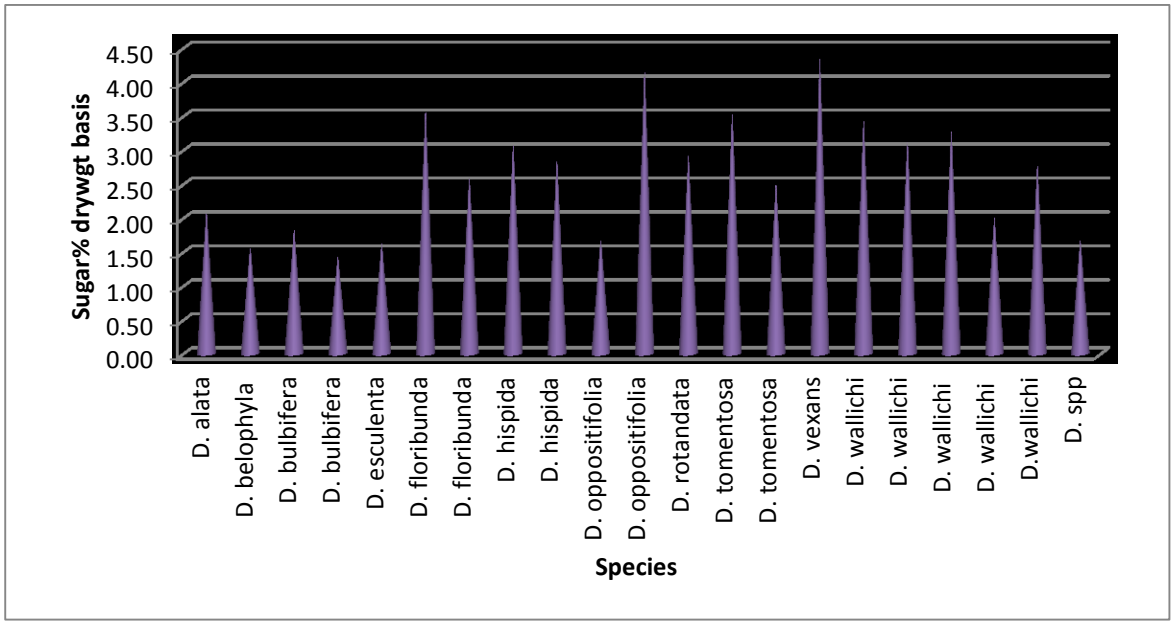


Figure 17. Variation for Crude protein content among wild yams

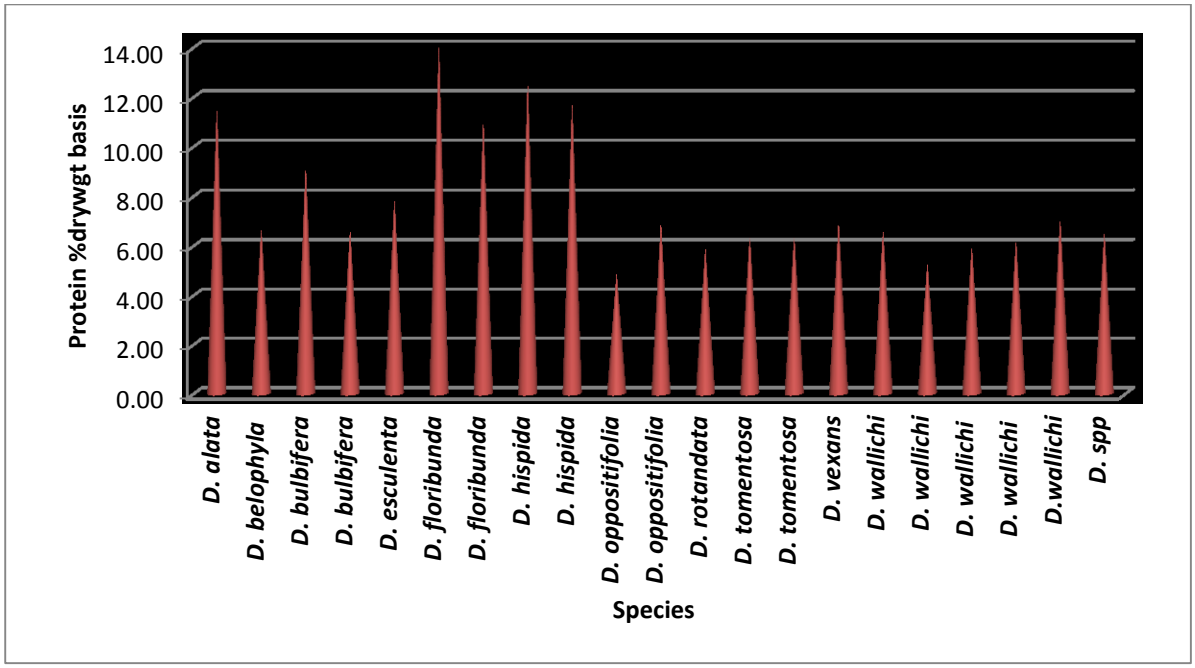


Figure 18. Variation for Crude fibre content among wild yams

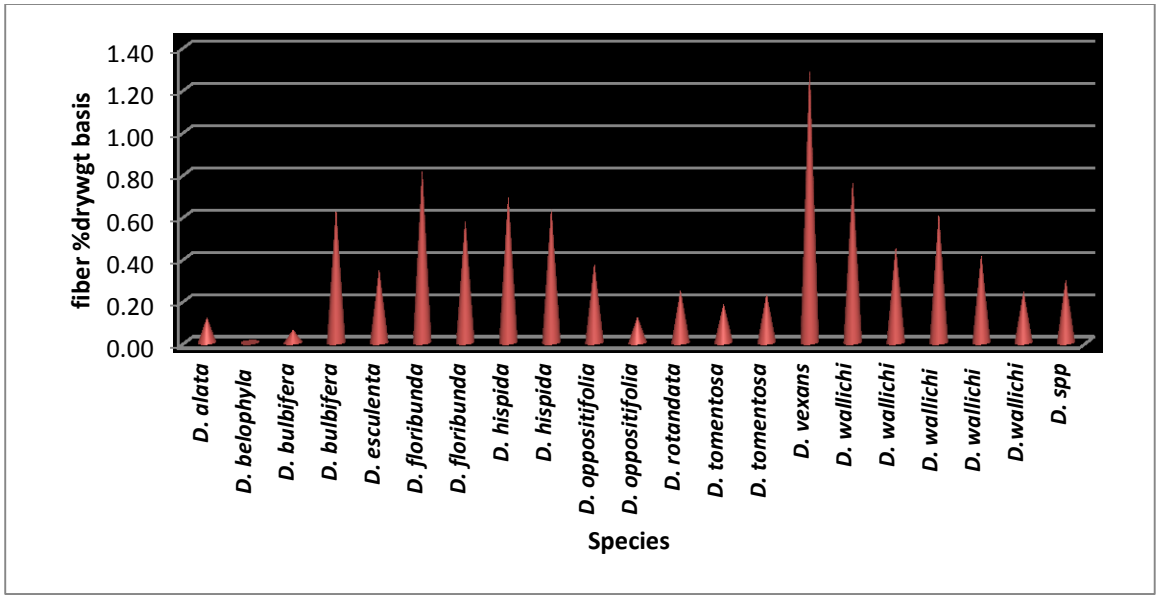


Figure. 19. Variation for fat content among wild yams

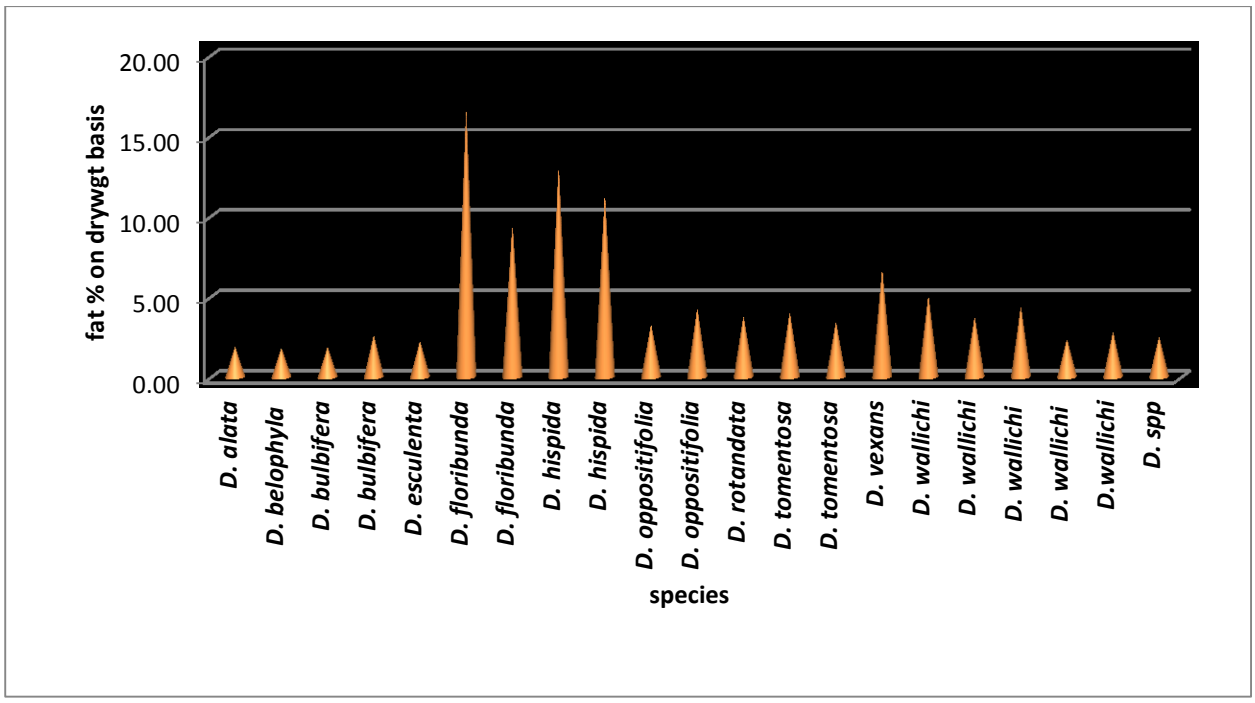


Table 11. Genotypic Correlation among biochemical traits in wild yams

CHARACTER	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11
X1	1										
X2	0.7093	1									
X3	0.7093	1.0000	1								
X4	-0.1310	-0.0026	-.0026	1							
X5	0.6892	0.5375	0.5375	0.5869	1						
X6	0.0643	-0.1301	-0.1301	0.3900	0.3505	1					
X7	0.6711	0.3143	0.3143	0.0777	0.6384	0.8843	1				
X8	-0.4045	-0.4512	-0.4512	0.7026	0.0886	0.4248	-0.0805	1			
X9	0.1874	-0.2026	-0.2026	0.5127	0.4868	0.5452	0.4492	0.7407	1		
X10	0.2887	0.0057	0.0057	0.5806	0.6645	0.7353	0.6930	0.5248	0.8249	1	
X11	0.5617	0.1976	0.1976	0.3223	0.7222	0.7369	0.8639	0.2074	0.6996	0.9342	1

Table 12. Phenotypic Correlation among biochemical traits in wild yams

CHARACTER	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11
X1	1										
X2	0.7064	1									
X3	0.7064	1.0000	1								
X4	-0.1326	-0.0049	-0.0049	1							
X5	0.6799	0.5300	0.5300	0.5932	1						
X6	0.0357	-0.0885	-0.0885	0.2514	0.2116	1					
X7	0.6712	0.3141	0.3141	0.0720	0.6315	0.6044	1				
X8	-.4051	-0.4477	-0.4477	0.6816	0.0879	0.2844	-0.0815	1			
X9	0.1856	-0.1993	-0.1993	0.4945	0.4806	0.3634	0.4472	0.7411	1		
X10	0.2834	0.0061	0.0061	0.5432	0.6426	0.5917	0.6825	0.5163	0.8116	1	
X11	0.5592	0.1971	0.1971	0.3073	0.7122	0.5348	0.8622	0.2072	0.6973	0.9287	1

Table 13. Evaluation of genetic parameters in wild yams for Biochemical trait

CHARACTERS	GCV	PCV	HERITABILITY	GA 5%	GA AS% OF MEAN
X1	29.3405	29.4819	0.9904	18.1989	60.1516
X2	38.4236	38.7005	0.9857	13.23	78.5861
X3	38.4236	38.7005	0.9857	13.23	78.5861
X4	42.9017	44.2747	0.9889	2.2051	85.6368
X5	50.6199	51.1237	0.9804	0.7922	103.2494
X6	24.3707	35.8862	0.4612	2.5378	34.0939
X7	55.0678	55.1148	0.9983	2.5114	113.3431
X8	112.7318	113.0166	0.995	1.0435	231.6422
X9	86.6807	87.0175	0.9923	0.2112	177.871
X10	87.1035	88.7394	0.9635	6.628	176.1255
X11	133.9909	134.4009	0.9939	3.3524	275.1794

Where,

X1 Drymatter%

X2 Starch (DW) %

X3 Starch (FW) %

X4 Sugar (DW) %

X5 Sugar (FW) %

X6 Protein (DW) %

X7 Protein (FW) %

X8 Fibre (DW) %

X9 Fibre (FW) %

X10 Fat (DW) %

X11 Fat (FW) %

4.4 Principal component analysis (PCA)

Twenty morphological traits were considered for PCA analysis and the Eigen values and cumulative variance of the first ten components is given in Table 14 and variability is depicted in Figure 20. The first six principal components of data accounted for 78.85% of the total variance among accessions. The first principal component (PC1) explained 27.88% of the total variance. The distribution of accessions in the scatter plot revealed high divergence of *D. bulbiferra* and *D. floribunda*. The cultivated greater yam (*D. alata*) was found to closely related to wild yam (*D. oppositifolia*).

The first principal component (figure 21) based on ISSR data revealed 28.63 percent of the variation. The first three components together explained 50.50 percent of variation and hence the diversity analysis based on ISSR data was found to be effective. Among the wild yams *Dioscorea vexans* found in Andaman's was found to be highly divergent one.

The first principal component (figure 22) based on SSR data revealed 38.39 percent of the variation. The first three components together explain 68.48 percent of variation and hence the diversity analysis based on SSR data was found to be more reliable. The cultivated African species *D. rotundata* was found to be highly divergent from wild yams in India. Among the wild yams, *D. pentaphylla* was found to be highly divergent one.

Table 14. Eigen values of the cumulative variance for morphological traits in wild yams

No	Eigenvalue	Proportion	Cumulative
1	5.29648632	0.2788	0.2788
2	3.15811176	0.1662	0.4450
3	2.35893509	0.1242	0.5691
4	1.61971987	0.0852	0.6544
5	1.47908828	0.0778	0.7322
6	1.06996896	0.0563	0.7885
7	0.99273411	0.0522	0.8408
8	0.81236904	0.0428	0.8835
9	0.60466452	0.0318	0.9154
10	0.38334370	0.0202	0.9355

Figure 20 Scatter plot for the first three principle components (PCs) for morphological traits in wild yams

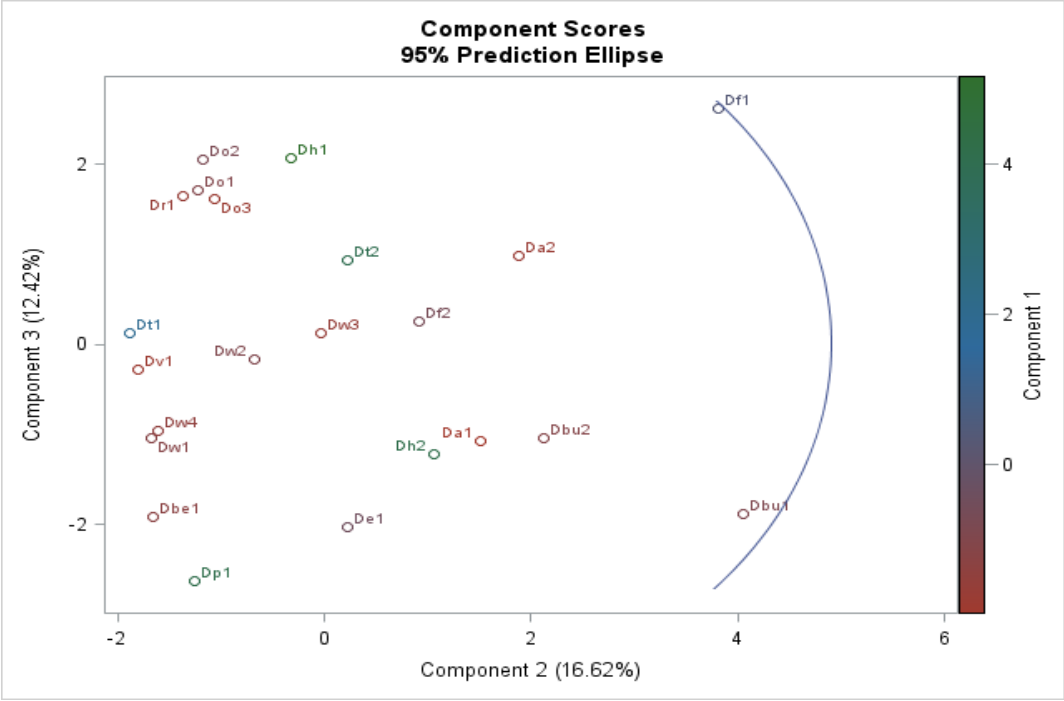


Table 15. Eigen values of the cumulative variance based on ISSR markers in wild yams

No	Eigenvalue	Difference	Proportion	Cumulative
1	83.5944585	48.9988154	0.2863	0.2863
2	34.5956432	5.3392307	0.1185	0.4048
3	29.2564125	2.0000040	0.1002	0.5050
4	27.2564085	7.4905264	0.0933	0.5983
5	19.7658821	6.6437707	0.0677	0.6660
6	13.1221115	1.8892381	0.0449	0.7109
7	11.2328733	0.6499441	0.0385	0.7494
8	10.5829292	2.2742218	0.0362	0.7856
9	8.3087074	0.6611900	0.0285	0.8141
10	7.6475173	0.4081407	0.0262	0.8403

Figure 21 Scatter plot for the first three principle components (PCs) for ISSR markers in wild yams

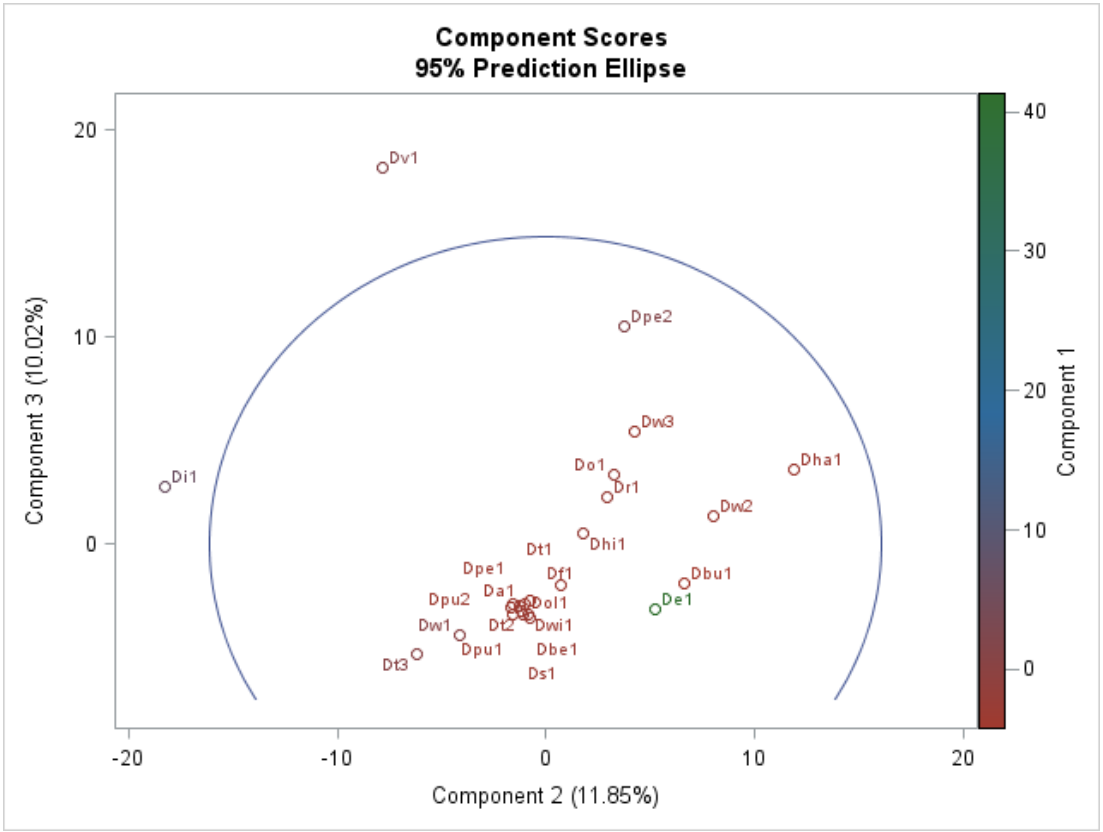
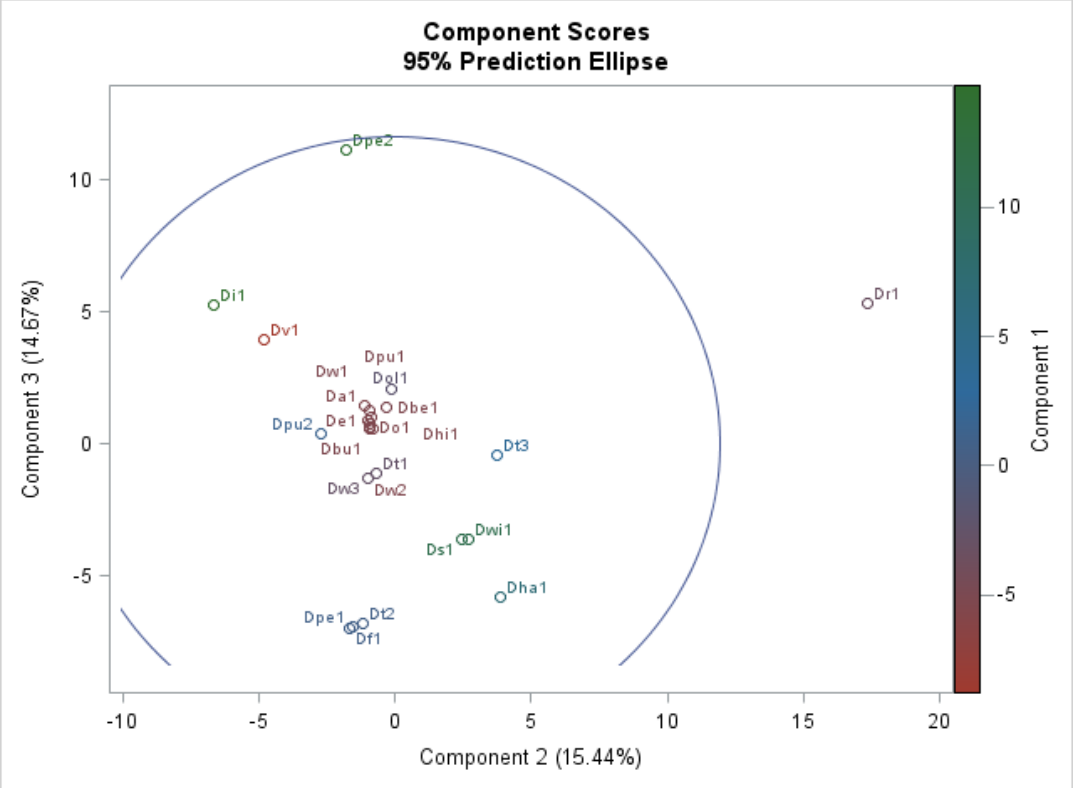


Table 16. Eigen values of the cumulative variance based on SSR markers in wild yams

No	Eigenvalue	Difference	Proportion	Cumulative
1	47.2146321	28.2247210	0.3839	0.3839
2	18.9899111	0.9465432	0.1544	0.5382
3	18.0433680	4.4064395	0.1467	0.6849
4	13.6369285	3.9955569	0.1109	0.7958
5	9.6413715	3.5776970	0.0784	0.8742
6	6.0636745	1.1584569	0.0493	0.9235
7	4.9052176	3.1088002	0.0399	0.9634
8	1.7964174	0.3595253	0.0146	0.9780
9	1.4368921	1.1938048	0.0117	0.9897
10	0.2430873	0.0563124	0.0020	0.9916

Figure 22 Scatter plot for the first three principle components (PCs) for SSR markers in wild yams



DISCUSSION

5. DISCUSSION

Yams (*Dioscorea*) belong to Dioscoreaceae family. They are herbaceous plants with twinning habit and 850 species were reported so far. Approximately eight *Dioscorea* species are eaten in various parts of the world due to their nutritional superiority when compared with other topical root crops. The wild yams make a significant contribution in the diets of the tribal people of India. The tubers were found with a high amount protein, a good proportion of essential amino acids and appeared as a fairly good source of many dietary minerals.

The National Repository on Tuber Crops germplasm at Central Tuber Crops Research Institute, India conserves a large number of accessions of wild yams as field genebank. Wide variability for tuber shape, tuber flesh colour, plant architecture and other morphological, biochemical and agronomic traits were recorded among wild yam accessions in the field gene bank.

Among the edible yams, *D. alata* (greater yam) is the most widely distributed species globally, while *D. rotundata* and *D. cayenensis* (guinea yam) are the most popular and economically important yams in West and Central Africa. Among the medicinal yams, the most widely cultivated are *D. floribunda* Mart. and Gal. (in Mexico and Central America), and *D. deltoidea* Wall. Being one of the hotspots of plant biodiversity, many *Dioscorea* species were reported from Western Ghats adjoining Kerala.

In the present study, evaluation of genetic diversity using molecular and morphological markers and their nutritive quality were carried out in wild yam accessions and it showed significant variations.

5.1 Morphological characterisation

Twenty two morphological traits were recorded using IPGRI descriptors. The frequencies of various types of tuber flesh colour among the 24 accessions of wild yams were recorded. The accessions were examined phenotypically based on their tuber flesh colour at central transverse cross-section exhibited wide range of polymorphism (five types, namely, white, white with purple, yellowish white/off-

white, yellow with purple and yellow flesh were observed). The predominant colour was off white tuber flesh colour. Varying tuber skin colours beneath bark were observed among the accessions. A majority of the total accessions had brown tuber skin colour. Majority of the accessions had sagitate leaf shape (Anokye *et al.,* 2014) varying from sagitate long, to sagitate broad and ovate leaf shape had the least relative frequency. The patterns of leaf arrangement were recorded and similarities were observed as recorded in Prain and Burkill, 1936.Various Quantitative characters like tuber weight, tuber length , girth and no of tubers per plant were observed and recorded.

Results of the morphological analysis confirmed that the wild yam accessions were genetically diverse for the morphological traits assessed. Factorial analysis using Principal Component Analysis (PCA) revealed 20 Principal components with Eigen values greater than 1.00 explaining 27% of the total variance in the samples assessed. However, the scree test, disclosed first one PCA were the most important accounting for 27% of variation. Thus, assessment was based on the first principal component axes. Tuber characteristics such as twining direction accounted for most of the observed variation in PCA.

The PCA analysis, using Jaccard's similarity matrix confirmed in general, that the accessions were grouped into two clusters (C1 and C2). The cluster formed were unique with *D. glabra* found to be more divergent, as a complete outlier. The cluster C2 was sub clustered and *D. hispida* was found to be divergent from all other accessions, hence the results obtained were unique from the research. Very less information is available on this aspect in literature.

5.2 Evaluation of biochemical traits

Wild yam accessions were evaluated for drymatter, starch, sugar, fat, crude, protein, and fibre content to assess their nutritional quality and variation. In the present study, the crude protein content in *D. alata* was found to be higher(10% to 12%) than the reports of Wanasundera and Ravindran (1994) and Shanthakumari *et al*. (2008). The starch percent of *D. alata* was found to be less when compared to Wanasundera and Ravindran (1994).The crude fibre content obtained for *D.*

esculenta, D. rotundata, D. alata, and D. bulbifera were found to less as compared to the earlier report by Polycarp *et al*., 2012.In the present study, the range in crude fat content obtained among wild yams was below the values reported by Polycarp *et al*. (2012). This may due to the difference in the area of sample collection, since wild yam species collected from Ghana (Africa) may have suitable climatic condition and since Africa is one of centre of origin of yams that may also constitute for increased nutritional content, in addition to that the methodology followed was also different for the analysis of crude fat and fibre content.

The crude protein content in *D. bulbifera* was found to be higher (2.5 on fw basis) than that reported by Shanthakumari *et al.* (2008). The range in crude protein content was below in *D. oppositifolia* than recorded in Shanthakumari *et al.* (2008). The value of *D. tomentosa* was found to be in conformity with the reports of Shanthakumari *et al.,* 2008. The percentage of fat content in *D. floribunda* was highest among the accessions studied which ranged from 17 % to 21.75%, which was a unique and informative value obtained and may be due to the presence of hormones, pigments etc. The crude fibre content in *D. floribunda* ranged from 0.8 to 0.7 percent, the protein content was 3.5 percent and the drymatter was found to be 48%.

The yam varieties were statistically analyzed for nutritional characteristics using genetic parameters. Negative correlations was observed for starch with fibre in genotypic correlation which means that as starch increases the fibre content in tubers decreases while crude protein, sugar, fat contents showed positive correlation with starch. There is not much information reported in wild yams on genetic parameter studies. These crops have great potentials to serve as sources of very necessary nutrients and thus, can be applied as food for humans and animals.

5.3 Molecular characterisation

Eighteen species and 24 accessions of wild yams were included in the study. Fifteen ISSR primers and 10 SSR primers (Tostain *et al.,* 2006) were chosen for genotypic analysis based on previous studies in yams. Jaccard's similarity matrix was utilized to construct the dendrogram showing genetic relationships using

UPGMA procedure. Principal component analysis (PCA) was performed to study the variation among the species in cluster.

Using SSR markers, the total alleles amplified were 123, with an average of 13.5 alleles per locus. The difference between the number of alleles ranged from MT 10(2) to Dab2D06 (19). The microsatellite marker Dab2D06was found to be highly polymorphic. Sheela *et al.* (2014) in the study of genetic diversity study on yams in India also reported the same in conformity with the present finding . In PCA analysis the cumulative Eigen value reached 92 percent with first six principle components analysed.

The fifteen ISSR primers produced total number of 293 alleles, with an average of 19.5 alleles per locus. Very high polymorphism was obtained with all the 15 primers. The number of polymorphic bands ranged from 13(UBC 848) to 24(UBC 808), which is high when compared to Wu *et al.* (2014) who studied genetic diversity analysis of yams *viz. D. opposita* and *D. fordii.* The cumulative Eigen value 59% was obtained with first four principle components analysed.

The number of studies in molecular based genotypic analysis including different species of wild yams using ISSR and SSR primers is very less. Hence in the present study was performed using ISSR and SSR markers and the divergence of 18 species of wild yams were studied and recorded. The SSR primer Dab2D06 and ISSR primer UBC 808 were found to be effective markers for wild yam genotypic analysis. The clustering of wild yams using SSR and ISSR markers showed greater similarity of greater yam (*D. alata*) to *D. oppositifolia.* The wild yams *viz*. *D. esculenta* (ISSR) and *D. hamiltonni* (SSR*)* were found to be highly divergent ones and they formed complete outliers in the cluster dendrogram.

The principal component analysis also depicted the genotypic similarity between *D. alata* and *D. oppositifolia*. Being the most prominent wild yam found in Western Ghats and used by the tribal community of Kerala, introgresion of useful genes into cultivated yams can be attempted in future using marker assisted selection.

SUMMARY

6. SUMMARY

Yams (*Dioscorea*) belong to Dioscoreaceae family is important for food, income and socio-cultural events. They are herbaceous plants with twining habit. It is a leading source of calories for over 300 million people in Africa, Asia, parts of South America, as well as the Caribbean and the South Pacific islands. Yam is a polyploid and clonally propagated crop that is cultivated for its starchy tubers.

Yams have been suggested to have nutritional superiority when compared with other topical root crops. They are reported as good sources of essential dietary nutrients.

The wild yams make a significant contribution to the diet of the tribal people of India. The most commonly consumed wild yam among the tribals are *D. pentaphylla* and *D. oppositifolia*. Many of the *Dioscoreae* species serve as a 'life saving' plant group to marginal farming and forest dwelling communities, during periods of food scarcity .The tubers were found with a high content of protein, a good proportion of essential amino acids and appeared as a fairly good source of many dietary minerals.

Members of this genus contain pharmacologically important component, diosgenin, which is a steroidal saponin and functions as phytoestrogen. The tubers of *Dioscorea* have, therefore, been used as food and medicine by many cultures worldwide. Diosgenin is used as a starting material for the production of contraceptive pills, hormones, corcosteroids etc. Being one of the hotspots of plant biodiversity, many *Dioscorea* species were reported from Western Ghats adjoining Kerala.

This research work attempts to combine morphological, molecular and biochemical data for greater understanding of the extent of genetic variation existing within and among the wild yams species collected from Western Ghats of India. The exploitation of the genetic diversity so determined serves to facilitate the development of better varieties conventional and marker assisted breeding programme.

In the present study, twenty four accessions collected from Western ghats and from different forest ecosystems were selected. The DNA was isolated using manual protocol and good quality of DNA was obtained with concentration ranging from 2000 to 7000ng/µl. The ISSR and SSR primers were chosen based on the review of previous literature. Fifteen ISSR and 10 SSR highly polymorphic primers were used for primer screening prior to PCR amplifications. In which all the 15 ISSR primers and only 9 SSR primers were chosen for PCR amplification.

Using allelic richness as a measure of genetic diversity, the wild forms exhibited greater allelic diversity than the cultigens. From 15 ISSR primers chosen for the study UBC 808 is the most efficient primer for wild yams for future studies. UBC 808 had greater number of polymorphic allele (24). From 10 SSR primers, Dab2D06 is the good primer for diversity study of wild yams and showed greater number of polymorphic alleles (19). Both ISSR and SSR markers revealed greater similarity of *D. alata* to *D. oppositifolia. D. esculenta (*ISSR*)* and *D. hamiltonni (SSR)* were the divergent wild yams identified in the present study. Hence these primers are identified for future genotypic analysis and for DNA fingerprinting.

About 22 morphological traits were chosen for the study wherein 18 qualitative and 4 quantitative traits are observed and scored using IPGRI descriptors. Dice dissimilarity matrix was utilized to construct a dendrogram showing genetic relationships using UPGMA procedure. Principle component analysis (PCA) was also performed to study the difference among the species in a cluster. These analyses were conducted using NTSYS pc, Darwin 6.0 and SAS1.0 softwares.

The first principal component (PC1) explained 27.88% of the total variance. The distribution of accessions in the scatter plot revealed high divergence of *D. bulbiferra* and *D. floribunda*. The cultivated greater yam (*D. alata*) was found to closely related to wild yam (*D. oppositttifolia*). The first principal component based on ISSR data revealed 28.63 percent of the variation. The first three components together explained 50.50 percent of variation and hence the diversity analysis based on SSR data was found to be effective. ISSR data revealed that among the wild

yams *Dioscorea vexans* found in Andaman's was found to be highly divergent one. The first three components together explain 68.48 percent of variation and hence the diversity analysis based on SSR data was found to be more reliable. The cultivated African species *D. rotundata* was found to be highly divergent from wild yams in India. Among the wild yams, *D. pentaphylla* was found to be highly divergent one.

The nutritional content of wild yams is rich in starch, sugar, fibre, fat and protein. The evaluations of nutritive traits among wild yams revealed that D. *belophylla* have higher sugar value than *D. alata*. *D. floribunda and D. bulbifera* have higher protein value than *D. alata. D. belophylla* have higher starch value than *D. alata.*

From the genetic parameters estimated, the fibre and sugar showed negative correlation to drymatter content whereas sugar, protein and fibre contents showed negative correlations to starch.

The highest genotypic covariance (GCV) of 133.9 was estimated in fat content (fw basis), phenotypic covariance (PCV) was also estimated to be high in fat (fw basis) of 134 and heritability was observed to be highest in protein content (0.998).

Hence this study was an attempt to study the genetic diversity of wild yams from different forest area and Western Ghats of Kerala using molecular and morphological markers. This study elucidated the relationship among the wild and cultivated yams and will provide information on phylogeny. Closely related wild yams identified can be used for the introgression of useful genes into cultivated yams. The study also resulted in DNA fingerprinting of important wild yams in Kerala. Since the wild yams play an important role in the diet of tribal communities, the information on nutritive quality traits will enhance further utilization of wild yams in Kerala.

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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)

Added freshly prior to grinding

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

Alcohol: Ether mixture (3:1)

Alcohol	30ml
Ether mixture	10ml

Mix 3 parts of ethyl alcohol (absolute) with 1 part of Diethyl ether (AR). Store in a tightly capped bottle.

APPENDIX VII

Chloroform : methanol mixture (1:1)

Chloroform	10ml
Methanol mixture	10ml

Mix 1 part of chloroform (AR) with 1 part of methanol (AR) and store in a tightly capped bottle.

APPENDIX VIII

Sulphuric acid – 1.25%.

Sulphuric acid 1.25g

Weigh 1.25g concentrated H_2SO_4 in a small beaker and increase with distilled water to 100ml

APPENDIX IX

Sodium hydroxide – 1.25%

Sodium hydroxide 1.25g

Weigh 1.25g NaOH and distilled water and increase to 10ml.

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 upto 9 litres

APPENDIX XII

Hydrochloric acid (2.5 N)

Hydrochloric acid	50ml

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

ABSTRACT

GENETIC DIVERSITY ANALYSIS OF WILD YAMS OF WESTERN GHATS

by

IRFA ANWAR
(2011-09-113)

Abstract of the thesis

Submitted in partial fulfilment of the Requirement for the degree of

MASTER OF SCIENCE (INTEGRATED) IN BIOTECHNOLOGY

Faculty of Agriculture
Kerala Agricultural University, Thrissur

B.Sc - M.Sc. (Integrated) Biotechnology

DEPARTMENT OF PLANT BIOTECHNOLOGY
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2016

ABSTRACT

Wild Yams belonging to the family Dioscoreaceae makes significant contribution to the diets of the tribal community in India. They also have immense medicinal properties due to the presence of secondary metabolites especially diosgenin and hence is of great interest to the pharmaceutical industry. It serves as a precursor for the production of corticosteroids, sexual hormones, oral contraceptives and possesses cancer chemotherapeutic activity. In the present study conducted at ICAR-CTCRI, Thiruvanathapuram, morphological, biochemical and molecular variation among the major wild yams were studied to establish their genetic relationship.

Morphological characterization of 25 accessions of wild yams (*Dioscorea* spp.) was done based on 21 IPGRI descriptors. Tuber weight/plant ranged from 40g (*D. belophylla*) to 5.66kg (*D. floribunda*) while tuber flesh color of the accessions varied from white to red purple.

The biochemical studies on starch, sugar, crude protein, crude fat and crude fiber was done keeping in mind the nutritive value of the wild yam tubers used by the tribal community. Among the accessions, *D. belophylla* recorded the highest starch content on D.W basis (98%) while *D. tomentosa* the lowest (25%). Crude protein content was highest in *D. floribunda* with 14% followed by *D. hispida* (12.46%) and lowest value was recorded in *D. wallichi* (4.81%). From the genetic parameters estimated the fibre and sugar showed negative correlation to drymatter whereas sugar, protein and fibre recorded negative correlations to starch. The highest heritability was observed for crude protein (0.998).

The wild yams were characterized at molecular level using 15 ISSR primers and 9 SSR primers, of which, UBC 808 and Dab2D06 showed high polymorphism for all the 18 species of wild yams studied. Principal component analysis was also performed to study the variation. The distribution of accessions in the scatter plot revealed high divergence of *D. bulbiferra* and *D. floribunda*. The cultivated greater yam (*D. alata*) was found to closely related to wild yam (*D. oppositifolia*). Hence the present study could identify the genetic relationship among wild yams and their nutritional quality.