MOLECULAR CHARACTERIZATION AND *IN VITRO* CONSERVATION OF TARO

(*Colocasia esculenta* (L.) Schott)

by

SREEVIDYA M. R.

(2012-09-123)

THESIS

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DEPARTMENT OF PLANT BIOTECHNOLOGY

COLLEGE OF AGRICULTURE

VELLAYANI, THIRUVANANTHAPURAM-695 522

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DECLARATION

I hereby declare that this thesis entitled **"Molecular characterization and *in vitro* conservation of taro (*Colocasia esculenta* (L.) Schott)** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title, of any other university or society.

Place: Sreekariyam
Date: 16-11-2017

SREEVIDYA M. R.
(2012-09-123)

भा कृ अनु प - केन्द्रीय कंद फसल अनुसंधान् संस्थान
(भारतीय कृषि अनुसंधान् परिषद, कृषि और् किसान कल्याण मंत्रालय, भारत सरकार)
श्रीकार्यम, तिरुवनन्तपुरम-695 017, केरल, भारत
ICAR- CENTRAL TUBER CROPS RESEARCH INSTITUTE
(Indian Council of Agricultural Research, Miniistry of Agricultural and Farmers Welfare, Govt. of India)
Sreekariyam, Thiruvananthapuram-695 017, Kerala, India
ISO 9001:2008

3

CERTIFICATE

Certified that the thesis entitled **"MOLECULAR CHARACTERIZATION AND *IN VITRO* CONSERVATION OF TARO (*Colocasia esculenta* (L.) Schott)"** is a record of research work done by **Ms. Sreevidya M. R. (2012-09-123)** under my guidance and supervision and that this has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

Sreekariyam
16.11.2017

(**Asha Devi A**)
(Chairperson, Advisory committee)
Principal Scientist
Division of Crop Improvement
ICAR- CTCRI, Sreekariyam
Thiruvananthapuram- 695017

Phone : +91-471-2598551 to 2598554
Director (Per) : +91-471-2598431
(Res) : +91-471-2597211
Sr. Admn. Officer : +91-471-2598193
Fax : +91-471-2590063
E-mail : director.ctcri@icar.gov.in
ctcritvm@gmail.com
Web : http://www.ctcri.org

CERTIFICATE

We, the undersigned members of the advisory committee of **Ms. Sreevidya M. R. (2012-09-123)**, a candidate for the degree of **B. Sc. – M. Sc. (Integrated) Biotechnology**, agree that the thesis entitled "**Molecular characterization and *in vitro* conservation of taro (*Colocasia esculenta* (L.) Schott)**" may be submitted by Ms. Sreevidya M. R., in partial fulfillment of the requirement for the degree.

Dr. Asha Devi A.
(Chairperson, Advisory Committee)
Principal Scientist
Division of Crop Improvement
ICAR- Central Tuber Crops Research Institute, Sreekariyam
Thiruvananthapuram- 695017

Dr. Swapna Alex
Professor and Head
Department of Plant Biotechnology
College of Agriculture, Vellayani
Thiruvananthapuram- 695 522

Dr. Shirly Raichal Anil
Senior Scientist
Division of Crop Improvement
ICAR- Central Tuber Crops Research Institute, Sreekariyam
Thiruvananthapuram- 695017

Dr. J. Sreekumar
Principal Scientist
Division of Social Science and Extension
ICAR- Central Tuber Crops Research Institute, Sreekariyam
Thiruvananthapuram- 695017

External Examiner:

Dr. K. K. Sabu
Scientist-E1, Biotechnology and Bioinformatics Division
JNTBGRI, Palode
Thiruvananthapuram-695562

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

%	- Percentage
µg	- Microgram
µl	- Microliter
µM	- Micro molar
2D	- 2 - Dimension
3D	- 3 - Dimension
A_{260}	- Absorbance at 260nm
A_{280}	- Absorbance at 280nm
ABA	- Abscisic acid
AFLP	- Amplified fragment length polymorphism
AgNO3	- Silver nitrate
ANOVA	- Analysis of variance
AP – PCR	- Arbitrarily primed - PCR
APS	- Ammonium per sulfate
B	- Benzylaminopurine
BA	- Benzyl adenine
Bind silane	- 3 - methacryloxpropyltrimethoxysilane
bp	- Base pair
CAPS	- Cleaved amplified polymorphic sequence
cm	- Centimeter
CRD	- Completely Randomized Design
CTAB	- Cetyl trimethyl ammonium bromide
CTCRI	- Central Tuber Crops Research Institute
DNA	- Deoxyribo nucleic acid
dNTP	- Deoxy nucleotide triphosphate

E	- East
EDTA	- Ethylene diamine tetra acetic acid
EST	- Expressed sequence tag
FAOSTAT	- Food and Agriculture Organization Statistical Database
g	- gram
h	- hour
ha	- hectare
$HgCl_2$	- Mercuric chloride
ICAR	- Indian Council of Agricultural Research
ICS	- International Core sample
IITA	- International Institute of Tropical Agriculture
INIVIT	- Research Institute on Tropical Roots and Tubers
IPC	- Integral plate chamber
ISSR	- Inter simple sequence repeats
IVAG	- *In vitro* active genebank
K	- Kinetin
KAU	- Kerala Agricultural University
kb	- Kilo base pairs
kg	- Kilogram
LAF	- Laminar air flow
M	- Molar
mg	- Milligram
$MgCl_2$	- Magnesium chloride
min	- Minute
ml	- Milliliter
mM	- Milli molar

mm	- Millimeter
MS	- Murashinge and Skoog
mtDNA	- Mitochondrial DNA
N	- North
Na2CO3	- Sodium carbonate
NAA	- α Napthalene acetic acid
NaCl	- Sodium chloride
ng	- Nanogram
nm	- Nanometer
°C	- Degree Celsius
OD	- Optical density
PAGE	- Poly acrylamide gel electrophoresis
PCA	- Principal component analysis
PCR	- Polymerase chain reaction
PEG	- Polyethylene glycol
pg	- Picogram
pH	- Power of hydrogen
PNG	- Papua New Guinea
psi	- Pounds per square inch
PVP	- Polyvinyl pyrolidone
RAPD	- Random amplified polymorphic DNA
rDNA	- Ribosomal DNA
RFLP	- Restriction fragment length polymorphism
RNA	- Ribonucleic acid
RNase	- Ribonuclease
rpm	- Revolutions per minute

s	- Second
SCAR	- Sequence characterized amplified regions
SNP	- Single nucleotide polymorphism
SSLP	- Simple sequence length polymorphism
SSR	- Simple sequence repeats
STR	- short tandem repeats
STS	- Sequence tagged site
t	- ton
Taq	- *Thermus aquaticus*
TaroGen	- Taro Genetic Resources: Conservation and Utilization
TBE	- Tris – borate EDTA buffer
TDZ	- Thidiazuron
TE	- Tris EDTA buffer
TEMED	- N, N, N', N', - tetramethylethylenediamine
T_m	- Melting temperature
Tris HCl	- Tris (hydroxymetyl) aminomethane hydrochloride
U	- Enzyme unit
USP	- University of South Pacific
UV	- Ultra violet
V	- Volt
V	- Volts
v/v	- Volume / volume
w/v	- Weight / volume

INTRODUCTION

1. INTRODUCTION

Taro (*Colocasia esculenta* (L.) Schott), an important tuber crop belonging to the monocotyledonous family Araceae is a staple food for 400 million people. Taro is consumed by people living mainly in the humid regions. The Araceae family consists of about 110 genera with over 2500 species. Taro is mainly grown for its corms, cormels, petiole and leaves. Taro is a widely sought after crop due to its adaptability. It originated mainly in the Indo–Malayan region, where it shows immense genetic diversity. Considerable variability has been recorded in the crop at morphological, agronomical and molecular level (Hussain and Tyagi, 2006). According to the corm size and shape, taro is classified into dasheen type and eddoe type.

The introduction of new pests and disease, intensified demands of land due to increasing population and the encouragement of developing commercial crops by the government has all resulted in the narrowing down of the diversity existing in taro. It has led to the loss of traditional diversity, which has led to problems like loss of allelic diversity. Thus there is a need to collect, evaluate and conserve taro genetic resources. Previous studies regarding diversity analysis of taro in India is limited and therefore characterization of taro accessions for diversity assessment becomes imperative. Characterization would facilitate the development of a core collection, study the breeding behavior of species, individual reproductive success and existence of gene flow. Molecular data helps in understanding taxonomy, domestication and evolution (de Vicente *et al.*, 2006). Analyzing the genetic diversity study is important to assess the extent of diversity present and use this information for crop improvement programs. ICAR–Central Tuber Crops Research Institute (ICAR-CTCRI) maintains around 590 accessions of taro in its field genebank, collected from different locations in India as well as some collections from abroad.

The development of molecular markers for characterizing taro and early progeny selection is highly desirable for developing an efficient breeding program to speed the integration of new genetic material into elite germplasm. Also, the characterization using molecular markers will give a knowledge about the genetic relationship between the accessions from wild and cultivated gene pool. This would help breeders to satisfy market needs and to respond to biotic and abiotic challenges.

In recent years, there has been a significant progress in the application of molecular markers for characterizing and evaluating plant genetic resources. Microsatellites have emerged as a marker of choice due to their co-dominant nature, high levels of polymorphism, abundant and uniform distribution throughout the genome, simplicity in detection and ease in transferability and reproducibility. Microsatellites have proven to be useful in characterizing root and tuber crops like cassava, taro, yams and other aroids.

There has been an increased threat of extinction of indigenous plant species, which has prompted public and various institutions into conserving them. Conservation should be in such a manner that the plant materials are not lost, but are also available for crop improvement programs. Conservation is mainly of two types, *in situ* and *ex situ*. For the plants which are propagated mainly by vegetative means like yams, taro, cassava, sweet potato, etc., *ex situ* type of conservation is mainly used. Field genebanks provides an easy access, but also faces threats of calamities, pest and disease, thus losing the whole material. The non-availability of large amount of land due to increasing population, use of land for cultivation of economic plants, etc. has also been a threat against having field genebank. The advancement in the field of biotechnology has provided with an alternative to this problem, which is to maintain cultures *in vitro* by means of tissue culture. *In vitro* methods mainly include slow growth and cryopreservation techniques. For short and medium

term conservation, slow growth technique is mainly used, whereas, for long term conservation, cryopreservation is mainly used.

For slow growth technique, temperature reduction is the main method used. In the case of temperate plants, the temperature is reduced from 20-25°C to 6-10°C and for tropical plants, the temperature is reduced from 25-30°C to 15-25°C. This reduction in temperature has favored slow growth and has extended the interval of sub culturing from days to years. Slow growth is also attained by manipulating the media by adding either osmotic inhibitors (mannitol, sorbitol, sucrose) or hormonal inhibitors (abscisic acid).

In the case of taro, alteration in temperature and addition of osmotic inhibitors has influenced slow growth. But the works regarding the *in vitro* conservation of taro was mostly done abroad or on exotic varieties. The *in vitro* conservation would help in establishing an *in vitro* active genebank (IVAG) for taro. Few studies have also been carried out in some Indian Institutions like, ICAR-National Bureau of Plant Genetic Resources (ICAR-NBPGR), which was dealing with *in vitro* conservation of many tuber crops including taro till quite recently. At ICAR–CTCRI, an IVAG is in existence, where many important accessions of taro are being maintained and conserved. The present study would augment the existing IVAG collection at the institute.

In short, the present study would help us in understanding the variability existing in the selected taro accessions as well as enable the selection of divergent accessions from divergent clusters as prospective parents in taro hybridization programs. Using this diversity, taro breeders could develop cultivars which could satisfy market needs and respond to biotic and abiotic challenges. The study would also facilitate conservation of taro germplasm using slow growth techniques for medium term conservation.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1. TUBER CROPS

Roots and tuber crops were domesticated in Southeast Asia, West-Central Africa and tropical Latin America thousands of years ago, but remained insignificant till the late 16th century. The major tuber crops include cassava, sweet potato, yams and aroids, which are of ancient origin, but remain staple food for people around the world even today. Roots and tuber crops belong to different families and were domesticated independently in separate regions. These crops get adapted to a condition very fast due to their high yield, resistance and earliness and in the dietary chart by the taste qualities (Leon, 1977).

The relative production of tuber crops is approximately 836 million tons, where Asia is the main producer followed by Africa, Europe and America (Chandrasekara and Kumar, 2016).

2.2. AROIDS

Aroids are plants belonging to the family Araceae which are distributed widely, mainly in the tropical areas. The geographical origin of these aroids are poorly defined (Mathews, 1995).

The edible aroids mainly include taro (*Colocasia esculenta* (L.) Schott), tannia (*Xanthosoma sagittifolium* (L.) Schott), giant taro (*Alocasia macrorrhiza* (L.) Schott), swamp taro (*Cyrtosperma spp.* Griff.), elephant foot yam (*Amorphophallus paeonifolius* (Dennst.) Nicolson) etc. These aroids mainly have corms or cormels. The centers of origin of these aroids are considered to be tropical America and tropical Asia. Some species could only be found in the Mediterranean and Africa. There is another possible center of origin for these aroids, which is considered to be Australia and the islands of Papua New Guinea (Ivancic and Lebot, 2000).

2.3. TARO

Taro (*Colocasia esculenta* (L.) Schott) is a tuber crop belonging to the family Araceae, grown traditionally for its edible corms and leaves. As per the studies of Plucknett (1976); Kuruvilla and Singh (1981), etc. taro is one of the earliest cultivated crop in the world dating back 9000 years. Taro was domesticated first in Southeast Asia and was later spread to Asia, Pacific, Africa and the Caribbean and remains an important crop there. Taro is a highly polymorphic species, cultivated mainly in the tropical areas, from equator to Japan (45°N) and has more than 10,000 existing landraces (Ivancic and Lebot, 2000).

Worldwide, taro ranks 14th among staple vegetable crops with about 10 million tons produced from about 1.5 million hectares with an average yield of 6.9 t/ha (FAOSTAT, 2014). In India, taro is grown in an area of 51,724 hectares producing 8,15,995 metric tons with a yield of 15.68 tons/hectare (Srinivas *et al.*, 2012).

There are two botanic varieties of taro characterized by their corm shape, which are *C. esculenta* var. *esculenta* (dasheen type) and *C. esculenta* var. *antiquorum* (eddoe type). The dasheen type taro has a small corm and a large number of smaller cormels (Purselove, 1972). Both the eddoe and dasheen types are common in India. Dasheen type is predominantly grown in India, especially in the hilly areas including Andaman Islands (Bose *et al.*, 2003). Eddoe type is more common, especially in South India (Devi, 2012). It is reported that *C. esculenta* var. *esculenta* is diploid and *C. esculenta* var. *antiquorum* is triploid (Kuruvilla and Singh, 1981; Irwin *et al.*, 1998). This classification is, however, controversial and it has not yet been demonstrated that all diploids belong to the var. *esculenta* and all triploids belong to var. *antiquorum*. This view is supported by reports that both diploids as well as triploids are present in eddoe type (Sreekumari, 1992; Kuruvilla and Singh,

1981; Nusaifa Beevi, 2009). Taro is an asexually propagated annual crop (Quero-Garcia *et al.*, 2004).

According to the habitat in which taro is grown, it varies again. The variations include upland taro, lowland taro (in India) and swamp taro, which grows in water logged conditions (West Bengal, Bangladesh). In India, taro is cultivated in 21 agro biodiversity hotspot regions out of 22 identified in the country. These regions include regions like eastern Himalayas, Brahmaputra valley, North eastern hills, upper Gangetic plains, Gangetic Delta, lower Gangetic plains, Chotanagpur, Bastar, Koraput (Gondwana tribal zone), Kaveri, Konkan, Malabar, Andaman – Nicobar Islands and Lakshadweep regions (Unnikrishnan *et al.*, 2013)

The basic chromosome number of taro is considered to be x = 14 (Yen and Wheeler, 1968; Kuruvilla and Singh, 1981. However, meiotic and karyomorphological data according to Krishnan and Magoon (1977) and Sreekumari (1997) favour the contention of x = 7 as the original basic chromosome number. But, recent investigations using fluorescent *in situ* hybridization with ribosomal DNA probe (Kokubugata and Konishi, 1999) showed evidence for basic chromosome number x = 14. *Colocasia* occurs mainly as two karyotypes, as diploids with 2n = 28 and as triploids with 2n = 42 chromosomes (Rattenbury, 1956; Yen and Wheeler, 1968; Ramachandran, 1978; Coates *et al.*, 1988). The majority of the cultivated taro are diploids, although some triploids could also be found (2n = 3x = 42) (Mace and Godwin, 2002). Earlier studies suggest that all dasheen types are diploids and most eddoe types are triploids, but there are no much data to prove this statement (Kuruvilla and Singh, 1981; Irwin *et al.*, 1998).

The natural geographic range depends upon factors including geography, availability of habitats, natural barriers to dispersal habitat preference (concerning temperature, daylight cycles, physiological availability of water, soil conditions, presence of pollinators, absence of plant competitors,

herbivores, pest and diseases), and the species inherent genetic capacity, which enables it to respond to a multitude of ecological and physiological stress or signals. The natural range of taro extends from India and Southeast Asia to Northern Australia and New Guinea. This conclusion was supported by considerations of taxonomic, ecological, ethnobotanical and geographic factors (Ahmed, 2014).

2.4. CENTER OF ORIGIN

Taro is said to have originated in the Indo–Malayan regions, probably in the North eastern India and Asia according to the studies of Kuruvilla and Singh (1981) and Yen and Wheeler (1968). From this center of origin, it is said to have dispersed to other parts. Lebot and Aradhya (1991) reported two gene pools of taro using isozyme analysis, one in Asia and the other in the Pacific. Studies of Noyer *et al.* (2004) and Krieke *et al.* (2004) confirmed this. The alternate hypothesis shows that taro was domesticated independently in New Guinea (Coates *et al.*, 1988; Yen, 1991,1993; Lebot, 1999). Fossil evidences (Loy *et al.,* 1992) and pollen (Haberle, 1995) from these regions has supported the second hypothesis.

2.5. NUTRITIONAL VALUE

Taro serves as a staple food for people around the world (Rao, 2010). Taro corm is an excellent source of carbohydrate, mainly starch. The edible parts include corm, cormels, rhizome, stalk, leaves and flowers and contain starch abundantly. Fresh corms are composed of moisture (69%), starch (25%), dietary fiber (1.5%), protein (1.1%) and sugar (1%). Taro contain high levels of protein and are also excellent source of carotene, potassium, calcium, phosphorus, iron, riboflavin, thiamine, niacin, vitamin A, vitamin C and dietary fiber (Bradbury and Halloway, 1998). The corms of taro are also said to possess medicinal values; they are used to treat tuberculosis, ulcers, pulmonary congestion and fungal infection (Singh *et al.,* 2011).

2.6. GENERAL PLANT MORPHOLOGY

Colocasia esculenta is a perennial, glabrous, herb growing to a height of 1m or more, with a massive, flesh corm at the base and lateral thick edible runners. The corm is located centrally lying just below the soil surface. Leaves grow upwards and roots grow downwards and cormels and runners (stolons) grow laterally from the corm. Taro has a fibrous root system which lies mainly in the top 1m of the soil. Corm is the edible part of the plant. In the dasheen type, it grows up to 30cm long and 15cm in diameter. In the eddoe type, the cormels constitute the edible harvest. The surface of the corm has rings showing the point of attachment of scale leaves or senesced leaves. Axillary buds are present at the nodal positions on the corm. The apex of the corm represents the growing point. Leaves arise in a whorl from the corm apex and determine the height of the plant. Leaf comprises an erect petiole and large lamina. Petiole is thick at the base and thin towards the point of attachment, and generally distinguishes taro from tannia, which has a hastate leaf, i.e., the petiole is attached to the basal edge of the lamina (Vinutha, 2014).

2.7. MOLECULAR CHARACTERIZATION

The development of molecular markers has led to a significant progress in the characterization and evaluation of plant genetic resources (Gupta and Varshney, 2000). Molecular markers are not influenced by environment and the detection occurs directly at the DNA level. This is the main advantage in the genetic diversity analysis or in identification of new cultivars, which are governed by complex genetic interactions (Jiang and Liu, 2011). Molecular data could be used in association with morphological analysis to reduce redundancy in collection and to maintain cost efficiency (Pissard *et al.,* 2008). Molecular markers show high degree of polymorphism and reproducibility by which genetic variation is obtained (Nguyen *et al.,* 2004). Molecular markers are used for the analysis of many crops.

2.7.1. Molecular markers

Molecular marker is a sequence of DNA, which are located with a known position on the chromosome (Kumar, 1999), or a gene whose phenotypic expression is frequently easily discerned and used to detect an individual, or as a probe to mark a chromosome, nucleus, or locus (King and Stansfield, 1990; Schulmann, 2007). Markers show polymorphism, which may arise due to alteration of nucleotide or mutation in the genome loci (Hartl and Clark, 1997) and make it possible to identify genetic differences between individual organisms or species (Collard *et al.*, 2005).

The introduction of molecular markers has led to the ease in analysis of polygenic characters that were difficult to analyze earlier using traditional breeding techniques. Through markers, it was possible to establish a genetic relationship between sexually incompatible crop plants. Molecular markers such as Random Amplified Polymorphic DNA – RAPD (Williams *et al.*, 1990), Restriction Fragment Length polymorphism – RFLP (Grodzicker *et al.*, 1974), Inter Simple Sequence Repeats – ISSR (Zietkiewics *et al.*, 1994), Simple Sequence Repeats – SSR (Akkaya *et al.*, 1992), Single Nucleotide Polymorphism –SNP (Jordan and Humphries, 1994), Amplified Fragment Length Polymorphism – AFLP (Vos *et al.*, 1995), Sequence Characterized Amplified Regions – SCAR (Paran and Michealmore, 1993), Cleaved Amplified Polymorphic Sequence – CAPS (Akopyanz *et al.*, 1992), Sequence Tagged Site – STS (Olsen *et al.*, 1989) and Arbitrarily Primed Polymerase Chain Reaction – AP-PCR (Welsh and McClelland, 1991), etc. are used to construct genetic map (Mohan *et al.*, 1997).

2.7.2. Simple sequence repeats

Microsatellites (Litt and Luty, 1989) are short sequence elements that are arranged in a simple internal repeat structure. They are a repeat of 2-6 base pairs (Chambers and MacAvoy, 2000) distributed ubiquitously throughout the

eukaryotic genome. They are found abundantly in plant genome and are thought to be the source of genetic variation. Due to the co-dominant nature, high levels of polymorphism, abundance and uniform distribution throughout the genome, simplicity in detection, ease of transferability and reproducibility, microsatellites have emerged as the marker of choice for plant genetic resources applications (Ijaz, 2011).

The flanking sequences are always conserved within the species or among and primers are designed complementary to it. The sequence is amplified by PCR and the amplicons are separated by electrophoresis. The factors responsible for generating polymorphism include size of the repeat unit, number of repeats, presence of variant repeats and the frequency of transcription in the area of DNA repeat (Somasundaram and Kalaiselvam, 2011). For inbreeding crops with low levels of intraspecific diversity, microsatellites have proven to be useful (Roder *et al.,* 1995). Microsatellites are particularly useful in root crops like cassava (Chavarriaga-Aguirre *et al.,* 1998; Roa *et al.,* 2000), sweet potato (Buteler *et al.*, 1999), yam (Terauchi and Konuma, 1994) and taro (Mace and Godwin, 2002; Singh *et al.*, 2008).

2.7.3. Molecular characterization of taro

Characterization of taro was previously done by many researchers using morphological and isozyme markers. Morphological characterization was done by Lebot *et al.,* (2004); Quero-Garcia *et al.*, (2004); Trimanto *et al.,* (2010); Singh *et al.,* (2011) in taro. Morphological markers are highly influenced by the environment and taro being a vegetatively propagated crop for thousands of years, the fixation of somatic mutations could result in morphotypes to be quite distinct (Kuruvilla and Singh, 1981), even when they share the same genetic content. In the case of biochemical markers, the first studies were done by Lebot and Aradhya (1991), analyzing 1417 cultivars from Southeast Asia and Oceania. This study supported the existence of two independent gene pools for taro, one in Southeast Asia and the other in

Melanesia. Prana *et al.* (2000) worked on 6 isozyme mediated characterization on 328 taro samples from various parts of West Java, Indonesia. Manzano *et al.* (2001) used both morphological as well as isozyme markers on 42 accessions of taro from the gene bank of the Research Institute on Tropical Roots and Tubers (INIVIT), Cuba. Trimonto *et al.* (2010) worked on 18 taro accessions from Central Java using both morphological and molecular markers (Devi, 2012). But similar to morphological markers, biochemical markers are also influenced by environment or the developmental stage of the plant, and as a result, molecular or DNA markers are used abundantly for characterization (Collard *et al.*, 2005).

Taro was characterized using many molecular markers like RFLP, RAPD, AFLP, ISSR and SSR. Mathews *et al.* (1992) examined Japanese taro cultivars using RFLP in the rDNA and mtDNA. Again Matsuda and Nawata (2002) investigated rDNA polymorphism using RFLP in 227 accessions of taro from Japan and other parts of Asia. Irwin *et al.* (1998) evaluated 44 taro and 2 tannia and 1 *Colocasia gigantea* accessions using RAPD markers. Schnell *et al.* (1999) evaluated 18 accessions of *Xanthosoma* and 2 accessions of *Colocasia esculenta* (L.) Schott using RAPD data. Lakhanpaul *et al.* (2003) evaluated 32 Indian taro accessions using 22 RAPD primers. Ochiai *et al.* (2001) used isozyme and RAPD markers to find the geographical differentiation and phylogenetic relationships of Asian taro which showed that RAPD markers are better suited for studying genetic differentiation. Singh *et al.* (2012) studied the genetic diversity of taro accessions from Andaman Islands using morphological, RAPD and ISSR markers. AFLP markers were used by Krieke *et al.* (2004); Quero-Garcia *et al.* (2004); Lebot *et al.* (2004); Caillon *et al.* (2006), to study the genetic diversity of taro collected from different places.

Due to the co-dominant nature, transferability, reproducibility and amenability to high throughput, SSR marker has evolved to be the marker of

choice in taro too (Devi, 2012). Mace and Godwin (2002) identified 16 polymorphic SSR markers and screened them on a restricted set of taro genotypes from Southeast Asia and Oceania, which resulted in the detection of 3.2 alleles on each locus. Out of these markers identified, Godwin *et al.* (2003) used 7 primers to evaluate the genetic diversity of 511 taro accessions and subsequently rationalize ten national collections from the Pacific Island Countries. This work resulted in the amplification of 38 alleles from the 7 SSR loci. The microsatellite enriched library constructed by Bastide (2000) was used by Noyer *et al.* (2004) to analyze a subset of samples earlier characterized by Krieke *et al.* (2004). A differentiation between the Southeast Asian and Melanesian taros was observed in this study, which confirmed the AFLP and isozyme results of Krieke *et al.* (2004). A regional core collection was developed by Mace *et al.* (2006) based on phenotypic and molecular characterization from germplasm collected by TaroGen (Taro Genetic Resources: Conservation and Utilization) from 10 countries in Oceania viz., Papua New Guinea, Solomon Islands, Vanuatu, New Caledonia, Fiji, Palau, Nue, Tonga, Cook Islands and Samoa. SSR markers was used to genotype 515 accessions. The DNA fingerprint data showed that great allelic diversity existed in Papua New Guinea and Solomon Islands. Rare alleles of taro were identified from Solomon Islands, which were not detected in any other collections. Singh *et al.* (2008) collected 859 taro accessions from 15 provinces of Papua New Guinea for characterization using 7 SSR primers and established a core collection of 81 accessions on the basis of this characterization data. Kan *et al.* (2009) isolated 11 microsatellite markers from taro and from the result, it was found to have polymorphism on 30 individuals from a taro population in China. Zhonglei *et al.* (2011) developed 19 novel microsatellite markers from taro germplasm collected from the Yunan Province, South-Western China, which was found to be useful in studying the taro germplasm management and population evolution in South-Western China. Sardos *et al.* (2012) collected a sample of 344 taro landraces, which

was referred to as the National Sample from Vanuatu, in South-West Pacific. The genetic diversity of these samples were analyzed using 9 microsatellite markers and was compared with an International Core Sample (ICS). Based on this study, hypothesis regarding the genetic diversification of taro in Vanuatu was formulated. Microsatellite marker and molecular dataset revealed that these were effective tools to monitor the diversity and evolution of taro in the future. Nunes *et al.* (2012) investigated the genetic diversity of Brazilian taro cultivars from 7 regional core collections by using 7 microsatellite loci. Molecular data demonstrated the primitiveness of the clones cultivated in Brazil and also showed that SSR markers could be used for allelic identification.

2.8. *IN VITRO* CONSERVATION OF TARO

The increased threat of extinction of indigenous plant species has prompted public and various institutions into conservation of these plant species. Conservation should be in such a manner that the plant materials are not lost, yet available for crop improvement programs. Conservation can be *in situ* and *ex situ* of which, the *ex situ* type of conservation is adopted for plants like yam, taro, cassava, sweet potato, etc. which are propagated mainly by vegetative means. Although field genebanks provide easy access, they face the risk of natural calamities, pest and disease. Another problem for field genebanks is the non-availability of large area of land for planting these materials and the high labor cost involved. Advances in the field of biotechnology has provided us with an alternate *in vitro* conservation method through tissue culture techniques. *In vitro* methods usually include slow growth conservation and cryopreservation. Slow growth technique is mainly used for short term and medium term conservation, whereas, for long term conservation, cryopreservation is the main method adopted. (Rajasekharan and Sahijram, 2015). Cryopreservation was successfully adopted by Sant *et al.* (2006) for preserving *in vitro* grown shoot tips of tropical taro.

2.8.1. *In vitro* conservation

Vegetatively propagated crops including roots and tuber crops like cassava, taro, yams and other aroids are conserved in the form of clones. *In vitro* culture, originated as a means of rapid clonal propagation, using nutritional and environmental conditions conducive to rapid growth. In *in vitro* maintenance, the cultures need to be regularly transferred to fresh medium after a certain number of days. The transfer may cause infection from foreign microbes and thus loss of culture. To prevent this to a certain extent, the whole procedure should be carried out in a sterile atmosphere. *In vitro* cultures could also be termed as laboratory version of field genebanks (Withers, 1991).

Storage could be made possible by altering the culture conditions. By reducing the culture temperatures from 20-25°C (in the case of temperate plants) and 25-30°C (in case of tropical plants) to 6-10°C (for temperate plants) and 15-25°C (for tropical plants), slow growth could be attained and the period of sub culture could be extended up to 1-2 years (Banerjee and De Langhe, 1985; Roca, 1985). The main disadvantage of using reduced temperature for storage include the need for additional controlled environment. The alternative to this problem was found out by modifying the culture medium for conservation. Additives like osmotic inhibitors (mannitol and sorbitol) or hormonal inhibitors (abscisic acid) could be used to modify the culture medium (Withers, 1987). Medium and long term conservation were previously researched in crops like cassava (Ng and Ng, 2002), sweet potato (IITA, 1980; Ng and Hahne, 1985), yams (Malaurie *et al.*, 1998; Borges *et al.*, 2004; Keller *et al.*, 2006) and aroids like taro (Staritsky *et al.*, 1986; Bessembinder *et al.*, 1993).

2.8.2. *In vitro* conservation in taro

Micropropagation systems in taro has also been developed that can be used for a wide range of cultivars (Mandal *et al.*, 2000; Chand *et al.*, 1999).

The feasibility of *in vitro* storage with minimum growth condition is reported by Arditti and Strauss (1979). The addition of osmotic agents such as sucrose, mannitol and sorbitol could reduce the growth effectively and extend storage life of many vegetatively propagated crops (Krahu, 1997; Shibli, 1990; Shibli *et al.,* 1992). A few reports are also available on *in vitro* conservation of taro at low temperatures (at 9^0C and 4^0C) (Bessembinder *et al.*, 1993; Zhou *et al.*, 1999).

The storage of plantlets under growth retarding conditions is mostly suitable for active collections (Withers, 1980). Marau and Maria (1993) reported that for taro, slow growth could be attained with the use of mannitol. Bessembinder *et al.* (1993) reported that taro could be stored for more than 8 years at 9°C having subculture interval of 3 years. Staritsky *et al.* (1986) also reported that taro could be stored for 3 years at 9°C and could still be viable. A research that was carried out in USP, Samoa, demonstrated that the most practical method for slowing down growth is temperature reduction. By altering temperature to 20°C, taro could be maintained for 9-12 months, but the time period should be altered according to the variety. The research also investigated upon other parameters including reduced light and supplementing the culture with osmoticum. This study showed that the inclusion of mannitol did suppress the growth, but also resulted in some morphological changes in the plant. When the plants were transferred to field, some phytotoxic effect was observed (Taylor *et al.,* 2003). As part of the TaroGen project, a pilot study was carried out, for an *in vitro* genebank using temperature reduction to inhibit growth rate. The overall objective of the study was to assess and demonstrate the technical and logistical aspects of establishing and operating *in vitro* active genebank using taro as a model plant (Taylor *et al.,* 2003). Song *et al.* (1994) reported that green stem taro (*Colocasia esculenta* Schott var. *carmosa* Chang) a common variety of China, was successfully conserved in MS medium containing 0.2 mg/l NAA and 4 mg/l BA with the addition of 20 g/l mannitol and 2 mg/l ABA under 1000 lux light. Unnikrishnan and Sheela

(2000) reported that taro could be stored for a period of 10 months under a light intensity of 3000 lux on MS basal medium containing sucrose and 3% mannitol without vitamins or plant growth regulators.

In this study, we have undertaken to assess the genetic diversity of 36 taro accessions including germplasm, variety and breeding lines using ten SSR markers. These diverse collections were then transferred to slow growth media for conservation purpose. Here, apart from the routine conservation protocol, we tried to incorporate a multiple shoot induction step also, so that even rare genotypes/mutants if any available in the genebank, can be multiplied first before being conserved in the slow growth media.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The study entitled "Molecular characterization and *in vitro* conservation of taro (*Colocasia esculenta* (L.) Schott)" was carried out at the Division of Crop Improvement, ICAR–Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram during 2016-2017. The details regarding the experimental materials and methods used for the study are elucidated here.

3.1. GERMPLASM COLLECTION

The plant material for molecular characterization comprised of 36 accessions of *Colocasia esculenta* (L.) Schott collected from the germplasm maintained at the field genebank of ICAR – CTCRI, Sreekariyam.

For the *in vitro* conservation part, the corms/cormels of the accessions taken for molecular characterization were collected from the germplasm collection at ICAR – CTCRI, Sreekariyam.

Table 1. Geographical locations of the taro accessions

Sl. No.	Accession No. / Variety name	Germplasm type	Place of collection
1.	C-96	Germplasm	Unknown
2.	C-557	Germplasm	NEH region
3.	JAS 7	Germplasm	Wayanadu, Kerala
4.	NEH 8	Germplasm	West Garo, Meghalaya
5.	CAU CO1	Germplasm	Imphal, Manipur
6.	C-622	Germplasm	Unknown
7.	DT 1	Germplasm	Andaman and Nicobar Islands
8.	IC012601 (L 14)	Germplasm	Unknown
9.	NEH 21	Germplasm	Imphal East, Manipur

10.	NEH 32	Germplasm	Senapati, Manipur
11.	NEH 109	Germplasm	West Siang, Arunachal Pradesh
12.	Line 4	Breeding line	-
13.	Line 18	Breeding line	-
14.	Line 38	Breeding line	-
15.	Line 48	Breeding line	-
16.	NEH 117	Germplasm	Dimapur, Nagaland
17.	Bhu Kripa	Variety	Cuttak, Orissa
18.	C-84	Germplasm	Unknown
19.	C-89	Germplasm	Unknown
20.	NEH 22	Germplasm	Imphal East, Manipur
21.	C-621	Germplasm	Barapani, Meghalaya
22.	C-717	Germplasm	Jowai, Meghalaya
23.	*Colocasia* Nicobar	Germplasm	Andaman and Nicobar Islands
24.	Line 29	Breeding line	-
25.	Line 33	Breeding line	-
26.	Line 43	Breeding line	-
27.	Mukthakeshi	Variety	Cuttak, Orissa
28.	NEH 34	Germplasm	Zunheboto, Nagaland
29.	NEH 77	Germplasm	Wokha, Nagaland
30.	Line 8	Breeding line	-
31.	Line 11	Breeding line	-
32.	Line 14	Breeding line	-
33.	Line 42	Breeding line	-
34.	C-322	Germplasm	Cuttak , Orissa
35.	C-556	Germplasm	Unknown
36.	Bhu Sree	Variety	Orissa

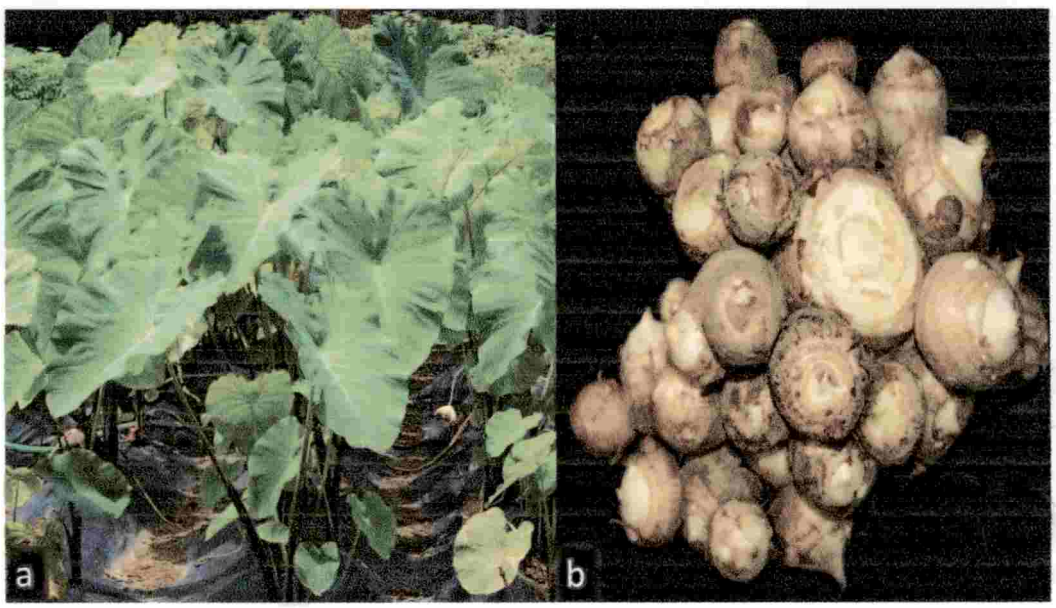

Plate 1: Taro plants and tubers

(a) Taro plant

(b) Taro tubers

3.2. MOLECULAR CHARACTERIZATION

In order to study the genetic variability among the 36 taro accessions, molecular characterization was carried out using SSR markers.

3.2.1. DNA ISOLATION PROTOCOL

The presence of polyphenol and mucilage in the DNA isolated from taro leaves mostly hinders the quality. In order to overcome these difficulties, four different protocols were tried, of which, each are listed below:

a. DNA isolation using DNeasy Plant Mini Kit (Qiagen) method

100 mg of fresh leaf sample was taken and ground using a mortar and pestle. 400µl of buffer AP1 was added to the ground sample and was further transferred to 2ml tubes. To these tubes 4µl RNase was added, vortexed and was incubated at 65°C for 10 minutes. Tubes were inverted 2-3 times during incubation. The lysate was centrifuged at 12,000 rpm for 5 minutes at 15-25°C. The lysate was pipetted into a QIA shredder spin column placed on a 2ml collection tube and was centrifuged at 14,000 rpm for 2 minutes at 15-25°C. The flow-through was transferred into a new tube without disturbing the pellet, if present. 1.5 volumes of buffer AW1 was added and was mixed by pipetting. 650µl of the mixture was transferred into a DNeasy Mini spin column placed in a 2ml collection tube and was centrifuged at 8,000 rpm for 1 minute. The flow-through was discarded and the same step was repeated with the remaining sample. The spin column was placed into a new collection tube and 500µl of buffer AW2 was added. The mixture was centrifuged at 8000 rpm and the flow through was discarded. Another 500µl of buffer AW2 was added to the tube and was centrifuged at 14,000 rpm for 2 minutes. The spin column was transferred to a new 1.5ml or 2ml centrifuge tube. 100µl of buffer AE was added for elution. The mixture was incubated at room temperature for 5 minutes (15-25°C). The mixture was centrifuged for 1 minute at 8,000 rpm. This step was repeated again to obtain the maximum yield of DNA.

b. CTAB method I

CTAB extraction method (Sharma *et al.*, 2008) was used to obtain good quality DNA.

Young leaf tissues were collected in the early hours of the morning and was kept in a moist condition. Extraction chemicals were prepared as shown in the appendix I.

300 mg of the leaf sample was weighed and was ground into a fine powder form in a mortar and pestle using liquid nitrogen. 1ml of pre-warmed extraction buffer was added to the powder and was ground again into a paste form. All these mixtures were transferred to 2ml Eppendorf tubes and 5µl proteinase K (10 mg/ml) was added. The tubes were incubated at 37°C for 30 minutes followed by incubation at 65°C for another 30 minutes with intermittent mixing. After the incubation, the tubes where centrifuged at 8,000g for 10 minutes at room temperature and the resultant supernatant was transferred into another fresh 2ml Eppendorf tube. To these tubes, equal volumes of phenol:chloroform:isoamyl alcohol (25:24:1) was added and was mixed by gentle inversion for 30-40 times. Again the samples were incubated at 8000g for 10 minutes at room temperature. The supernatant was once again transferred to fresh 2ml tubes. To these tubes 200µl of 2M NaCl solution containing 4% PEG was added and was incubated at 4°C. This step helps in increased recovery of DNA yield. The samples were further centrifuged at 8,000g for 10 minutes at room temperature. The supernatant was transferred to a fresh tube and was precipitated by adding 2/3rd volume of ice cold isopropanol. The nucleic acid was precipitated and was washed with wash solution. The pellet was air dried to remove the ethanol content and was dissolved in 100µlof TE buffer. The nucleic acid was further treated with ribonucleases, incubated at 37°C for 30 minutes and was stored at -20°C for future use.

c. CTAB method II

CTAB extraction method by Vinutha (2014) was done.

300mg of freshly collected leaves were ground into a fine powder using liquid nitrogen in an autoclaved mortar and pestle. 2ml of the extraction buffer (Appendix I) was added to the powder before it gets thawed. The mixture was added to 2ml Eppendorf tubes and was mixed thoroughly. 5µl of RNAse was added and was mixed properly. The tubes were incubated at 37°C for 1 hour with intermittent mixing. Again the tubes were kept for incubation at 65°C in a water bath with frequent mixing for 30 min. The homogenates were further centrifuged at 10,000 rpm for 10 min at room temperature. The supernatant was collected and to it equal volume of chloroform:isoamyl alcohol (24:1) was added. The tubes were mixed by inversion for 30-40 times and was centrifuged at 10,000 rpm for 10 min at room temperature. The upper aqueous layer was transferred to 1.5ml Eppendorf tubes and to it 2/3rd volume of ice-cold isopropanol was added. The tubes were mixed gently until the DNA was observed as a thread. The DNA was precipitated by centrifuging at 10,000 rpm for 5 min. The precipitated DNA was washed with 70% ethanol and was kept to air dry to remove the ethanol content. The DNA was dissolved in 100µl TE buffer and was stored in -20°C for further use.

d. CTAB Method III

Sharma *et al.* (2008) was modified slightly to obtain good quality DNA.

160mg of fresh leaf sample was weighed and was ground into a powdered form using liquid nitrogen in an autoclaved mortar and pestle. 2ml of freshly prepared extraction buffer (Appendix III) was added to the powdered sample before it got thawed and the contents were homogenized. The mixture was transferred into 2ml Eppendorf tubes. 5µl of proteinase K (10mg/ml) was added to the tubes and was incubated at 37°C with intermittent mixing for 30 min. The tubes were again incubated at 65°C water bath for

another 30 min with intermittent mixing. The tubes were centrifuged at 12,000 rpm for 15 minutes and the supernatant was transferred to fresh 2ml Eppendorf tubes. To these tubes, 5µl of RNase (10mg/ml) was added and was incubated for 1 hour at 37°C. After incubation, equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the tubes and was mixed by thorough inversion. The tubes were allowed to stand at room temperature for 5 minutes to allow the phase separation. The tubes were further centrifuged at 12,000 rpm for 15 minutes at room temperature. The upper aqueous phase was transferred to fresh 2ml Eppendorf tubes using cut tips. To this, equal volume of chloroform:isoamyl alcohol (24:1) was added and was mixed by gentle thorough inversion. The tubes were centrifuged at 12,000 rpm for 15 minutes at room temperature. The resultant upper aqueous phase was again transferred to fresh 1.5ml Eppendorf tubes. To this, equal volume of isopropanol was added and was mixed gently till DNA threads were obtained. These threads were precipitated by centrifugation at 10,000 rpm for 10 min. The precipitated DNA was washed using 70% ethanol 2-3 times. The pellets were air dried to remove the traces of ethanol and the pellet was dissolved in 100µl TE buffer. The DNA was stored in -20°C freezer for further use.

3.2.2. ANALYSIS OF THE EXTRACTED DNA

The DNA extracted was analyzed to check the quality and quantity using electrophoresis and spectrophotometric measurements for further studies.

a. Agarose gel electrophoresis

The isolated DNA was checked on 1% agarose gel electrophoresis. 1g of agarose (Sigma Aldrich) was weighed and was dissolved in 100ml of 1X TBE buffer by boiling. Once the agarose dissolves and the solution attains a bearable temperature, 1µl of ethidium bromide was added. After gentle and

thorough mixing of the ethidium bromide by gentle rotation of the flask the gel was poured on to the casting tray and the comb was placed appropriately. The gel was allowed to solidify for 20 – 30 minutes. When the gel has solidified, it was placed on to the electrophoresis tank, in a manner where the wells are in the vicinity of the cathode. 1X TBE running buffer was poured on to the tank so that the wells were properly submerged. The comb was removed carefully without disrupting the wells.

2µl of 6X loading dye and 3µl of DNA was mixed thoroughly using a micropipette. 5µl of the mixture was carefully added to each of the wells. When all the samples were loaded, the tray was closed properly, and the voltage of 80V was given. The gel was allowed to run for 15 – 20 min, till the dye front almost reached 2/3rd of the total gel length and the image was taken using the gel documentation system (G: Box Gel documentation system M/s. Syngene).

b. Quantification of the DNA

The quality and quantity of the isolated DNA was assessed by measuring the absorbance of the DNA at 260nm and 280nm using a Nano spectrophotometer (NANODROP® ND-1000). The software was switched on by clicking on the icon. From the "user" folder for storage of data, the "nucleic acid" button was selected. The Module startup panel came up on the screen. For the first step, the pedestals were cleaned and a water sample was loaded in order to initialize the instrument. In order to prepare a report of all readings, the "recording" button was pressed. The report can log either 12 or 32 measurements. Before doing "DNA", "RNA" or other analysis, the "blank" measurement was chosen. A blank must be measured and stored before measuring the samples. TE buffer was used as blank. 1µl of the TE buffer was placed on the pedestal and pressed "blank". The pedestal was cleaned using kim wipes. A fresh sample was placed on the pedestal and the concentration was measured by clicking the "measure" icon. The concentration of the DNA,

A260 value, A_{260}/A_{280} ratio was displayed by the instrument. These measurements could be used to check the quality and quantity of the DNA. "exit" was pressed to exit the instrument.

The concentration of the DNA present in the sample was calculated by the following formula:

$$concentration\ (\mu g\ ml^{-1}) = absorbance\ at\ 260nm \times 50 \times dilution$$

Where, the value 50 gives the concentration of DNA in µg/ml of unit absorbance;

Dilution factor = total volume / volume of sample taken

The purity was determined by measuring the ratio of OD at 260nm to that of OD at 280nm.

3.2.3. PCR STANDARDIZATION

A few taro DNA samples were taken to standardize the PCR conditions. The composition of the reaction mixture was as follows:

10X Taq buffer (with 15 mM $MgCl_2$)	: 2µl
$MgCl_2$	: 0.8 µl
dNTPs (10 mM each)	: 0.6µl
Forward primer (10 µM)	: 0.5 µl
Reverse primer (10 µM)	: 0.5 µl
Template DNA (10 ng μl^{-1})	: 4 µl
Taq polymerase (5 U μl^{-1})	: 0.2 µl
Nuclease free water	: 11.4 µl

Total volume : 20 µl

PCR was carried out in Proflex (ABI Biosystems) thermal cycler with the program of an initial denaturation at 94°C for 5 min followed by 35 cycles with denaturation at 94°C for 30 s, annealing at 58-68°C for 2 min and extension at 72°C for 2 min. The final extension was done at 72°C for 10 min followed by infinite hold at 4°C.

The products were resolved using 2% agarose gel using 100bp DNA ladder for checking the amplification and was visualized using a gel documentation system (G: Box Gel documentation system M/s. Syngene).

3.2.4. PRIMER SCREENING

Two SSR primer series namely Uq (Mace and Godwin, 2002) and Ce1 (Noyer *et al.*, 2004) series were selected for screening:

Uq series : Uq 84 – 207, Uq 110 – 283, Uq 88B – 94, Uq 97 – 256, Uq 201 – 302, Uq 115 – 71, Uq 132 – 147 and Uq 73 - 164

Ce1 series : Ce1 F12, Ce1 F04, Ce1 B12, Ce1 D12, Ce1 H12, Ce1 B03

Gradient PCR for T_a optimization

According to the data obtained from the preliminary screening, standardization of the annealing temperatures using gradient PCR for the selected primer series were found necessary. Gradient PCR was carried out for the primer series Uq with a temperature range 60 - 68°C to obtain the most accurate annealing temperature giving specific bands with minimum errors.

3.2.5.SSR ANALYSIS ON TARO

After screening, 10 primers, given below, were selected for characterization based on polymorphism and quality of electrophoretic

patterns. SSR analysis was done for the selected 36 accessions of taro with the optimized annealing temperature. The amplicons were resolved using 2% agarose gel to check the amplification.

Table 2. List of primers selected for molecular characterization

Sl. No.	SSR ID	Primer sequence	Repeat type	Expected product size
1	Uq 84-207	F-CCCATTGGAGAGATAGAGAGAC R-AGGACAAAATAGCATCAGCAC	$(CT)_{18}$	207
2	Uq 110-283	F-GCCCAGTATATCTTGCATCTCC R-AGCCACGACACTCAACTATC	$(TGA)_6$ $(TGGA)_4$	283
3	Uq 88B-94	F-CACACATACCCACATACACG R-CCAGGCTCTAATGATGATGATG	$(CAT)_9$	94
4	Uq 97-256	F-GTAATCTATTCAACCCCCCTTC R-TCAACCTTCTCCATCAGTCC	$(CA)_8$	256
5	Uq 201-302	F-CTAAGGAGAGGAGATCCGAAC R-CAAGACGATGCTGAACCAC	$(C)_{15}$	283
6	Uq 115-71	F-CCCCTCTTTTGTAATAATCC R-GTTTAAATGACTTGTTCTGC	$(T)_6$ A $(G)_6(A)_{12}$	71
7	Uq 132-147	F-ACCCCGAAAAAGCCAATG R-CTATCACTTGTTCCTCCTTCTC	$(TTTGAA)_4$	147
8	Uq 73-164	F-CGTCTAGCTTAGGACAACATGC R-ATGCCAATGGAGGATGGCAG	$(CT)_{15}$	164
9	Ce1F12	F-GATGCCTGTCCTTATGTTT R-CTTAGCTTGTTCCCTAC	$(TC)_{16}$ TT $(TC)_{10}$	162
10	Ce1F04	F-ACGAGGGAAGAGTGTAAA R-AGGGAATACAATGGCTC	$(CT)_{29}$	142

3.2.6. PCR PRODUCT DETECTION

a. Polyacrylamide gel electrophoresis

7 M urea denaturing PAGE (6% polyacrylamide) was performed using BioradSequi – Gen® Cell (38 X 50 cm) with heat denatured sample and detected by silver staining.

The outer glass plate was cleaned using labolene and was washed thoroughly using distilled water. The integral plate chamber (IPC) was cleaned with distilled water. Further, both IPC and glass plate was wiped clean with

absolute alcohol using kim wipes. The outer glass plate was coated with a thin film of bind silane, uniformly applied throughout the plate. The IPC was coated with a thin layer of repellant, Labolene, uniformly throughout the plate. The spacers, comb, caster base and side clamps were cleaned with alcohol. Spacers were kept uniformly on both the edges of the IPC. The outer glass plate was kept on top of the spacer in such a manner that the coated side faces down, towards the spacer and the un-coated side faces outwards. The unit were kept proper and was made to stand vertically. Both the side clamps were attached accordingly to both the sides and was secured using the lever attached to the side clamp. The assembly was further placed in the caster base and was locked by locking the cam pegs. The entire unit was laid upside down on the bench top, such that the drain port faces the user.

6% acrylamide solution was prepared as given in appendix VII and was injected into the injection port in the caster base. As the gel gets to the opposite end of the plate, the comb was inserted in between the plates. The gel was allowed to polymerize for 15-30 min.

The IPC assembly was removed from the caster base and was fit into the universal base using stabilizing bar. The buffer chambers (upper and lower) were filled with 1X TBE running buffer. The top and bottom safety covers were positioned. The gel was kept for a pre-run for 20 min at 100W. After the pre-run, the power supply was stopped, the top safety cover was removed and the wells were thoroughly rinsed by pipetting to remove urea deposition in them. 6µl of the denatured sample was loaded into the wells along with 100bp DNA ladder. The safety cover was kept back and power supply was provided, electrophoresis was done at 100W for 35-40 minutes. After completion of the process, the power was turned off, the safety cover was removed, running buffer was drained partially from the upper chamber through the drain port on the IPC unit and the IPC assembly was removed from the universal base. The rest of the running buffer in the upper chamber

was removed by inverting. The side clamps were removed from the IPC assembly and the outer glass plate was removed carefully from the IPC unit.

The glass plate was transferred to a large trough kept on a shaker containing fixer (Appendix IX). The plate was kept shaking for 20 min. After fixation, the plate was kept for shaking in distilled water for washing. The plate was kept in water until the oily appearance from the plate was removed. After the wash, the plate was kept in Silver stain (Appendix IX) for 20 min. Subsequent to staining, the plate was given a quick wash and was kept for developing in a developer solution (Appendix IX). The plates were gently rocked to and fro until the bands were visualized. Immediately after visualization, the plate was kept in fixer for 15 minutes. After fixation, the plates were washed and the gel was allowed to dry.

When the gel gets dried, the plate was scanned using a scanner attached to a computer. The image obtained was labeled properly and was saved.

b. Agarose gel electrophoresis

The products of the PCR done with the selected 10 primers were analyzed using 4% agarose gel. 4 g agarose (Sigma Aldrich) was weighed and was dissolved in 100ml 1X TBE buffer. The mixture was kept undisturbed for the agarose to spread uniformly throughout the buffer. It was then heated in the oven to dissolve the agarose completely. The melted agarose was allowed to cool to a hand bearable temperature. 1µl EtBr was added to the mixture and was mixed thoroughly and cautiously without any bubble formation. The agarose was poured onto the casting tray, comb was placed and was allowed to set. 8µl 6X loading dye was added to the PCR product and was allowed to mix properly. When the gel was finally solidified, it was transferred to the electrophoresis tank. 1X TBE running buffer was poured onto the gel in such a manner that the wells get immersed. The comb was removed carefully without

disrupting the wells. 12µl of the mixture (PCR product + 6X loading dye) was added carefully to appropriate wells. 5µl of 100bp DNA ladder (M/s. Origin) was added for comparison purpose. Electrodes were connected and electrophoresis was done at 150V for 30 min. The gel image was taken using the gel documentation system (G: Box Gel documentation system M/s. Syngene).

3.2.6. ANALYSIS OF MOLECULAR DATA

Various parameters are estimated to quantify the genetic variability of 36 accessions using 10 SSR primers.

a. Band scoring

The clear and reproducible images of the resolved PCR products were taken for scoring. Scoring was done by assigning the value "1" for the presence of band and the value "0" for the absence of the band. This data was entered in Excel and was used as the input for the statistical analysis.

b. Heterozygosity

Heterozygosity (H) is a method to measure the degree of polymorphism. Its unbiased estimator and formula are well known (Nei and Roychoudhury, 1974). Heterozygosity is defined as the probability that a random population is heterozygous at a locus and is given in a randomly mating population by

$$H = 1 - \sum_i p_i^2$$

Where, p is the frequency of the i^{th} allele in the population.

c. Polymorphism information content

Polymorphism information content (PIC) is another measure of polymorphism (Botstein *et al.,* 1980) commonly used as a measure of polymorphism for a codominant marker locus used in linkage analysis

$$PIC = 1 - \sum_i p_i^2 - \sum_{i,j} p_i^2 p_j^2$$

Where, p is the frequency of the allele in a population.

d. Shannon's diversity index

Shannon's index (H_i) (Shannon and Weaver, 1949) is calculated as

$$H_i = -\sum_{i=1}^{n} p_i \ln p_i$$

Where, p is the frequency of the presence or absence of a band in a locus for all the individuals composing a group.

e. Average no. of alleles per locus

The average no. of alleles per locus is calculated as

$$n = (\frac{1}{K}) \sum n_i$$

Where,

n_i is the no. alleles per locus

p is the no. of loci

f. Cluster analysis

Clustering is a technique for grouping individuals into unknown groups to assess the relationship between the groups. In diversity studies, cluster analysis has been used to classify breeds or strains into groups on the basis of their genetic characteristics.

Apart from R package, NTSYS pc was also used to generate similarity matrix as well as cluster. The binary data were scored and statistically analyzed using NTSYS pc Ver.2.2. Pair-wise distance (similarity) matrices was computed using sequential, agglomerative, hierarchical and nested (SAHN) clustering

option of the NTSYS-PC (Numerical Taxonomy and Multivariate Analysis System, Biostatistic, New York, U.S.A., Software Version 2.02 package (Rohlf, 1998). The program also generated a dendrogram, which grouped the accessions on the basis of Nei genetic distance (Nei, 1979) using unweighted pair group method with arithmetic average (UPGMA) cluster analysis (Sneath and Sokal, 1973).

g. Principal component analysis

R – package was used for PCA. Based on the molecular scoring data 2D and 3D plots were obtained.

h. SM matrix

Sample matching matrix were drawn to understand the similarity between 2 accessions. The matrix was drawn using NTSys-PC. The similarity between 2 accessions were represented as a number less than 1.0.

3.2. *IN VITRO* CONSERVATION

The 36 taro accessions selected were kept for *in vitro* conservation in half and full strength MS media using sprouts from tubers as the explant.

3.2.1. MEDIA PREPARATION

The media composition is given in the appendix X. The components were mixed thoroughly and the pH was checked before adding agar. The pH was adjusted to be between 5.6 and 5.8. Agar was added to the medium and the medium was heated to dissolve the agar. The medium was poured in to sterile test tubes and was plugged using sterile cotton plugs. The media was stored for 2 days to check for contamination and was then used for inoculation.

3.2.2. STERILIZATION OF MEDIA, GLASSWARES AND INSTRUMENTS

Media was sterilized in an autoclave for 15 – 20 min at a temperature of 121°C and a pressure of 15 psi. Glasswares were sterilized by autoclaving them at a temperature of 121°C and a pressure of 15 psi for 20-40 minutes. Instruments like laminar air flow cabinet should be sterilized by wiping with alcohol and by using UV light. Before work, forceps, blades, petri plates, etc. should be wiped with ethanol and flame sterilized.

3.2.3. PREPARATION OF EXPLANT

Explant used for culture is sprouts from tubers. Cormels were used to get the sprouts. Cormels were cleaned thoroughly in running tap water using a soft brush so as not to cause any physical damage to the cormels and were kept in a moist atmosphere to allow it to sprout. These sprouts were taken as explants for tissue culture. The sprouts were removed carefully using a scalpel, without disturbing the meristem. They were rinsed properly with water. These sprouts were transferred to a solution of bavistin (1%) and was shaken well for 25 min to remove any fungal contaminants. The excess bavistin was removed by rinsing with water 4 – 5 times. Further, the explants were kept in a solution of labolene (1%) for 10 min with constant shaking, to remove any adhering dirt or fungal spores from its surface. The excess labolene was removed by rinsing with water till the foam disappears and the water becomes clear. The explant was further cleaned with distilled water. It was then transferred to the laminar air flow cabinet, which was previously UV sterilized. In the sterile laminar air flow, the explants were transferred to a solution of $HgCl_2$ (0.1%) for surface sterilization for an interval of 5 min. $HgCl_2$ is toxic to the plants as well as humans. Therefore, complete care has to be taken while dealing with this chemical. The traces of the chemical have to be removed completely by repeated washing with double distilled water. The explants thus treated were ready for inoculation.

Table 3: Sterilization conditions used for standardization

Variety	Bavistin (1%)	Labolene (1%)	$HgCl_2$ (0.1%)	Total tubes inoculated
CAUCO1	15	8	4	8
	20	10	6	7
	25	10	5	14
	30	10	5	14
Line14	20	10	5	7
	25	10	5	8
	30	10	5	9
Line 18	20	10	5	19
	25	10	5	18
	30	10	5	4
Line 33	20	10	5	6
	25	10	5	7
	30	10	5	3

3.2.4. CULTURE ESTABLISHMENT

The explant was inoculated on to basal MS media to establish initial growth. The explant was taken using a sterile forceps and the tube mouth was flame sterilized to remove any microbes, if present. The explant was inoculated in to the media carefully using the forceps, at the center of the tube. The tubes were again plugged using cotton plugs and were transferred to the culture room.

The condition inside the culture room was maintained at 25±1°C under 16/8 h photoperiod and at 70 – 80% humidity and 3000 lux light intensity provided by cool white fluorescent tubes (Phillips).

3.2.5. SHOOT MULTIPLICATION

When the shoot has reached a 2 leaved stage, which will be after 20 – 30 days, they were inoculated onto media containing hormones which favors shoot multiplication. Various shoot multiplication media were tried in MS medium fortified with various plant growth regulators (PGR) viz., BA (1 – 5mg/l); Kinetin (1mg/l) and TDZ (0.1 – 1mg/l). The details of the combinations and concentrations tried are given in the table 4. The method of preparation of these PGRs are given in appendix XI. The hormones break the apical dormancy and induces lateral dormancy thus producing multiple shoots.

The shoots in the initial establishment medium was transferred to test tubes containing shoot multiplication media using sterile forceps and by excising the root and the tip of the shoot which has grown from the shoot tip. The tubes were plugged with cotton plugs near the flame. Number of multiple shoots initiated after 20 days was recorded and it was subjected to CRD analysis using SAS software for finding out the best media combination and the most responsive genotype for the induction of shoot multiplication.

Table 4: Standardization of multiplication media

Media	Accession	Replications
MS+B (1mg/l)	CAU CO1	5
	Line14	3
	Line18	3
	Line33	3
	Line42	3
MS+K (1mg/l)	CAU CO1	5
	Line14	3
	Line18	3
	Line33	3
	Line42	3

MS+TDZ (0.1mg/l)	CAU CO1	5
	Line14	3
	Line18	3
	Line33	3
	Line42	3
MS+TDZ (0.2mg/l)	CAU CO1	5
	Line14	3
	Line18	3
	Line33	3
	Line42	3

3.2.6. MAINTENANCE OF CULTURE IN SLOW GROWTH MEDIA

The multiple shoots obtained in the different shoot multiplication media were transferred to basal MS media after 20 days of inoculation for further elongation. To each tube, a single shoot was inoculated. When these shoots have achieved enough growth, it was further transferred to tubes containing slow growth media. The slow growth media included half strength and full strength MS medium containing 2% sucrose and 2% mannitol (Appendix XII), respectively.

The culture was transferred carefully in the laminar air flow with sterile forceps to tubes containing slow growth media. The tubes were plugged with cotton plug near the flame. The tubes were then transferred to culture room and observations were made at 15 days' interval.

RESULTS

4. RESULT

The result of the study titled "Molecular characterization and *in vitro* conservation of taro (*Colocasia esculenta* (L.) Schott)", which was carried out in the Division of Crop Improvement, ICAR – Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram during 2016-2017 are explained in this chapter.

4.1. MOLECULAR CHARACTERIZATION

The result of molecular characterization of the 36 taro accessions comprising germplasm, variety as well as breeding lines collected from the germplasm maintained at ICAR – CTCRI using ten SSR primers are explained in this section.

4.1.1. STANDARDIZATION OF DNA ISOLATION PROTOCOL

Four different methods were adopted to obtain DNA of good quality and quantity. For protocol standardization, 3 accessions namely, Mukthakeshi, Line-38 and C-322 were used. DNA isolation using DNeasy Plant Mini Kit method yielded sheared and poor quality DNA. The second method adopted was the method proposed by Vinutha (2014). Unsheared bands were obtained in this protocol but the quantity of the DNA was less. The spectrophotometric readings gave low values. The DNA isolation protocol proposed by Sharma *et al.* (2008) using CTAB yielded good quantity DNA but the bands obtained were sheared. This method was modified slightly by including extraction using 25:24:1 ratio of phenol:chloroform:isoamyl alcohol. This method yielded good quality, pure as well as unsheared DNA. The spectrophotometric readings showed higher quantity of DNA with a purity range of 2.06 – 2.09. The DNA profile of these 4 methods on 0.8% agarose are given in plate 1 and table 5 shows the corresponding spectrophotometric readings.

The modified CTAB method was used to isolate DNA from the complete set of 36 samples. The DNA extracted from these samples using the standardized method are represented in plates 1-4 and the corresponding spectrophotometric readings are given in the table 6.

Table 5. Quantity and purity of the DNA extracted through various methods

Method	Sample	Absorbance (A_{260} nm)	A_{260}/A_{280}	DNA yield (ng/µl)
DNeasy Plant Mini Kit method	Mukthakeshi	0.085	2.04	427
	Line-38	0.081	2.01	408
	C-322	0.084	2.02	419
CTAB method I (Vinutha, 2014)	Mukthakeshi	0.121	2.03	606
	Line-38	0.102	2.02	514
	C-322	0.135	2.00	676
CTAB method II (Sharma *et al.*, 2008)	Mukthakeshi	0.256	2.23	1281
	Line-38	0.274	2.18	1371
	C-322	0.192	2.02	959
CTAB method III (modified Sharma *et al.* (2008)	Mukthakeshi	0.420	2.09	2103
	Line-38	0.271	2.06	1356
	C-322	0.210	1.84	2103

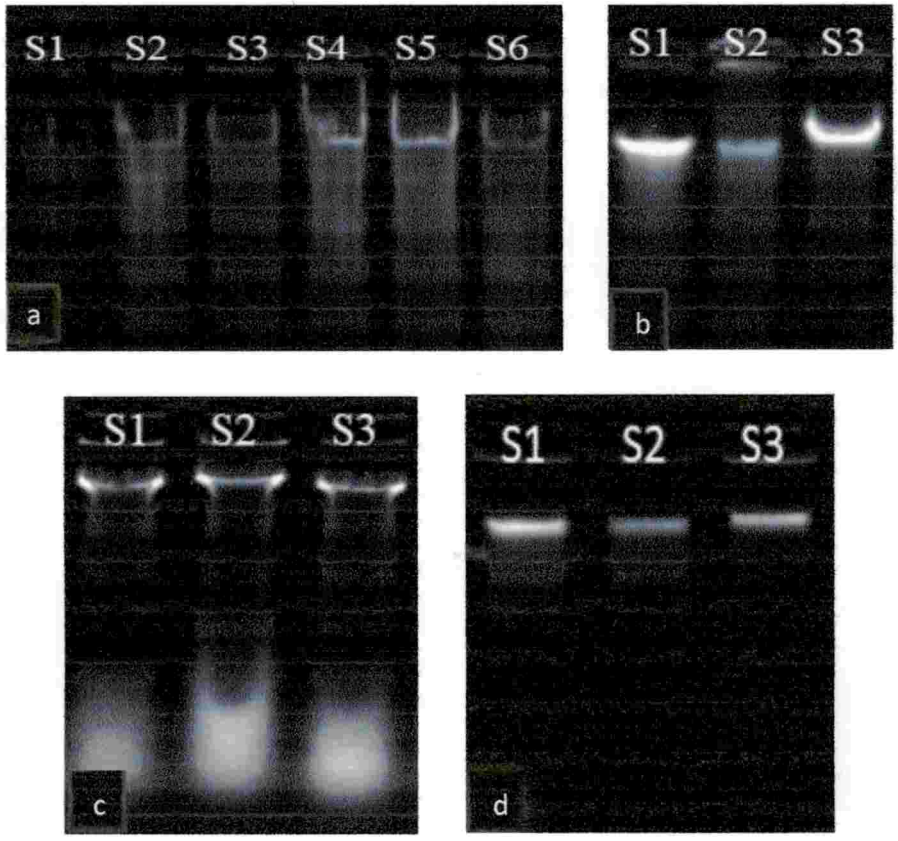

Plate 2: DNA isolated from various methods

(a) DNA isolated from DNeasy Plant Mini Kit method

S1 – Mukthakeshi 1st elute, S2 – Mukthakeshi 2nd Elute, S3 – Line 38 1st elute, S4 – Line 38 2nd elute, S5 – C-322 1st elute, S6 – C-322 2nd elute.

(b) DNA isolated using CTAB method I (Vinutha, 2014)

S1 – Mukthakeshi, S2 – Line 38, S3 – C-322

(c) DNA isolated using CTAB method II (Sharma *et al.,* 2008)

S1 – Mukthakeshi, S2 – Line 38, S3 – C-322

(d) DNA isolated using CTAB method III (modified Sharma *et al.* (2008))

S1 – Mukthakeshi, S2 – Line 38, S3 - C-322

Table 6. Quantity and purity of DNA from 36 taro accessions using modified CTAB method III (Modified Sharma *et al.*, 2008)

Sl. No.	Sample	Absorbance (A_{260} nm)	A_{260}/A_{280}	DNA yield ng/µl
1.	C-96	0.077	1.99	386
2.	C-557	0.081	2.12	407
3.	JAS 7	0.106	2.14	531
4.	NEH 8	0.076	2.12	381
5.	CAUCO1	0.073	2.06	367
6.	C-622	0.344	2.07	1722
7.	DT 1	0.457	1.96	2285
8.	IC012601	0.279	2.16	1398
9.	NEH 21	0.326	1.98	1630
10.	NEH 32	0.288	2.10	1440
11.	NEH 109	0.217	1.82	1084
12.	Line 4	0.080	1.96	799
13.	Line 18	0.119	2.13	1191
14.	Line 38	0.136	2.22	1357
15.	Line 48	0.088	1.89	877
16.	NEH 117	0.101	2.04	1010
17.	Bhu Kripa	0.078	2.00	679
18.	C-84	0.077	2.05	771
19.	C-89	0.176	2.09	1757
20.	NEH 22	0.180	2.03	1802
21.	C-621	0.066	2.00	660
22.	C-717	0.050	2.05	496
23.	*Colocasia* Nicobar	0.126	2.04	1256
24.	Line 29	0.082	1.80	818
25.	Line 33	0.110	2.13	1097
26.	Line 43	0.669	2.23	693
27.	Mukthakeshi	0.107	1.97	1065
28.	NEH 34	0.118	2.17	1180
29.	NEH 77	0.112	2.08	1124
30.	Line 8	0.122	2.12	1226
31.	Line 11	0.137	2.01	372
32.	Line 14	0.163	2.03	1329
33.	Line 42	0.072	1.97	717
34.	C-322	0.210	1.84	2013
35.	C-556	0.141	1.98	1408
36.	Bhu Sree	0.154	1.99	1544

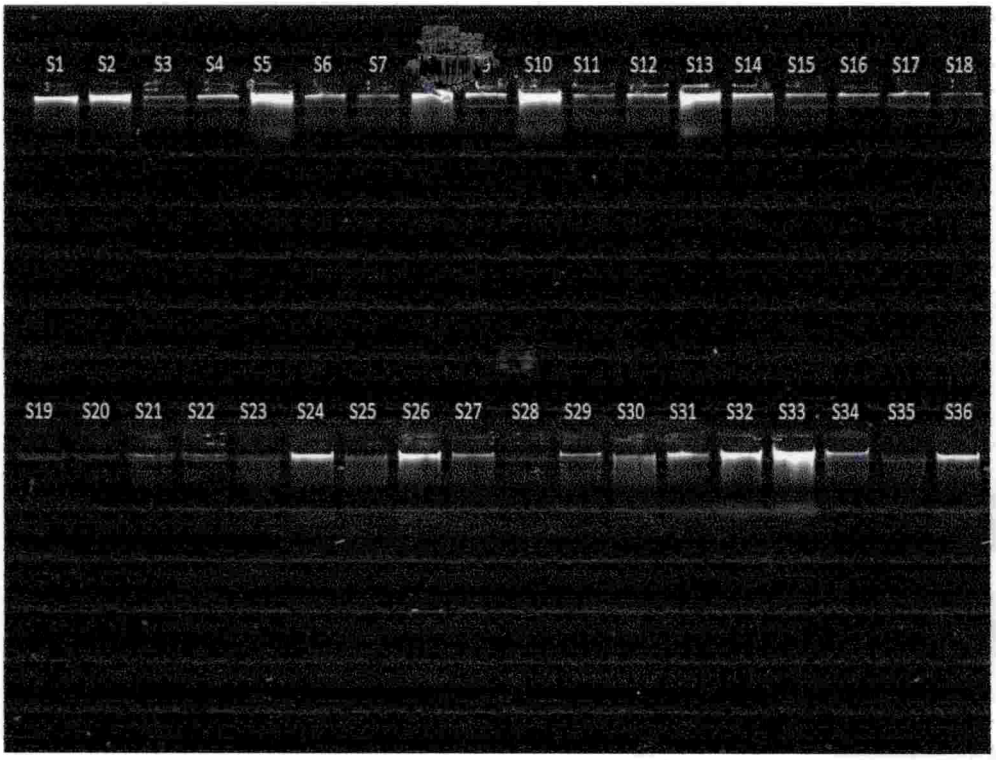

Plate 3: DNA from 36 samples isolated using CTAB method III (modified Sharma *et al.*, 2008)

S1 – C-96, S2 – C-557, S3 – JAS 7, S4 – NEH 8, S5 – CAUCO1, S6 – C-622, S7 – DT 1, S8 - IC012601, S9 – NEH 21, S10 – NEH 32, S11 – NEH 109, S12 – Line 4, S13 –Line 18, S14 – Line 38, S15, Line 48, S16 – NEH 117, S17 – Bhu Kripa, S18 – C-84, S19 – C-89, S20 – NEH 22, S21 – C-621, S22 – C-717, S23 – *Colocasia* Nicobar, S24 – Line 29, S25 – Line 33, S26 – Line 43, S27 – Mukthakeshi, S28 – NEH 34, S29 – NEH 77, S30 – Line 8, S31 – Line 11, S32 – Line 14, S33 – Line 42, S34 – C-322, S35 – C-556, S36 – Bhu Sree

4.1.2. PCR STANDARDIZATION

PCR conditions were standardized to obtain clear and consistent amplicons under reduced cycle duration. PCR profile obtained using SSR primers with original reaction conditions and modified reaction conditions are given in plate 3. Initially, the PCR condition used was the following:

Initial denaturation	94℃	5min
Denaturation	94℃	1min
Annealing	56 - 60℃	2min
Extension	72℃	2min
Final extension	72℃	10min

After standardization the following condition was standardized,

Initial denaturation	94℃	5min
Denaturation	94℃	30 s
Annealing	60 - 68℃	1min
Extension	72℃	2min
Final extension	72℃	8min

4.1.3. PRIMER SCREENING

16 primers were selected initially for screening of which 10 were selected based on their polymorphism and repeatability. Gradient PCR was done to find the proper annealing temperature of the primers. The primers which showed great amount of unwanted bindings and those which did not show any polymorphism were eliminated and the ones which showed proper bands at the proper annealing temperature were selected for further analysis. The annealing temperature was standardized for the selected ten primers and the same is given in table 7.

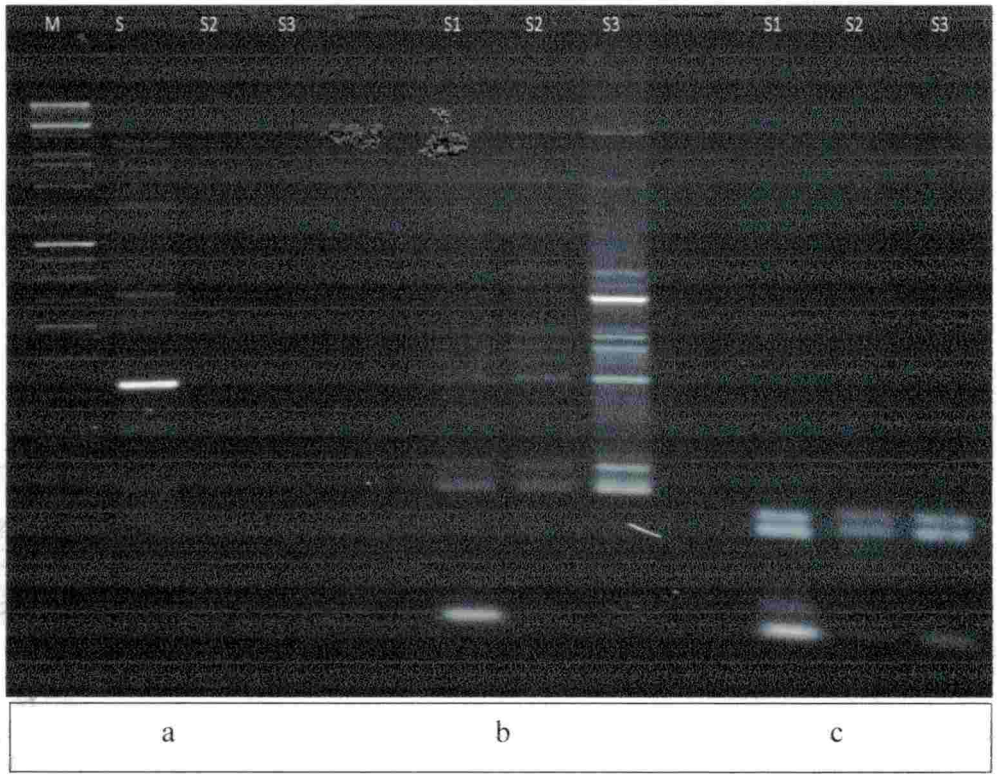

Plate 4: Standardization of PCR conditions

(a and b) Gel obtained under various PCR conditions

(c) Gel obtained under modified PCR conditions

4.1.4. SSR ANALYSIS OF TARO ACCESSIONS

The selected primers were used to characterize the complete set of 36 taro accessions. The amplicons were resolved at first in 2% agarose gel to check the presence of bands of the expected product size. The amplicons were resolved further in 6% PAGE and 4% agarose gel to obtain more resolution. The plates 4 to 10 show the PCR profile of the standardized SSR primers. The banding pattern obtained in both the 6% PAGE and 4% agarose gels were similar and hence 4% agarose was used for resolving the PCR products considering the ease of preparation.

4.1.5. ANALYSIS OF MOLECULAR DATA

The various parameters estimated for the quantification of genetic variability using 10 SSR markers are given in table 7.

a. Band scoring

From the gel profile obtained by resolving the SSR PCR amplicon, band scoring was done by assigning the value "1" for the presence of band and the value "0" for the absence of band. This score value is used for further statistical analysis.

b. Heterozygosity

Heterozygosity, which indicates the measure of genetic variation within a population. The observed heterozygosity is defined as the percentage of loci heterozygous per individual or the number of individuals heterozygous per locus. The heterozygosity observed in the current study varied from 0.65 – 0.82, where primer Uq 115 – 71 gave the least value whereas primer Ce1F04 gave the highest value. The He values are given in table 7.

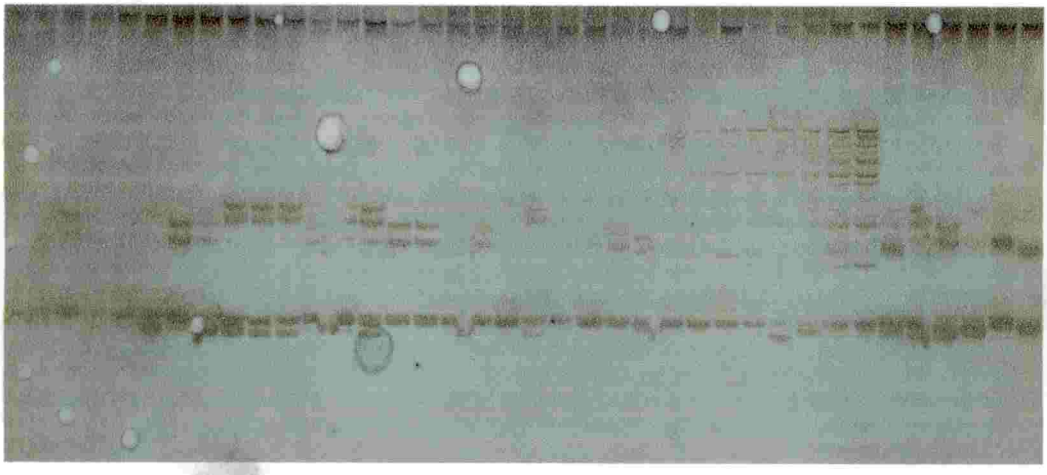

Plate 5: 6% Poly acrylamide gel profile of the primer Ce1F12.

S1 – C-96, S2 – C-557, S3 – JAS7, S4 – NEH8, S5 – CAUCO1, S6 – C-622, S7 – DT1, S8 - IC012601, S9 – NEH21, S10 – NEH32, S11 – NEH109, S12 – Line 4, S13 – Line 18, S14 – Line 38, S15, Line 48, S16 – NEH117, S17 – Bhu Kripa, S18 – C-84, S19 – C-89, S20 – NEH22, S21 – C-621, S22 – C-717, S23 – *Colocasia* Nicobar, S24 – Line 29, S25 – Line 33, S26 – Line 43, S27 – Mukthakeshi, S28 – NEH34, S29 – NEH77, S30 – Line 8, S31 – Line 11, S32 – Line 14, S33 – Line 42, S34 – C-322, S35 – C-556, S36 – Bhu Sree

Plate 6: 4% Agarose gel profile of the primer Uq 84 – 207.

S1 – C-96, S2 – C-557, S3 – JAS 7, S4 – NEH 8, S5 – CAUCO1, S6 – C-622, S7 – DT 1, S8 - IC012601, S9 – NEH 21, S10 – NEH 32, S11 – NEH 109, S12 – Line 4, S13 – Line 18, S14 – Line 38, S15, Line 48, S16 – NEH 117, S17 – Bhu Kripa, S18 – C-84, S19 – C-89, S20 – NEH 22, S21 – C-621, S22 – C-717, S23 – *Colocasia* Nicobar, S24 – Line 29, S25 – Line 33, S26 – Line 43, S27 – Mukthakeshi, S28 – NEH 34, S29 – NEH 77, S30 – Line 8, S31 – Line 11, S32 – Line 14, S33 – Line 42, S34 – C-322, S35 – C-556, S36 – Bhu Sree

Plate 7: 4% Agarose gel profile of the primer Uq 201 - 302.

S1 – C-96, S2 – C-557, S3 – JAS 7, S4 – NEH 8, S5 – CAUCO1, S6 – C-622, S7 – DT 1, S8 - IC012601, S9 – NEH 21, S10 – NEH 32, S11 – NEH 109, S12 – Line 4, S13 – Line 18, S14 – Line 38, S15, Line 48, S16 – NEH 117, S17 – Bhu Kripa, S18 – C-84, S19 – C-89, S20 – NEH 22, S21 – C-621, S22 – C-717, S23 – *Colocasia* Nicobar, S24 – Line 29, S25 – Line 33, S26 – Line 43, S27 – Mukthakeshi, S28 – NEH 34, S29 – NEH 77, S30 – Line 8, S31 – Line 11, S32 – Line 14, S33 – Line 42, S34 – C-322, S35 – C-556, S36 – Bhu Sree

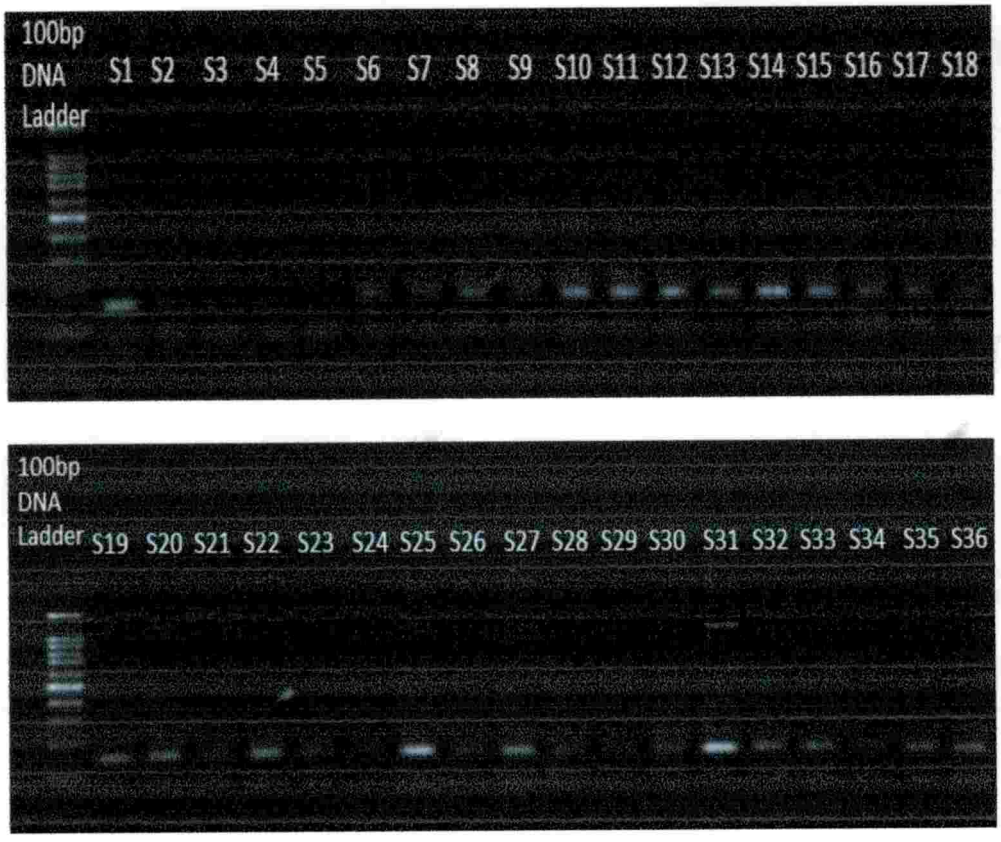

Plate 8: 4% Agarose gel profile of the primer Uq 132 – 147.

S1 – C-96, S2 – C-557, S3 – JAS7, S4 – NEH8, S5 – CAUCO1, S6 – C-622, S7 – DT1, S8 - IC012601, S9 – NEH21, S10 – NEH32, S11 – NEH109, S12 – Line 4, S13 – Line 18, S14 – Line 38, S15, Line 48, S16 – NEH117, S17 – Bhu Kripa, S18 – C-84, S19 – C-89, S20 – NEH22, S21 – C-621, S22 – C-717, S23 – *Colocasia* Nicobar, S24 – Line 29, S25 – Line 33, S26 – Line 43, S27 – Mukthakeshi, S28 – NEH34, S29 – NEH77, S30 – Line 8, S31 – Line 11, S32 – Line 14, S33 – Line 42, S34 – C-322, S35 – C-556, S36 – Bhu Sree

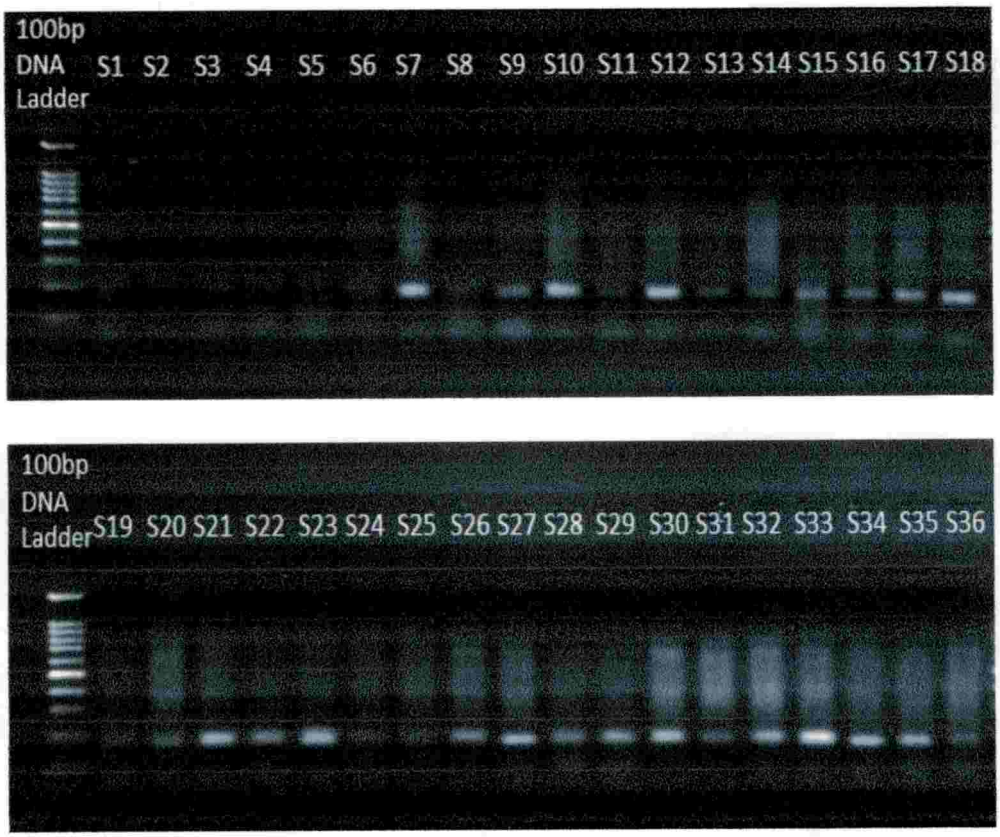

Plate 9: 4% Agarose gel profile of the primer Uq 73 – 164.

S1 – C-96, S2 – C-557, S3 – JAS7, S4 – NEH8, S5 – CAUCO1, S6 – C-622, S7 – DT1, S8 - IC012601, S9 – NEH21, S10 – NEH32, S11 – NEH109, S12 – Line 4, S13 – Line 18, S14 – Line 38, S15, Line 48, S16 – NEH117, S17 – Bhu Kripa, S18 – C-84, S19 – C-89, S20 – NEH22, S21 – C-621, S22 – C-717, S23 – *Colocasia* Nicobar, S24 – Line 29, S25 – Line 33, S26 – Line 43, S27 – Mukthakeshi, S28 – NEH34, S29 – NEH77, S30 – Line 8, S31 – Line 11, S32 – Line 14, S33 – Line 42, S34 – C-322, S35 – C-556, S36 – Bhu Sree

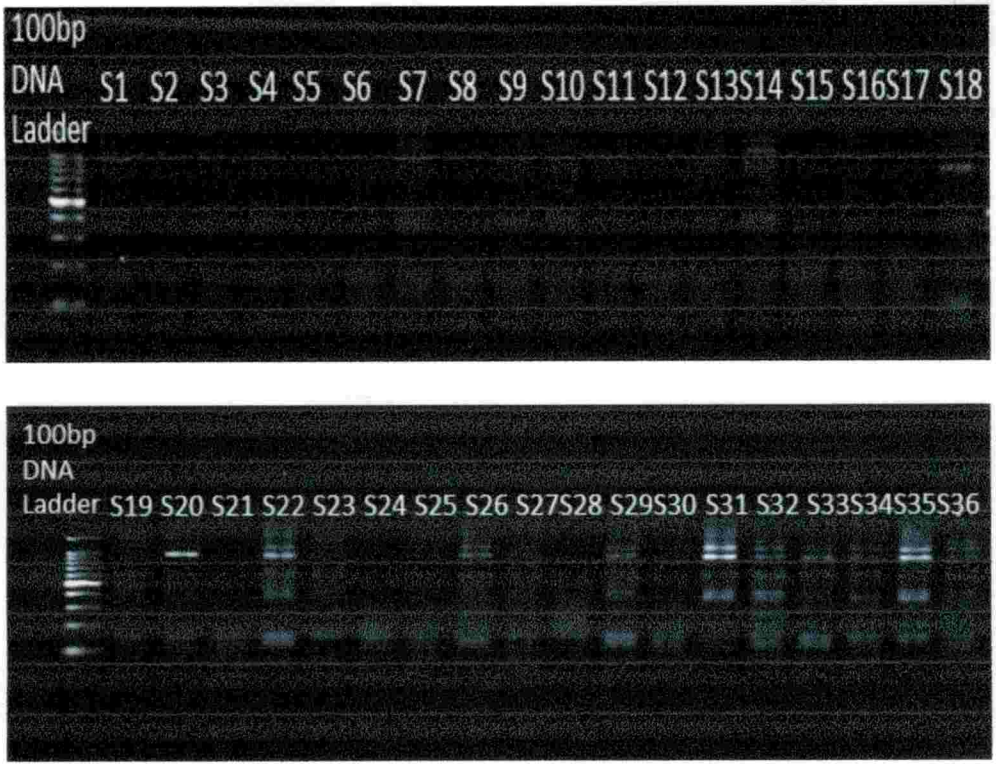

Plate 10: 4% Agarose gel profile of the primer Ce1F12.

S1 – C-96, S2 – C-557, S3 – JAS7, S4 – NEH8, S5 – CAUCO1, S6 – C-622, S7 – DT1, S8 - IC012601, S9 – NEH21, S10 – NEH32, S11 – NEH109, S12 – Line 4, S13 – Line 18, S14 – Line 38, S15, Line 48, S16 – NEH117, S17 – Bhu Kripa, S18 – C-84, S19 – C-89, S20 – NEH22, S21 – C-621, S22 – C-717, S23 – *Colocasia* Nicobar, S24 – Line 29, S25 – Line 33, S26 – Line 43, S27 – Mukthakeshi, S28 – NEH34, S29 – NEH77, S30 – Line 8, S31 – Line 11, S32 – Line 14, S33 – Line 42, S34 – C-322, S35 – C-556, S36 – Bhu Sree

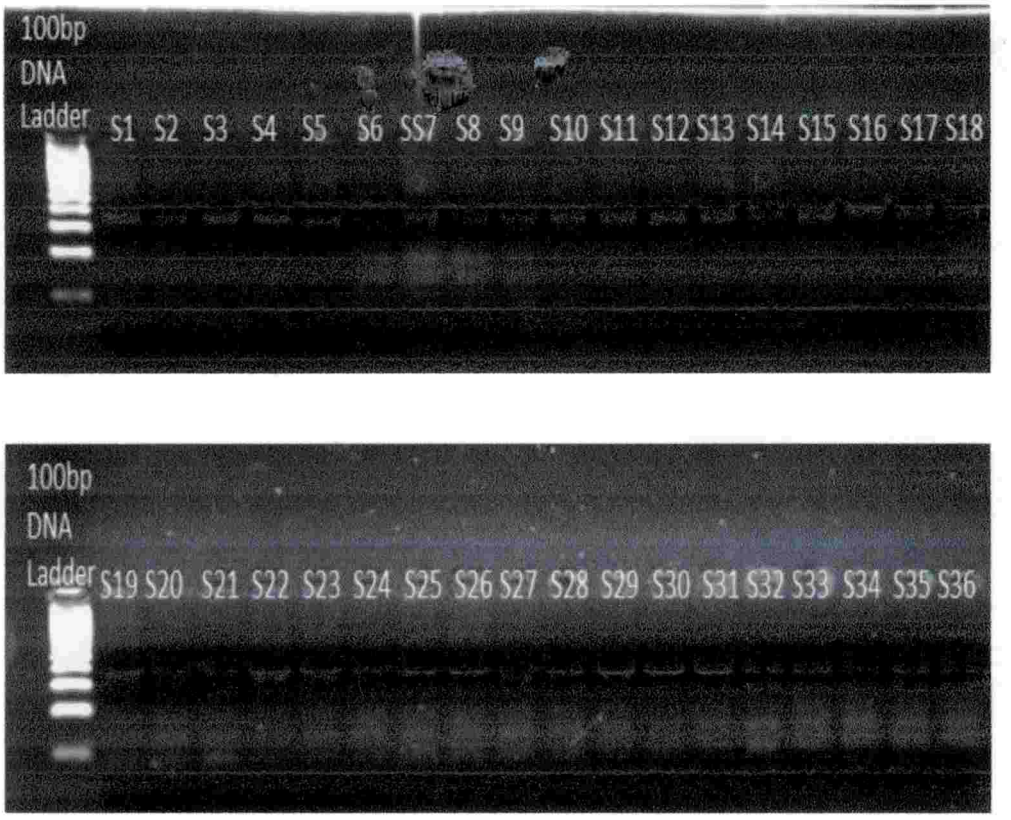

Plate 11: 4% Agarose gel profile of the primer Ce1F04.

S1 – C-96, S2 – C-557, S3 – JAS7, S4 – NEH8, S5 – CAUCO1, S6 – C-622, S7 – DT1, S8 - IC012601, S9 – NEH21, S10 – NEH32, S11 – NEH109, S12 – Line 4, S13 – Line 18, S14 – Line 38, S15, Line 48, S16 – NEH117, S17 – Bhu Kripa, S18 – C-84, S19 – C-89, S20 – NEH22, S21 – C-621, S22 – C-717, S23 – *Colocasia* Nicobar, S24 – Line 29, S25 – Line 33, S26 – Line 43, S27 – Mukthakeshi, S28 – NEH34, S29 – NEH77, S30 – Line 8, S31 – Line 11, S32 – Line 14, S33 – Line 42, S34 – C-322, S35 – C-556, S36 – Bhu Sree

c. Polymorphism information content (PIC)

The polymorphism information content (PIC) value is commonly used in genetics as a measure of polymorphism for a marker locus. PIC value ranged from 0.58 – 0.80, where primer Uq 115 – 71 gave the least value and primer Ce1F04 gave the highest value. The values are given in table 7.

d. Shannon's diversity index

Shannon's diversity index is usually calculated to estimate the species diversity in a community. It accounts for both abundance and evenness of the species. The primer Ce1F04 gave the highest value of Shannon's diversity index (2.10), which ranged from 0.88 – 2.10. The lowest value was shown by Uq 115 – 71. The values are given in table 7.

e. Average no. of alleles per locus

The average number of alleles observed over a range of loci for different populations is considered to be a reasonable indicator of genetic variation. The average no. of alleles per locus varied from 1.81 – 2.75, with the highest no. of alleles per locus shown by the primer Uq 97 – 256 and the least by Uq 115 – 71. The values are given in table 7.

Table 7. Measures of diversity estimated for SSR primers

Primer	T_a (°C)	Average no. of alleles per locus	Shannon's diversity index	Polymorphism Information Content	Heterozygosity
Uq 84-207	63	2.33	1.87	0.76	0.79
Uq 110-283	68	2.11	1.25	0.68	0.73
Uq 88B-94	66	2.06	1.35	0.70	0.74
Uq 97-256	66	2.75	1.50	0.74	0.78
Uq 201-302	68	2.00	1.03	0.60	0.66
Uq 115-71	60	1.81	0.88	0.58	0.65
Uq 132-147	62	2.31	1.41	0.70	0.74
Uq 73-164	66	2.64	1.52	0.78	0.80
Ce1F12	60	2.67	1.72	0.75	0.79
Ce1F04	60	2.53	2.10	0.80	0.82

f. Cluster analysis

The genetic distance measures are used to construct the dendrograms, also called phylogenetic trees.

Clustering of the 36 taro accessions was done on the basis of Nei genetic distance using UPGMA cluster analysis in the NTSYS software. The complete set of 36 accessions were grouped into two major clusters, I and II. Cluster I had only five accessions, whereas, all the rest 31 accessions were grouped in the cluster II, which was sub divided into four sub clusters having 8, 8, 10 and 5 accessions, respectively. The details of the accessions clustered under the different clusters and sub-clusters are given in table 8. The

maximum percentage of similarity was 83% between accessions C-557 and NEH 8; NEH 32 and Line 4 as well as Lines 29 and 33. In cluster I, three taro accessions from the NEH region was grouped together with JAS 7, which is a collection from Northern parts of Kerala. The accession CAU CO1 was found to be divergent in that cluster. In cluster II, few accessions were found to be divergent viz., Lines 48, 18, 38, 42, C-84 and *Colocasia* Nicobar. Of these, the last one, *Colocasia* Nicobar from Andaman and Nicobar Islands was found to be the most divergent one. All the three varieties used namely, Bhu Kripa, Bhu Sree and Muktakeshi released from Regional Centre, Bhubaneswar grouped together in sub-cluster 2c, whereas in the sub-cluster 2d, of the five accessions grouped together, four were from the NEH region and the most divergent one was from A&N Islands. Cluster I was related to Cluster II at 53% (Figure 1).

Table 8: Details of accessions grouping under various clusters

Cluster		No. of accessions	Accessions grouped
1		5	C-96, Jas 7, C-557, NEH 8, CAUCO1
2	a	8	C-622, IC012601, NEH 32, Line 4, NEH 109, DT 1, NEH 21, Line 48
	b	8	Line 18, Line 8, Line 11, Line 29, Line 33, Line 38, C-89, NEH 22
	c	10	NEH 117, Bhu Kripa, C-84, Line 14, Bhu Sree, Line 43, Mukthakeshi, Line 42, C 32, C 556
	d	5	C 621, C 717, NEH 34, NEH 77, *Colocasia* Nicobar

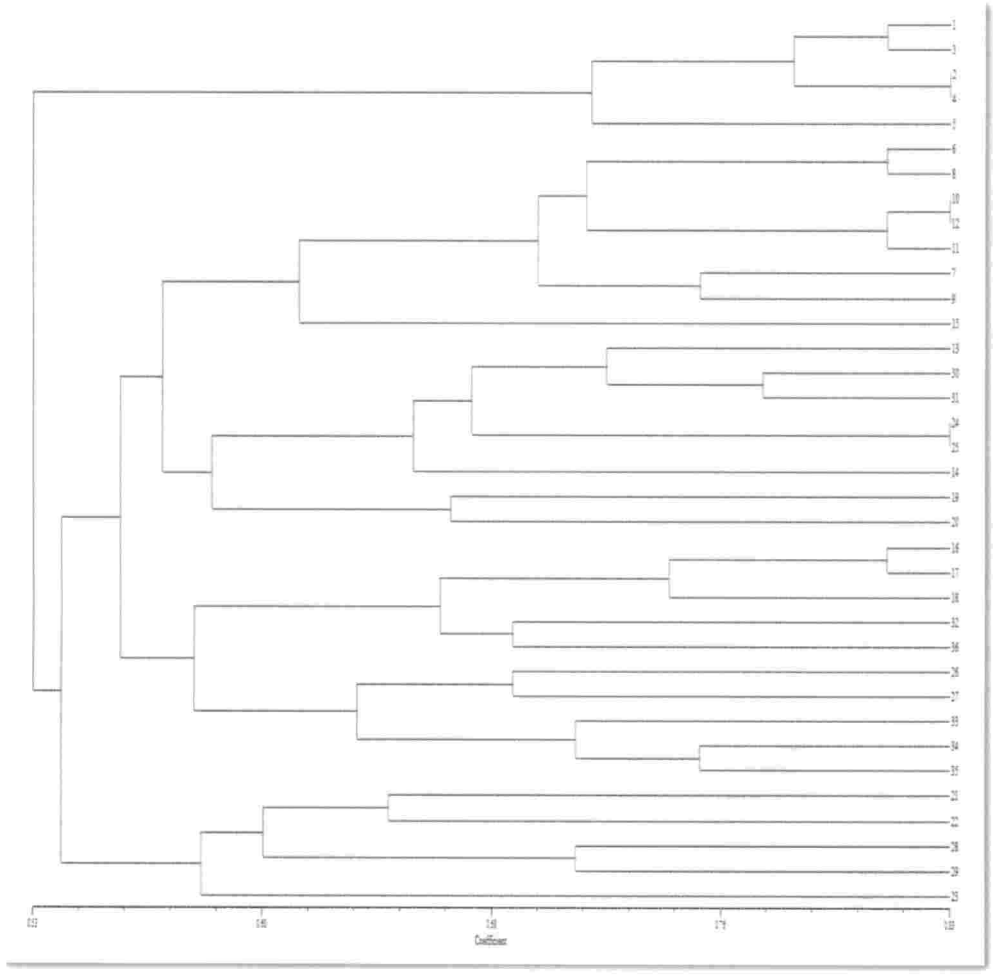

Figure 1: Dendrogram of 36 accessions obtained from NTSys – PC.

S1 – C-96, S2 – C-557, S3 – JAS 7, S4 – NEH 8, S5 – CAUCO1, S6 – C-622, S7 – DT 1, S8 - IC012601, S9 – NEH 21, S10 – NEH 32, S11 – NEH 109, S12 – Line 4, S13 – Line 18, S14 – Line 38, S15, Line 48, S16 – NEH 117, S17 – Bhu Kripa, S18 – C-84, S19 – C-89, S20 – NEH 22, S21 – C-621, S22 – C-717, S23 – *Colocasia* Nicobar, S24 – Line 29, S25 – Line 33, S26 – Line 43, S27 – Mukthakeshi, S28 – NEH 34, S29 – NEH 77, S30 – Line 8, S31 – Line 11, S32 – Line 14, S33 – Line 42, S34 – C-322, S35 – C-556, S36 – Bhu Sree

g. Principal component analysis

Principal components can be used objectively to evaluate variation in measurements and to increase understanding of structural relationships as an entity rather than as a series of individual and independent relationships. PCA was done using R package with the molecular data. The bi-plot data are shown in figure 2.

h. Similarity matrix

Variations between the accessions was obtained by determining the SM coefficient using Jaccards coefficient using sequential, agglomerative, hierarchical and nested (SAHN) clustering option of NTSYS-PC. The similarity between the accessions was represented by a number. As the value increases, the diversity decreases between the accessions. The SM coefficient of the 36 accessions ranged between 0.35 (between NEH 34 and C-557) to 0.83 (between Mukthakeshi and Line 29). The details are shown in table 9.

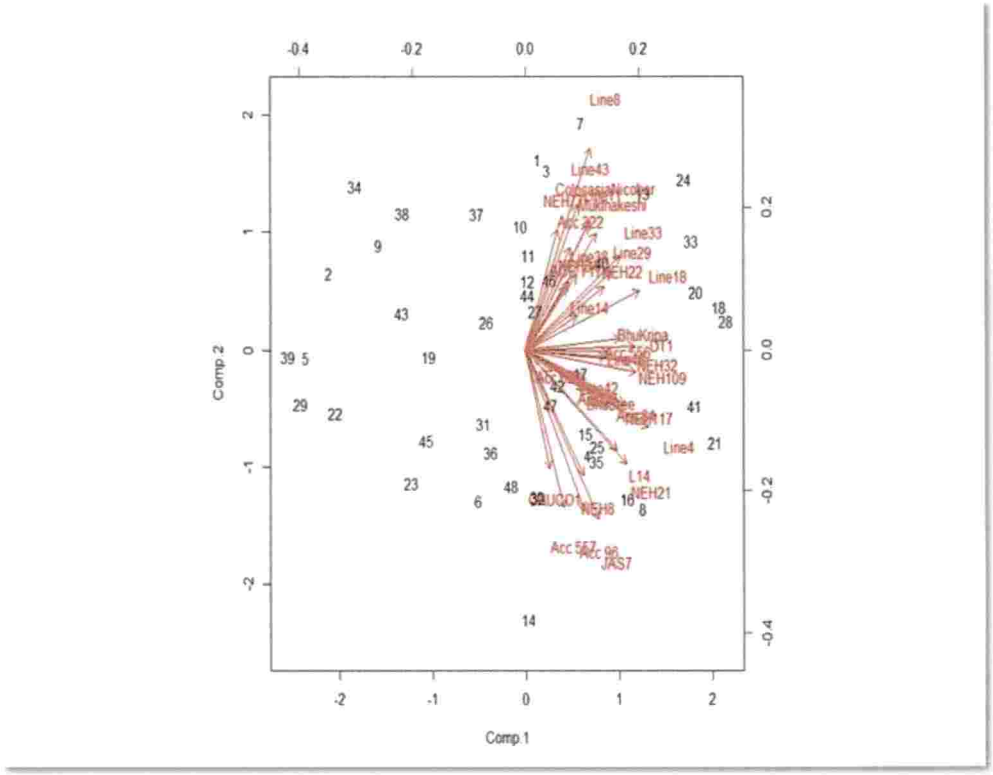

Figure 2: Biplot showing the special distribution of the various markers and accessions

Table 9: Similarity matrix

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
1	1.00																																			
2	0.77	1.00																																		
3	0.81	0.79	1.00																																	
4	0.77	0.83	0.79	1.00																																
5	0.65	0.71	0.75	0.75	1.00																															
6	0.50	0.52	0.52	0.48	0.56	1.00																														
7	0.50	0.52	0.52	0.48	0.40	0.71	1.00																													
8	0.52	0.54	0.58	0.54	0.58	0.81	0.73	1.00																												
9	0.71	0.69	0.69	0.60	0.52	0.63	0.75	0.73	1.00																											
10	0.56	0.63	0.58	0.58	0.50	0.69	0.73	0.71	0.60	1.00																										
11	0.54	0.56	0.52	0.56	0.52	0.71	0.75	0.77	0.67	0.81	1.00																									
12	0.60	0.67	0.67	0.67	0.63	0.65	0.69	0.75	0.73	0.83	0.81	1.00																								
13	0.58	0.52	0.52	0.60	0.40	0.58	0.71	0.60	0.63	0.73	0.71	0.69	1.00																							
14	0.46	0.48	0.52	0.52	0.40	0.58	0.58	0.52	0.42	0.69	0.63	0.56	0.71	1.00																						
15	0.54	0.52	0.52	0.44	0.44	0.54	0.63	0.65	0.67	0.56	0.63	0.65	0.67	0.50	1.00																					
16	0.63	0.52	0.65	0.65	0.48	0.46	0.63	0.60	0.58	0.60	0.63	0.65	0.71	0.58	0.58	1.00																				
17	0.56	0.38	0.58	0.54	0.54	0.52	0.52	0.58	0.56	0.54	0.65	0.63	0.69	0.56	0.65	0.81	1.00																			
18	0.67	0.52	0.73	0.60	0.56	0.54	0.54	0.56	0.58	0.52	0.50	0.56	0.50	0.54	0.58	0.71	0.77	1.00																		
19	0.63	0.56	0.56	0.60	0.56	0.46	0.54	0.52	0.50	0.60	0.67	0.60	0.58	0.63	0.58	0.63	0.56	0.63	1.00																	
20	0.58	0.52	0.52	0.52	0.44	0.54	0.71	0.48	0.58	0.52	0.63	0.52	0.67	0.58	0.63	0.54	0.56	0.67	0.67	1.00																
21	0.60	0.46	0.50	0.50	0.54	0.48	0.52	0.54	0.60	0.38	0.44	0.54	0.52	0.40	0.60	0.56	0.58	0.60	0.52	0.60	1.00															
22	0.63	0.56	0.56	0.56	0.52	0.46	0.50	0.44	0.63	0.52	0.50	0.56	0.54	0.50	0.54	0.46	0.48	0.50	0.50	0.58	0.65	1.00														
23	0.48	0.50	0.42	0.50	0.54	0.52	0.54	0.52	0.50	0.60	0.58	0.60	0.58	0.52	0.58	0.42	0.56	0.52	0.48	0.56	0.58	0.63	1.00													
24	0.52	0.58	0.58	0.58	0.50	0.60	0.65	0.58	0.60	0.67	0.56	0.58	0.69	0.69	0.52	0.60	0.50	0.56	0.52	0.60	0.50	0.60	0.60	1.00												
25	0.48	0.50	0.58	0.50	0.50	0.52	0.69	0.50	0.60	0.67	0.56	0.63	0.69	0.65	0.56	0.60	0.54	0.56	0.52	0.65	0.46	0.56	0.56	0.63	1.00											
26	0.44	0.50	0.46	0.50	0.46	0.48	0.52	0.50	0.52	0.58	0.56	0.50	0.56	0.56	0.52	0.44	0.46	0.52	0.52	0.56	0.42	0.60	0.65	0.67	0.67	1.00										
27	0.46	0.44	0.52	0.48	0.56	0.50	0.63	0.52	0.50	0.56	0.54	0.56	0.50	0.54	0.63	0.50	0.56	0.58	0.50	0.63	0.44	0.63	0.63	0.56	0.69	0.69	1.00									
28	0.46	0.35	0.52	0.48	0.56	0.54	0.42	0.52	0.50	0.48	0.54	0.56	0.54	0.46	0.58	0.58	0.77	0.63	0.46	0.50	0.65	0.63	0.58	0.48	0.48	0.56	0.58	1.00								
29	0.46	0.44	0.48	0.52	0.52	0.50	0.50	0.52	0.46	0.44	0.54	0.48	0.54	0.50	0.58	0.50	0.56	0.50	0.46	0.54	0.52	0.63	0.54	0.56	0.52	0.65	0.67	0.71	1.00							
30	0.40	0.42	0.46	0.50	0.42	0.44	0.56	0.42	0.44	0.58	0.52	0.54	0.73	0.65	0.52	0.52	0.63	0.52	0.48	0.65	0.50	0.65	0.65	0.63	0.67	0.75	0.69	0.60	0.65	1.00						
31	0.50	0.48	0.48	0.56	0.48	0.54	0.58	0.44	0.50	0.56	0.54	0.52	0.71	0.58	0.42	0.58	0.60	0.58	0.50	0.67	0.56	0.50	0.56	0.69	0.69	0.60	0.54	0.54	0.58	0.77	1.00					
32	0.52	0.42	0.54	0.50	0.50	0.44	0.56	0.46	0.52	0.46	0.48	0.46	0.56	0.52	0.52	0.65	0.63	0.69	0.56	0.56	0.58	0.52	0.50	0.46	0.58	0.58	0.65	0.56	0.52	0.58	0.60	1.00				
33	0.50	0.56	0.60	0.52	0.52	0.46	0.58	0.52	0.63	0.48	0.50	0.60	0.50	0.42	0.63	0.63	0.60	0.71	0.54	0.54	0.52	0.46	0.38	0.44	0.52	0.56	0.63	0.50	0.54	0.56	0.54	0.69	1.00			
34	0.48	0.42	0.42	0.42	0.46	0.48	0.56	0.46	0.56	0.46	0.52	0.54	0.44	0.40	0.52	0.56	0.54	0.56	0.56	0.56	0.54	0.65	0.56	0.54	0.54	0.63	0.73	0.60	0.69	0.58	0.60	0.63	0.69	1.00		
35	0.56	0.50	0.54	0.54	0.58	0.56	0.60	0.63	0.60	0.54	0.56	0.63	0.52	0.44	0.60	0.65	0.67	0.69	0.60	0.56	0.50	0.52	0.52	0.50	0.50	0.58	0.69	0.56	0.56	0.58	0.52	0.63	0.73	0.75	1.00	
36	0.58	0.44	0.65	0.52	0.60	0.58	0.46	0.65	0.63	0.52	0.50	0.56	0.58	0.54	0.58	0.63	0.69	0.71	0.58	0.50	0.60	0.46	0.48	0.56	0.69	0.56	0.50	0.63	0.46	0.52	0.54	0.69	0.58	0.48	0.56	1.00

4.2. *IN VITRO* CONSERVATION

The 36 taro accessions selected for the study was kept for *in vitro* conservation in half and full strength MS media using sprouts as the explant. The results of the *in vitro* conservation studies are given in this section.

4.2.1. STANDARDIZATION OF STERILIZATION CONDITIONS

The sterilization conditions for the explants were standardized for time of incubation and also for the concentration. Bavistin was used mainly to avoid fungal contamination. Bavistin treatment was given to the explants at 4 different time periods – 15, 20, 25 and 30 min. The treatment for 15 min and 20 min showed the percentage of survival to be approximately 20% and 40%, respectively. The cultures after treatment at both these time periods showed high fungal contamination. The treatment for 30 min showed survival percentage to be approximately 25%. The cultures could not tolerate the exposure to that much time and was found to be dead within days. The exposure time of 25 min was found to be optimum, which had a survival percentage of 80-85%. As a result, the treatment period of 25 min was adopted for surface sterilization using Bavistin. Labolene treatment was given at 3 different time periods – 8, 10 and 12 min. Labolene is a neutral detergent mainly used to avoid surface contamination. There wasn't much difference in the survival percentage for the treatment at all three time intervals. Therefore, the treatment period of 10 min was adopted for the surface sterilization using Labolene. $HgCl_2$ is used as an antimicrobial agent. This chemical is toxic to plants as well as humans. Therefore, it has to be washed many times to remove its traces completely. $HgCl_2$ treatment was given for 3 different time periods – 4, 5 and 6 min. At 4 min treatment the survival rate of the explant was very less and majority of the culture was lost due to contamination (Table 10). 6 min treatment was not tolerated by the explant as evidenced by the blackening of the explant within a few days. The optimum treatment time was observed to be 5 min. Hence, a final sterilization step of initial washing with 1% Bavistin

for 25 min. followed by a wash with 1% Labolene for 10 min and a final step of washing with 0.1% $HgCl_2$ for 5 min was adopted. The survival percentage of the plantlets were 82% and the plantlets remained healthy.

Table 10: Standardization of explant sterilization

Variety	Bavistin (1%)	Labolene (1%)	$HgCl_2$ (0.1%)	Total number of test tubes inoculated	Number of test tubes showing no contamination after 15 days	Percentage survival of explant (%)
CAUCO1	15	8	4	8	1	13
	20	10	6	7	0	0
	25	10	5	14	12	86
	30	10	5	14	8	57
Line14	20	10	5	7	6	86
	25	10	5	8	6	75
	30	10	5	9	5	56
Line 18	20	10	5	19	13	68
	25	10	5	18	17	94
	30	10	5	4	3	75
Line 33	20	10	5	6	3	50
	25	10	5	7	6	86
	30	10	5	3	1	33

4.2.2. STANDARDIZATION OF SHOOT MULTIPLICATION MEDIA

For the shoot multiplication studies, various cytokinins were added to basal MS media, which promoted shoot multiplication. The shoot multiplication media studied included MS+TDZ (0.1mg/l), MS+TDZ (0.2mg/l), MS+B (1mg/l), MS+B (5mg/l) and MS+K (1mg/l). Of the 5 media used, MS+TDZ (0.1mg/l) gave statistically superior to the other combinations

viz., MS+K(1mg/l), which was second best. Both MS+B(1mg/l) and MS+TDZ(0.2mg/l) were the least responsive (Table 11).

Table 11: Comparison of treatment effect on number of shoots induced

Sl. No.	Treatment	Number of shoots
1.	MS+TDZ0.1^{A}	1.96
2.	MS+K1^{B}	1.24
3.	MS+B1^{C}	0.68
4.	MS+TDZ0.2^{C}	0.52

Out of the two selected media, MS+TDZ (0.1mg/l) showed better results in the cultures. Approximately 80% of the cultures in this medium showed multiplication whereas in MS+K (1mg/l), approximately 50% cultures showed multiple shoots. It was also found from CRD analysis that all the five accessions tested were statistically at par with regards to its response to multiple shoot induction (Table 12).

Table 12: Effect of accessions on multiple shoot induction

Sl. No.	Accession	Number of shoots
1.	Line42^{A}	1.20
2.	Line14^{A}	1.20
3.	CAU CO1^{A}	1.20
4.	Line18^{A}	0.95
5.	Line33^{A}	0.95

The result showed that CAU CO1 inoculated in MS+TDZ (0.1 mg/l) showed the maximum number of multiple shoots (2.00), which was statistically significant. This same combination was also statistically at par with all the other accessions also *viz.*, Line14, Line18, Line42 and Line33

resulting in the induction of an average of 1.95 multiple shoots. Apart from this, MS+K (1 mg/l) also produced the same effect. CAU CO1 in MS+K (1 mg/l) produced 1.40 multiple shoots which was also at par with the results obtained in MS+TDZ (0.1 mg/l) (Table 13). From the above results, for further shoot multiplication studies, the medium MS+TDZ (0.1mg/l) was selected for the rest of the accessions.

Table 13: Standardization of multiplication media

Sl. No.	Accession	Treatment	Number of shoots
1.	CAU CO1	MS+TDZ0.1	2.00^{A}
2.	Line14	MS+K1	1.95^{AB}
3.	Line14	MS+TDZ0.1	1.95^{AB}
4.	Line18	MS+TDZ0.1	1.95^{AB}
5.	Line42	MS+TDZ0.1	1.95^{AB}
6.	Line33	MS+TDZ0.1	1.95^{AB}
7.	CAU CO1	MS+K1	1.40^{ABC}
8.	Line42	MS+TDZ0.2	0.95^{BCD}
9.	Line42	MS+K1	0.95^{BCD}
10.	Line18	MS+K1	0.95^{BCD}
11.	Line14	MS+B1	0.95^{BCD}
12.	Line42	MS+B1	0.95^{BCD}
13.	Line33	MS+K1	0.95^{BCD}
14.	Line18	MS+TDZ0.2	0.95^{BCD}
15.	Line33	MS+B1	0.95^{BCD}
16.	CAU CO1	MS+TDZ0.2	0.80^{CD}
17.	CAU CO1	MS+B1	0.60^{D}
18.	Line14	MS+TDZ0.2	-0.50^{D}
19.	Line33	MS+TDZ0.2	-0.50^{D}
20.	Line18	MS+B1	-0.50^{D}

Plate 12: Initiation of growth in basal MS media

Plate 13: Multiple shoots produced in the medium MS+TDZ (0.1mg/l)

4.2.3. MAINTENANCE IN SLOW GROWTH MEDIA

The cultures kept in shoot multiplication media was sub cultured in slow growth media which were half strength MS and full strength MS having 2% sucrose and 2% mannitol. The cultures in the tubes containing mannitol dried up and hence for further culturing full and half strength MS with 2% sucrose only was used. After the interval of 15 days, the growth in the tubes containing half strength MS with 2% sucrose was found to be lesser when compared to the cultures in the tubes containing full strength MS media with 2% sucrose. Similar results were observed after the intervals of 30 days and 45 days. These observations are being taken at every 15-day interval.

Table 14: Percentage survival in full and half strength MS media with 2% sucrose

Media	Tubes	Time interval (days)	Percentage of survival (%)
Full strength MS+2% sucrose	46	15	83.3
		30	75.0
Half strength MS+2% sucrose	50	15	91.7
		30	83.3

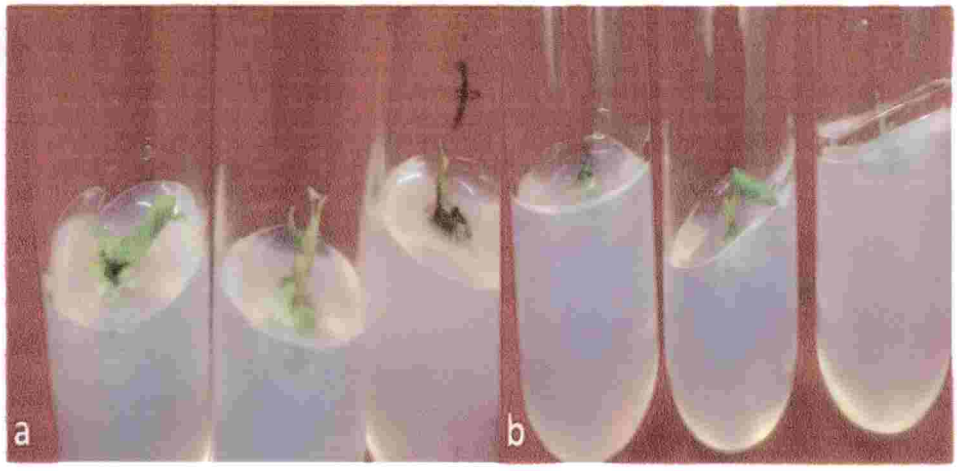

Plate 14: Explants in slow growth media after 15 days

(a) full strength MS+2% sucrose

(b) half strength MS+2% sucrose

a b

Plate 15: Explants in slow growth media after 30 days

(a) Full strength MS+2% sucrose

(b) Half strength MS+2% sucrose

DISCUSSION

5. DISCUSSION

Taro is an ancient crop, presumably originated from the Indo Malayan region, probably in the regions of India and Bangladesh. North Eastern India, being one of the center of origin is blessed with immense genetic wealth of taro. Along with the North Eastern states, Andaman and Nicobar Islands are also blessed with immense genetic wealth of taro. The evolutionary forces have created a variation in the biochemical and morpho–physio traits in the taro germplasm (Stebbins, 1957) including geographic speciation. The intense exposure to ultra violet radiation to the places near to the equator has also led to variations through mutations.

Genetic diversity of a species is important for its survival and adaptability. Little or lack of genetic diversity reduces the biological fitness and increases the chance of species extinction. The genetic diversity could be assessed through various means like morphological, biochemical and molecular characterization and evaluation. Taro, being one of the oldest cultivated crop possess a great threat in its diversity due to the loss of species owing to the disease and pest attack, natural catastrophes and changes in climate, culture and population structure. Due to these increasing threat, assessing the genetic diversity existing in a crop becomes important.

There have been numerous studies on the characterization of the genetic diversity of *C. esculenta* using different characters like morphological, biochemical and molecular. But the reports regarding the characterization of taro species from one of the centers of origin, the Indian sub-continent, are indeed limited. Genetic diversity analysis could help in understanding the diversity present in the crop and further in conserving the existing diversity in the germplasm for posterity. The morphological and biochemical method of characterization alone would not be advised for characterization because, they are highly influenced by the environment. Molecular markers, due to their neutral nature are used for characterization, to overcome this problem.

In the present study, the first part dealt with the characterization of 36 taro accessions, comprising germplasm collected from different parts of the country, varieties as well as breeding lines using 10 selected SSR primers.

Conservation of taro germplasm has become important due to the significant loss of germplasm as a result of pest and disease, natural calamities, unavailability of land and labor and the greater expense of maintenance. These are the main drawbacks of maintaining taro collections in field gene banks. *In vitro* storage was adopted to overcome this problem and was found extremely suitable for the storage of taro germplasm in a disease free manner, under controlled conditions. The advancement in biotechnology has resulted in the fine tuning of the tissue culture techniques which is greatly helpful in establishing and maintaining a repository. Slow growth conditions are used for medium term conservation. Slow growth could be attained through altering the culture conditions like lowering temperature, light intensity or time of exposure; or by adding osmoticum like sucrose, mannitol or sorbitol. The second part of the work was to maintain these 36 taro accessions *in vitro* for medium term storage and augmenting the existing IVAG collection at ICAR-CTCRI.

5.1. MOLECULARCHARACTERIZATION

5.1.1. STANDARDIZATION OF DNA ISOLATION PROTOCOL

Taro has a high composition of viscous secondary metabolites which composes of polar polymer of glycoprotein, present in tubers, seeds and stems. The non-performance of the commercial kit in obtaining the expected good quality DNA may be due to the presence of mucilage in taro leaves. The presence of mucilage hinders in pipetting of DNA and thus shows variation even in the quantification. The secondary metabolites co – precipitates with DNA and inhibits the action of Taq polymerase (Jose and Usha, 2000; Ghosh *et al.,* 2009). CTAB method of DNA isolation followed by Vinutha (2014) resulted in the presence of comparatively less amount of isolated DNA. CTAB

protocol for tuber crops developed by Sharma *et al.,* 2008 was found suitable in obtaining good concentration and pure DNA by providing some modifications in the protocol to remove the polysaccharides. In order to remove the secondary metabolites, the concentration of PVP and β – mercaptoethanol was altered. PVP and β – mercaptoethanol are useful to prevent oxidation of the secondary metabolites in the disrupted samples (Prittila *et al.,* 2001; Warude *et al.,* 2003), avoiding the brown pigmentation of the sample, thus increasing the yield and quality of the DNA. Presence of phenol in the extraction procedure increased the yield of DNA twice. Phenol is used in the extraction procedure mainly to remove the protein and lipids. Phenol:chloroform:isoamyl alcohol (25:24:1) has been reported to increase the purity of DNA in many plant species (Aras *et al.,* 2003; Sablok *et al.,* 2009; Lele, 2011). It was in 1953 that Grassmann and Defner first described the efficacy of phenol at extracting proteins from aqueous solution. Utilizing this finding, Kirby demonstrated the use of phenol to separate nucleic acids from proteins in 1956. Phenol should be completely removed after the step as oxidation of the phenol, if it happens will cause nicking of the DNA and degradation of RNA. For long term storage of the DNA, it was dissolved in TE buffer and stored at -80^{0}C.

DNA is considered to be pure if the ration of absorbance between the 260nm to that at 280nm is in the range 1.8-2.0 (Weising *et al.,* 2005). The DNA isolated using modified Sharma *et al.,* (2008) protocol was considered pure as its A260/A280 ratio ranged from 1.9–2.2. Young leaves are preferred over mature leaves for DNA isolation due to the presence of high concentrations of polysaccharides, polyphenols and other secondary metabolites in the older leaves as compared to younger ones, which can make it tough for DNA isolation (Debo *et al.,* 1993; Zhang and Steward, 2000). Hence young unopened or just opened leaves were used for DNA isolation.

5.1.2. PCR STANDARDIZATION AND PRIMER SCREENING

SSR marker was chosen to be the marker of choice for plant genetics due to the high levels of polymorphism. SSR is a single locus marker with multiple alleles, which provide an effective means for discriminating between genotypes (Powell *et al.,* 1996; Li *et al.,* 2001). Microsatellites are rather inexpensive and are easy to handle since they require only small quantity of template DNA. Despite these advantages, SSR markers show a few disadvantages. The microsatellite alleles may differ as far as one base pair which requires the samples to be run at high resolution agarose or poly acrylamide gel. The primers used for amplification vary in size, and nucleotide composition and needs to be optimized for each locus (Ogliari *et al.*, 2000).

SSRs could be calculated into neutral and non – neutral (functional) markers based on the structure of sequence used for marker development. Markers which are derived from genic region are functional and are less subjected to variation as they are under selection pressure. On the other hand, neutral markers are derived from genomic regions and are under less selection pressure. As they are less amenable to mutative variations, they are preferred for diversity studies. SSRs derived from ESTs or cDNA often fail to produce PCR products as their primer binding site tends to be present on the splice sites (Park *et al.,* 2009).

For the selection of SSR primers, along with polymorphism, quality of electrophoretic patterns should also be considered where the best ones are selected based on clarity and repeatability (Smulders *et al.,* 1997; Ramsey *et al.,* 2000). Based on these factors, 10 primers were shortlisted for analysis which were Uq 84 – 207, Uq 110 – 283, Uq 88B – 94, Uq 97 – 256, Uq 201 – 302, Uq 115 – 71, Uq 132 – 147, Uq 73 – 164, Ce1 F12 and Ce1 F04.

Often, the reaction temperatures differed from the recommended temperatures from the literature. Amplifications may not be obtained from the temperatures in the literature. This may be because of different brands / types

of thermocycler reaction or even minor differences in the thickness of the walls of the PCR tubes (Dograr and Akkaya, 2001). Most of the times, annealing temperatures should be empirically found out by performing touch down or gradient PCR. The standardized annealing temperature varied greatly from the ones found in the literature. The reasons for these might be because, at elevated temperatures, the nucleation of the primer hybridization becomes more difficult, thus non – specific target sites with some mismatched base pair competes with the specific target site for primer hybridization. At high temperatures, the ratio of the primers binding to the mismatched binding to the specific target site decreases when compared to the mismatched binding and at higher temperatures, extension becomes easier. Thus, at high temperatures, there would be non–specific polymerization. At lower annealing temperatures, primer hybridization becomes easier and there would be no or a few non–specific binding and amplification would occur accurately. Dograr and Akkaya (2001) made similar observation while using wheat SSR markers. Stutter bands was observed in some other studies including taro using SSR markers, however, in these cases scoring was done only for the bands of the expected product size (Nunes *et al.*, 2012, Rallo *et al.*, 2000). In the present study, the annealing temperatures ranged from 60°C to 68°C.

5.1.3. SSR ANALYSIS OF TARO ACCESSIONS

The amplicons were initially resolved in 2% agarose gel, which was used to screen for amplification at the expected product size. But these gel profiles could not show clearly the band separation for the taro accessions to assign binary scoring. Hence, two methods were tried for resolving the PCR products, by using 6% polyacrylamide gel electrophoresis and 4% high resolution agarose gel electrophoresis (Sigma Aldrich). The result obtained from PAGE profile was found to be similar to that obtained from 4% AGE and as a result, 4% AGE was used to resolve rest of the primer amplicons, which

required lesser time and labor. In the 4% agarose gel profile, the fragments resolved by differing in very few base pairs.

5.1.4. ANALYSIS OF MOLECULAR DATA

5.1.4.1. **Quantification of Genetic Diversity**

SSR polymorphisms are mostly length polymorphisms differing in the number of repeat units, which are reflected in the average number of alleles per locus. In the present study, the average number of alleles ranged from 1.81 to 2.67. The value is nearer to the result observed by Mace and Godwin (2002), which was 3.2 alleles per locus. The low values obtained in our study suggests that there is only low degree of variability, which could be due to the fact that of the 36 accessions of taro selected for the study, 11 were breeding lines obtained from few parental lines. Probably more divergent parents need to be crossed to obtain a higher level of heterosis. In a recent study by Mezhii, *et al.* (2017), which dealt with the analysis of genetic diversity in 50 accessions of taro from 11 districts of Nagaland, India, the average no. of alleles was found to be 1.89 alleles per locus. In our study also many lines used, belonged to the NEH region including Nagaland. The initial inference length variation in minisatellites was because of the occurrence of unequal crossing over between repeat units during meiosis (Jeffreys *et al.*, 1998) while the length variation in microsatellites was because of the DNA replication slippage (Strand *et al.*, 1993). Several studies have demonstrated that both type of repeats could be derived by either of the mechanism (Richard *et al.*, 2008).

The heterozygosity is defined as the probability that a random individual chosen from the population is heterozygous at a locus. In the present study, the heterozygosity ranged from 0.65 to 0.82. This value inferred that the 36 accessions selected for the studies are highly heterozygous.

Polymorphism of the marker is indicated by various indices like Shannon's diversity index and Polymorphism Information content. In the

present study, the Shannon's diversity ranged from 0.88 – 2.09. this shows that high degree of polymorphism is shown by the primer Ce1F04 and the least polymorphism was shown by primer Uq 115-71. The PIC value of the primers ranged from 0.58, shown by the primer Uq115-71 to 0.80, shown by primer Ce1F04. In the study be Mezhii *et al.* (2017) on Indian taro, The PIC value was found to be in the range of 0.41 – 0.93. this range indicates high specificity and discriminatory power of the markers.

5.1.4.2. **Cluster Analysis**

Clustering was done using NTSYS–pc using Jacard's coefficient. The 36 accessions were grouped into 2 main clusters (I and II). The major cluster II was further grouped into 4 sub clusters. The clusters formed a similarity index range of 53% to 83%. This indicates that the accessions selected did not show any duplicates and were genetically divergent. JAS7 from Wayanad, Kerala and C-557, from NEH region were found to be sharing 83% similarity. Similar results were shown by NEH 32 from Manipur and the breeding Line 4; and between the breeding lines Line 29 and Line 33.The Cluster I and Cluster II had a similarity index of approximately 53%. The Cluster II was further sub divided into four sub-clusters. Cluster I was small and had only 5 accessions, but Cluster II had 31 accessions in it. Line 18, Line 8, Line 11, Line 29, Line 33 and Line 38 were found in one cluster along with C-89 and NEH 22. This might indicate that these accessions might be having some common ancestry. The NEH varieties, which are having geographical similarities were found in different clusters, which emphasized on the fact that similarity may not be based on geographical origins.

In the studies of Lakhanpaul *et al.* (2003), the clustering did not show any strict relationship between geographic distribution and geographic diversity, as accessions from different geographic regions were placed nearly in all clusters. Similar work was done by Sharma *et al.* (2008b) where the data showed no correlation between the clusters and the geographical origins of the

accessions. Similar results were obtained by Vinutha (2014) also where around 30 taro accessions from the NEH region was characterized using SSR markers. Here also no similarity was shown between the clusters and the geographical origins.

The cluster analysis would help in selecting divergent parents for crop improvement programs. The parental selection should be made on the basis of relative merits of each cluster for each trait depending on the objective of breeding program (Mulaualem *et al.,* 2013).

Similarity matrix coefficient obtained for the accessions showed its variation with each other. The coefficient of an accession with itself is 1.00 and that with any other accession will be less than 1.00. For greater similarity between two accessions, the value was greater. The value was low when there was greater diversity present between two accessions. In the PCA, Line 8, C-557, C-96 and JAS7 were found to be different from the 2 clusters formed.

5.2. *IN VITRO* CONSERVATION OF TARO

5.2.1. STERILIZATION OF EXPLANTS

Tuber crops, being present underground has a high amount of microorganisms attached to it. To remove these microorganisms and to establish a contamination free culture, thorough sterilization was done. The sprouts which were taken as explants was washed first with bavistin to remove fungal contamination. 25 minutes of exposure gave good results when compared to the other time periods taken for standardization. Labolene, a neutral detergent is used commonly used to remove surface contamination. The explants were given a labolene treatment for 10 minutes. $HgCl_2$ was used as a surface sterilant and is used in the final step of sterilization. The time for the treatment of $HgCl_2$ was standardized at 5 minutes. The explants which were sterilized at a lesser time than 5 min, was found to be contaminated within 10-15 days and the explants which were inoculated for more time was

found to be dead in less than 10 days. The sterile cultures were kept in basal MS media for shoot initiation.

5.2.2. MULTIPLICATION OF EXPLANTS

Various shoot multiplication media were tried in MS medium fortified with various plant growth regulators (PGR) viz., BA (1 – 5mg/l); Kinetin (1mg/l) and TDZ (0.1 – 1mg/l). The cultures in the tube having the media composition MS+B5 dried up within 20 days of observation. This could be because, at higher concentrations, BA could be inhibitory to the growth. Similar results were found by Yam *et al.* (1990) in the regeneration of South Pacific taro. At lower concentrations, BA induced rooting and multiple shoots. MS+K (1mg/l) gave on an average 2 shoots per explants. Similar result was found in *Dioscora opositifolia* by Behera *et al.* (2009), which produced 10±0.51 shootlets in the media composition MS+Kinetin (2.0 mg/l) + BAP (1.0mg/l), NAA (0.5mg/l) + ascorbic acid (100mg/l). In his study, the second best result was shown by medium MS+K (1.5mg/l)+BAP (1.0mg/l)+ NAA(0.5mg/l)+ascorbic acid (100mg/l) which gave 5.5±0.48 shoot multiplication. Cytokinins like kinetin is a critical factor in shoot multiplication (Balachandran *et al.*, 1990). At higher concentrations, kinetin was found to induce callusing.

Thidiazuron is a synthetic hormone, which is recently being investigated for its cytokinin like activity. It is found to be better than zeatin, when added to tissue culture medium at low concentrations, but depends upon the type of crop plant, parts of plant, phase of development, concentration of growth regulators, interaction between hormones and environment factors (Salisbury and Ross, 1992). Higher concentration of TDZ are said to induce callusing. In the study of Deo *et al.* (2009), TDZ at the concentration 1mg/l was found to induce 85.8% callusing. At lower concentrations, TDZ induced multiple shooting. In the present study, callusing was found at TDZ concentrations of 1mg/l and 0.5mg/l. Good results were obtained in TDZ

(0.2mg/l) and Kinetin (1mg/l), but the best result was obtained by TDZ at a concentration of 0.1mg/l.

5.2.3. ESTABLISHMENT IN SLOW GROWTH MEDIUM

Application of tissue culture is highly useful in the case of rapid multiplication, safe exchange and conservation of germplasm of many vegetatively propagated crops. For conservation purpose, the two methods mainly used are reducing the temperature and photoperiod and by providing osmoticum. At reduced temperatures, taro was found to give good conservation results as it was evident from the works of Straitsky *et. al.* (1986); Bessembinder *et. al.* (1993), where the transfer intervals were extended to three years. In the present study, temperature reduction was not done, but the media was supplemented with osmoticums, which were said to help in growth reduction. Half and full strength MS having 2% sucrose and 2% mannitol was used as conservation medium. Inclusion of mannitol in the medium suppressed growth, but some morphological changes were observed (Bissembinder *et al.*, 1993; Taylor *et al.*, 2003). This was confirmed by Bhuiyan *et al.*, 2016. In our study, the plants dried a few days after inoculation. The cultivars in the present study was hence established in full strength and half strength MS having 2% sucrose. Half strength media induced slow growth more than full strength media, may be because of the presence of less amount of nutrients. Plants were cultured in half strength MS + 2% sucrose for conservation and thus was used for IVAG of taro.

SUMMARY

6. SUMMARY

The study entitled "Molecular characterization and *in vitro* conservation of taro (*Colocasia esculenta* (L.) Schott)" was carried out in the Division of Crop Improvement, ICAR – Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram during the period 2016-17. The main objective of the study was to analyze the genetic diversity of 36 taro accessions including germplasm, variety and breeding lines using ten SSR markers and to maintain them in slow growth conditions for medium term conservation. In the conservation protocol a step for shoot multiplication was also included so that rare genotypes/mutants in the germplasm collection can be initially multiplied and then maintained *in vitro*. The 36 taro accessions were collected from the taro field genebank maintained at ICAR – CTCRI for molecular characterization and *in vitro* studies.

Young leaf samples were taken to isolate DNA and of the four different protocols tried, the modified Sharma *et al.* (2008) method (CTAB method III) was found to give good quality (showing OD values 0.050 to 0.457) and pure DNA. This protocol also gave high quantities of DNA (372ng to 2285 ng/µl). Standardization of SSR – PCR conditions was attempted to obtain consistently good amplification under reduced cycle duration. 16 SSR primers, were used for initial screening out of which only 10 were selected, which gave good amplification at the expected product size, for further analysis. The selected primers included 8 from the Uq series (Mace and Godwin, 2002) and 2 from the Ce1 series (Noyer *et al.*, 2004). These primers were selected based on their quality of polymorphic bands and the electrophoretic patterns of bands at the expected product size. The annealing temperatures of these primers varied from the literature and was standardized using gradient PCR. The PCR was carried out using the annealing temperatures standardized in our lab using gradient PCR. The presence of amplicons at the expected size was confirmed by resolving them in 2%

agarose gel. Further PCR gel profiling was done using 6% PAGE and 4% high resolution agarose (Sigma Aldrich) gel electrophoresis. Both 6% PAGE and 4% AGE gave similar results and hence, 4% agarose gel electrophoresis was adopted for resolving the rest of the amplicons, which required less time and labor.

All the selected primers gave high polymorphism, which was explained by average number of alleles per locus which ranged from 1.81 to 2.67; Shannon's diversity index which ranged from 0.88 to 2.10 and Polymorphism Information Content (PIC) which ranged from 0.58 to 0.80. All the 36 accessions were found to show diversity which was explained by the heterozygosity value which ranged from 0.65 to 0.82. The dendrogram drawn by NTSYS–PC, using Jaccard's coefficient, divided the 36 accessions into 2 main clusters, I and II, having 53% similarity. The similarity range of the dendrogram was from 53% to 83%. The maximum percentage of similarity was 83% between accessions C-557 and NEH 8; NEH 32 and Line 4 as well as Lines 29 and 33. The results shows that there were no duplicates present in the set studied and all the 36 accessions were divergent. The cluster II was further grouped into 4 sub clusters at 53% similarity. 5 accessions were found in Cluster I and 31 accessions in Cluster II. The accessions were not clustered based on geographical similarities. Accessions which were geographically similar could be found in separate clusters. The principal component analysis done by R package also separated the accessions into separate clusters.

For *in vitro* conservation studies, sprouts were taken as explants. The sterilization techniques were standardized and the explants were standardized by keeping for 25 min in bavistin, 10 min in labolene and 5 min in $HgCl_2$ with intermittent washing in distilled water. The explants were inoculated in basal MS media for establishment of contamination free cultures. Multiple shoots were induced by inoculating in shoot multiplication media (MS+TDZ (0.1mg/l)). Initially six shoot multiplication media was tried of which two

were selected finally based on statistical analysis. The best media for multiplication was found to be MS+ TDZ (0.1mg/l) and the second best was MS+K (1mg/l). The explants were subjected to shoot multiplication using these media, and was transferred to basal MS for a brief period, for establishment and then inoculated to the slow growth media for medium term conservation.

The conservation media used were full and half strength MS having 2% sucrose and 2% mannitol, each. The presence of mannitol induced slow growth but the shoots got dried and hence all further trials were carried out in 2% sucrose containing media only. The cultures in half strength MS was found to induce slow growth than the ones in full strength MS, which maybe as a result of the lesser quantity of nutrients present in the media. These cultures maintained in the slow growth media was added to the already existing *in vitro* active germplasm collection of tuber crops at ICAR – CTCRI, where many important accessions of the tuber crops are conserved *in vitro*.

REFERENCE

7. REFERENCES

Ahmed, I. 2014. Evolutionary dynamics in taro (*Colocasia esculenta* L.). *PhD thesis*, Massey University, Palmerston North, New Zealand, 128p.

Akkaya, M. S., Bhagwat, A. A., and Cregan, P. B. 1992. Length polymorphisms of simple sequence repeat DNA in soybean. *Genetics* 132: 1131-1139.

Akopyanz, N., Bukanov, N. O., Westblom, T. U., and Berg, D. E. 1992. PCR-based RFLP analysis of DNA sequence diversity in the gastric pathogen *Helicobacter pylori*. *Nucleic Acid Res.* 20: 6221-6225.

Aras, S., Duran, A., and Yenilme, G. 2003. Isolation of DNA for RAPD analysis from dry leaf material of some *Hesperis* L. specimens. *Pl. Mol. Biol. Rep.* 461a-461f.

Arditti, J. and Strauss, M. S. 1979. *Taro tissue culture Manual.* South Pacific Commission Information Document No. 44-1979, Noumea, New, Caledonia. 132p.

Balachandran, S. M., Bhat, S. R., and Chandel, K. P. S. 1990. *In vitro* clonal multiplication of turmeric (*Curcuma spp*) and ginger (*Zingiber officinalle* Rosc*) Pl. Cell Rep.* 8: 521-524.

Banerjee, N. and de Langhe, E. A. L. 1985. A tissue culture technique for rapid clonal propagation and storage under minimal growth conditions of *Musa* (banana and plantain). *Pl. Cell. Reports* 4: 351-354.

Batside, C. 2000. Creating banks microsatellites for yam (*Dioscorea alata, Dioscorea praehensilis* and *Dioscoria abyssinica*) and taro (*Colocasia esculenta*). Memory DUT, Dept. Biological Engineering Aronomy Option, IUT de Perpignan, France. p. 13.

Behera, K.K., Sahoo, S. and Prusti, A., 2009. Regeneration of Plantlet of Water Yam (Dioscorea oppositifoliaaL.) through *in vitro* Culture from Nodal Segments. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca* 37(1): 94-102.

Bessembinder, J. J. E., Staritsky, G., and Zandvoort, E. A. 1993. Long – term *in vitro* storage of *Colocasia esculenta* under minimal growth conditions. *Pl. Cell Tissue Org. Cult.* 33: 121-127.

Bhuiyan, M. K. R., Hossain, M. J., and Haque, M. M. 2016. *In vitro* conservation of taro (*Colocasia esculenta* var. *globulifera*) as influenced by mannitol. *Bangladesh. J. Agril. Res.* 41(1): 67-74.

Borges, M., Ceiro, W, Meneses, S., Aguilera, N., Vazquez, J., Infante, Z., and Fonseca, M. 2004. Regeneration of *Dioscorea alata* germplasm maintained *in vitro*. *Pl. Cell Tissue Org. Cult.* 44: 125-130.

Bose, T. K., Kabir, J., Maity, T. K., Parthasarathy, V. A., and Som, M. G. 2003. *Vegetable Crops: Vol 2.* Naya Udyog Publishers, Kolkatha, 568p.

Bradburry, J. H. and Halloway, W. D. 1998. *Chemistry of Tropical Root Crops: Significance for Nutrition and Agriculture in the Pacific.* ACAIR Monograph No. 6, ACIAR, Canberra, 201p.

Buteler, M. I., Jarret, R. L., and LaBonte, D. R. 1999. Sequence characterization of microsatellites in diploid and polyploidy *Ipomoea. Theor. Appl. Genet.* 99: 123-132.

Caillon, S., Quero-Garcia, J., Lescure, J. P., and Lebot, V. 2006. Nature of taro (*Colocasia esculenta* (L.) Schott) genetic diversity prevalent in Pacific Ocean Island, Vanua Lava, Vanuatu. *Genet. Resour. Crop Evol.* 53: 1273-1289.

CGIAR (Consultative Group on International Agricultural Research) System – wide Genetic Resources Programme (SGRP) 2011. *Refinement and Standardization of storage procedures for clonal crops.* Consultative Group on International Agricultural Research, Rome, Italy, 86p.

Chambers, G. K. and MacAvoy, E. S. 2000. Microsatellites: consensus and controversy. *Comp. Biochem. Phys. Part B* 126 (2000): 455-476.

Chand, H., Pearson, M. N., and Lovell, P. H. 1999. Rapid vegetative multiplication in *Colocasia esculenta* (L.) Schott (taro). *Pl. Cell Tissue Org. Cult.* 55: 223-226.

Chandrasekara, A. and Kumar, T. J. 2016. Roots and tuber crops as functional foods: A review on phytochemical constituents and their potential health benefits. *Int. J. Food Sci.* 2016: 1-15.

Chavarriaga-Aguirre, P., Maya, M. M., Bonierbale, M. W., Kresovich, S., Fregene, M. A., Tohme, J., and Kochert, G 1998. Microsatellites in Cassava (*Mannihot esculenta* Crantz): discovery, inheritance and variability. *Theor. Appl. Genet.* 97: 493-501.

Christensen, M., Sunde, L., Bolund, L. and Orntoft, T. F., 1999. Comparison of three methods of microsatellite detection. *Scandinavian J. Clin. Lab. investigation, 59*(3), pp.167-177.

Coates, D. J., Yen, D. E., and Gaffey, P. M. 1988. Chromosome vatiation in taro *Colocasia esculenta*: Implications of origin in the Pacific. *Cytologia* 53: 551-560.

Collard, B. C. Y., Jahufer, M. Z. Z., Brouwer, J. B., and Pang, E. C. K. 2005. An introduction to markers, quantitative trait loci (QTL) mapping and marker – assisted selection for crop improvement: The basic concepts. *Euphytica* 142: 169-196.

Dabo, S, M., Mitchell, E. D., and Melcher, U. 1993. A method for the isolation of nuclear DNA from cotton (*Gossypium*) leaves. *Anal. Biochem.* 210(1): 34-38.

de Vicente, M. C., Guzman, F. A., Engles, J., and Rao, V. R. 2006. Genetic characterization and its use in decision making for the conservation of crop germplasm. In: Ruane, J. and Sonnino, A. (eds), *The role of biotechnology in exploring and protecting agricultural genetic resources.* Food and Agriculture Organization, Italy, pp. 121-128.

Deo, P. C., Harding, R. M., Taylor, M., Tyagi, A. P., and Becker, D. K., 2009. Somatic embryogenesis, organogenesis and plant regeneration in taro (*Colocasia esculenta* var. *esculenta*). *Pl. Cell Tissue Org. Cult.* 99(1): 61-71.

Devi, A. A. 2012. Genetic diversity analysis in taro using molecular markers – An overview. *J. Root Crops* 38(1): 15-25.

Devi, A. A., Suja, G., and Sreekumar, J. 2013. Analysis of genetic diversity in edible aroid accessions of India based on morphological characters. *J. Root Crops* 39(2): 51-56.

Dograr, N. and Akkaya, M.S., 2001. Optimization of PCR amplification of wheat simple sequence repeat DNA markers. *Turkish J. Biol.* 25(2): 153-158.

FAOSTAT [Food and Agriculture Organization Statistical Database]. 2014 [on-line]. Available: http://faostat.fao.org. [03 Aug. 2017].

Ghosh, R., Paul, S., Ghosh, S. K., and Roy, A. 2009. An improved method of DNA isolation for PCR based detection of begomovirus from jute and other mucilaginous plants. *J. Virol. Met.* 159: 34-39.

Godwin, I. D., Mace, E. S., Mathur, P. N, and Izquierdo, L. 2003. Applications of DNA markers to management of taro (*Colocasia esculenta* (L.) Schott) genetic resources in the Pacific Island region. In: Guarino, L., Taylor, M., and Osborn, T. (eds), *Third Taro Symposium.* Proceedings of an International Scientific Meeting. Nadi, Fiji Islands. Secretariat of the Pacific Community and International Plant Genetic Resources Institute, pp. 174-180.

Grassman, W. and Deffner, G. 1953. Verteilungschromatographisches Verhalten von Proteinen und Peptiden in phenolhaltigen Lösungsmitteln. *Hoppe-Seylor's Z. Physiol. Chem.* 293: 89-98.

Grodzicker, T., Williams, J., Sharp, P., and Sambrook, J. 1974. Physical mapping of temperature sensitive mutants of adenovirus. *Cold Spring Harbor Symp Quant. Biol.* 39: 439-446.

Gupta, P. K. and Varshney, R. K. 2000. The development and use of microsatellite markers for genetic analysis and plant breeding with emphasis on bread wheat. *Euphytica.* 113: 163-185.

Haberle, S. G. 1995. Identificationof cultivated *Pandanus* and *Colocasia* in pollen records and the implications for the study of early agriculture in New Guinea. *Vegetn. Hist. Archaeobotany* 4: 195-210.

Hartl, D. L. and Clark, A. G. 1997. *Principles of population genetics* (3rd ed.). Sinauer Associates, Sunderland, Massachsetts, Unites States of America, 542p.

Hussain, Z. and Tyagi, R. K. 2006. *In vitro* corm induction and genetic stability of regenerated plants in taro [*Colocasia esculenta* (L.) Schott]. *Indian J. Biotechnol.* 4: 535-542.

IITA (International Institute of Tropical Agriculture) 1980. Sweet potato tissue culture and virus indexing. *Annual Report 1979.* International Institute of Tropical Agriculture, Ibada, Nigeria, 90p.

Ijas, S. 2011. Microsatellite markers: An important fingerprinting tool for characterization of crop plants. *Afr. J. Biotechnol.* 10(40): 7723-7726.

Irwin, S. V., Kaufusi, P., Banks, K. de la Pena, R., and Cho, J. J. 1998. Molecular characterization of taro (*Colocasia esculenta*) using RAPD markers. *Euphytica* 99: 183-189.

Ivancic, A. and Lebot, V. 2000. *The Genetics and Breeding of Taro.* CIRAD publication series, Montpellier, France, 194p.

Jeffreys, A. J., Murray, J. and Neumann, R., 1998. High-resolution mapping of crossovers in human sperm defines a minisatellite-associated recombination hotspot. *Mol. cell* 2(2): 267-273.

Jiang, Y. and Liu, J. P. 2011. Evaluation of genetic diversity in Piper spp. using RAPD and SRAP markers. *Genet. Mol. Res.* 10(4): 2934-2943.

Jordan, S. A. and Humphries, P. 1994. Single nucleotide polymorphism in exon 2 of the BCP gene on 7q31 – q35. *Hum. Mol. Genet.* 3: 1915.

Jose, J. and Usha, R. 2000. Extraction of geminiviral DNA from a highly mucilaginous plant (*Abelmoschus esculentus*). *Plant. Mol. Biol. Rep.* 18: 349-355.

Kan, H., Xing, F. H., Wei, D. K., and Yi, D. 2009. Characterization of 11 new microsatellite loci in taro (*Colocasia esculenta*). *Mol. Ecol. Resour.* 9: 582-584.

Keller, E. R., senula, A., Leunifna, S., and Grube, M. 2006. Slow growth storage and cryopreservation – tools to facilitate germplasm

maintenance of vegetatively propagated crops and living plant collections. *Int. J. Refrigern.* 29: 411-417.

King, R.C. and Stansfield, W.D., 1990. *Encyclopedic dictionary of genetics*. VCH Verlagsgesellschaft mbH., Wienham, Germany, 809p.

Kirby, K.S. 1956. A new method for the isolation of ribonucleic acids from mammalian tissues. *Biochem. J.* 64: 405.

Kokubugata, G. and Konishi, T. 1999. Implication of a basic chromosome number of x = 14 in seven cultivars of two varieties of *Colocasia esculenta* by fluorescent in situ hybridization using rDNA probe. *Cytologia*, 64: 77 - 83.

Krahu, S. T. 1997. Sucrose use in relation to shoot induction by sorbitol and cytokinin in apple. *J. Am. Soc. Hort. Sci.* 122: 476-480.

Krieke, C. M., Van Eck, H. J., and Lebot, V. 2004. Genetic diversity of taro, *Colocasia esculenta* (L.) Scott, in Southeast Asia and the Pacific. *Theor. Appl. Genet.* 109: 761-768.

Krishnan, R. and Magoon, M. L. 1977. Edible aroids – new insights into phylogeny. In: Leakey, C. L. A. (Ed.), *Proceedings of the Third International Symposium on Tropical Tuber Crops*. Ibadan, Nigeria, International Institute of Tropical Agriculture. pp. 58 – 60.

Kumar, L.S., 1999. DNA markers in plant improvement: an overview. *Biotechnol. Adv.* 17(2): 143-182.

Kuruvilla, K. M. and Singh, A. 1981. Karyotypic and electrophoretic studies on taro and its origins. *Euphytica* 30: 405-412.

Lakhanpaul, S., Velayudhan, K. C., and Bhat, K. V. 2003. Analysis of genetic diversity in Indian taro (*Colocasia esculenta* (L.) Schott) using

Random amplified polymorphic DNA (RAPD) markers. *Genet. Resour. Crop. Evol.* 50: 603-609.

Lebot, V. 1999. Biomolecular evidence for plant domestication in Sahul. *Genet. Resour. Crop Evol.* 46: 619-628.

Lebot, V. and Aradhya, M. 1991. Isozyme variation in taro (*Colocasia esculenta*(L.) Schott.) from Asia and Pacific. *Euphytica* 56: 55-66.

Lebot, V., Prana, M. S., Krieke, N., vanHeck, H., Pardales, J., Hue, H., Viet, N., andYap, T. C. 2004. Characterization of taro (*Colocasia esculenta* (L.) Schott) genetic resources in Southeast Asia and Oceania. *Genet. Resour. Crop Evol.* 51: 381-392.

Lele, S. S. 2011. Isolation and PCR amplification of genomic DNA from traded seeds of nutmeg (*M. fragrans*). *J. Biol. Agri. Healthcare* 1(3): 1-7.

Leon, J. 1977. Origin, evolution, and early dispersal of root and tuber crops. In: Cook, J., MacIntyre, R., and Graham, M. (eds), *Proceedings of the Fourth Symposium of the International Society for Tropical Root Crops*, 1-7 August 1976, Cali, Columbia. International Development Research Centre, Ottawa, pp. 20-36.

Li, C. D., Fatokun, C. A., Ubi, B., Singh, B. B., and Scoles, G. 2001.Determining genetic similarities among cowpea breeding lines and cultivars by microsatellite markers, *Crop. Sci.* 41: 189-197.

Li, H., Luo, J., Hemphill, J. K., Wang, J. T, and Gould, J. H. 2001. A rapid and high yielding DNA miniprep for Cotton (*Gossypium* spp.). *Pl. Mol. Biol. Rep.* 19: 183a-183e.

Litt, M. and Luty, J. M. 1989. A hypervariable microsatellite revealed by *in vitro* amplification of a dinucleotide repeat within the cardiac muscle actin gene. *Am. J. Hum. Genet.* 44: 397-401.

Loy, T. H., Spriggs, M., and Wicklet, S. 1992. Direct evidence for human use of plants 28,000 years ago: starch residues on stone artefacts from Solomon Islands. *Antiquity*. 66: 898-912.

Mace, E. S. and Godwin, I. D. 2002. Development and characterization of polymorphic microsatellite markers in taro, *Colocasia esculenta* (L.) Schott. *Genome* 45: 823-832.

Mace, E. S., Mathur, P. N, Izquierdo, L., Hunter, D., taylor, M. B., Singh, D., DeLacy, I. H., Jackson, G. V. H., and Godwin, I. D. 2006. Rationalization of taro germplasm collections in the Pacific Island region using simple sequence repeat (SSR) markers. *Plant Genet. Resour.: Charac. Util.* 4: 210-220.

Malaurie, B., Truslot, M. F., Chabrillange, N., and Engelmann, F. 1998. Survival and recovery of yam (*Dioscorea spp.*) apices after cryopreservation using encapsulation dehydration. In: Engelmann, F. and Takagi, H. (eds), *Cryopreservation of Tropical Plant Germplasm: Current Research Progress and Application.* Japan International Research Centre for Agricultural Sciences, Tsukuba, Japan, pp 414-417.

Mandal, B. B., Tyagi, R. K., Pandey, R. P., Sharma, N., and Agarwal, A. 2000. *In vitro* conservation of agri – horticultural crops at NBPGR: An overview. In: Razdan, M. K. and Cocking, E. C. (eds), *Conservation of plant genetic resource in vitro: Applications and Limitations: Vol 2.* Oxford and IBH, New Delhi. Pp. 279-308.

Manzano, A. R., Nodals, A. A. R., Gutierrez, M. I. R., Mayor, Z. F., and Alfonso, L. C. 2001. Morphological and isozyme variability of taro (*Colocasia esculenta* (L.) Schott) germplasm in Cuba. *Pl. Genet. Resour. Newsl.* 126: 31-40.

Matews, P., Matsushita, Y., Sato, T., and Hirai, M. 1992. Ribosomal and mitochondrial DNA variation in Japanese taro (*Colocasia esculenta* (L.) Schott). *Jpn. J. Breed.* 42: 825-833.

Matsuda, M. and Newata, E. 2002. Geographical distribution of ribosomal DNA variation in taro (*Colocasia esculenta* (L.) Schott), in Eastern Asia. *Euphytica* 128: 165-172.

Matthews, P. J. 1995. Aroids and the Austroneseans. *Tropics* 4 (2): 105-126.

Maurai, I. R. and Maria, C. F. 1993. *In vitro* conservation of *Colocasia esculenta* (L.) Schott germplasm. I Jardim – Museu Agricola Tropical / IICT, Largo dos Jeronimos, Portugal, 65p.

Mezhii, T. L., Changkija, S., Pattanayak, A., Chaturvedi, H. P., Devi, S. V., and Kole, P. R. 2017. Genetic characterization of locally cultivated taro germplasm from eleven district of Nagaland, India. *Int. J. Curr. Microbiol. Appl. Sci.* 6(8): 3338-3348.

Mohan, M., Nair, S., Bhagwat, A., Krishna, T. G., Yano, M., Bhatia, C. R., and Sasaki, T. 1997. Genome mapping, molecular markers and marker-assisted selection in crop plants. *Mol. Breed.* 3: 87-103.

Mulualem, T., WeldeMicheal, G., and Belachew, K. 2013. Genetic diversity of taro (*Colocasia esculenta* (L.) Schott) genotypes in Ethiopia based on agronomic traits. *Time J. Agric. Vet. Sci.* 1(2): 23-30.

Ng, S. Y. C. and Hahn, S. K. 1985. Application of tissue culture to tuber crops at IITA (International Institute of Tropical Agriculture). In: Burn, K.

and Mandoza, C. V. (eds), *Biotechnology in International Agricultural Research.* Proceedings of the Inter – Center Seminar on International Agricultural Research Centers (IARCs) and Biotechnology. 23-27 April 1984, Los Banos, Philippines. International Rice Research Institute, Philippines, pp. 29-40.

Ng. S. Y. C. and Ng, N. Q. 2002. Genetic resources and conservation. In: Hillocks, R. J., Thresh, J. M., and Belloti, A. C. (eds), *CAB (Centre for Agriculture and Biosciences) International Cassava: Biology, Production and Utilization.* Centre for Agriculture and Biosciences International, Egham, United Kingdom, pp. 167-177.

Nguyen, T. T., Taylor, P. W. J., Redden, R. J., and Ford, R. 2004. Genetic diversity estimates in *Cicer* using AFLP analysis. *Plant Breed.* 123: 173-179.

Noyer, J. L., Billot, C., Weber, A., Brottier, P., Quero-Garcia, J., and Lebot, V. 2004. Genetic diversity of taro (*Colocasia esculenta* (L.) Schott) assessed by SSR markers. In: Guarino, L., Taylor, M., and Osborn, T. (eds), *Third Taro Symposium.* Proceedings of an International Scientific Meeting. Nadi, Fiji Islands. Secretariat of the Pacific Community and International Plant Genetic Resources Institute, pp. 174-180.

Nunes, R. S. C., Pinhati, F. R., Golinelli, L. P., Reboucas, T. N. H., Paschoalin, V. M. F., and da Silva, J. T. 2012. Polymorphic microsatellites of analysis in cultivars of taro. *Horticul. Bras.* 30: 106 – 111.

Nusaifa Beevi, P. 2009. Biosystematic studies in taro (*Colocasia esculenta* (L.) Schott.). *Ph.D. Thesis*. University of Kerala, Thiruvananthapuram, India. 280p.

Ochiai, T., Nguyen, V. X., Tahara, M., and Yoshinno, H. 2001. Geographical differentiation of Asian taro, *Colocasia esculenta* (L.) Schott., detected by RAPD and isozyme analysis. *Euphytica* 122: 219-234.

Ogliari, J. B., Boscariol, R. L., and Camargo, L. E., 2000. Optimization of PCR amplification of maize microsatellite loci. *Genet. Mol. Biol.* 23(2): 395-398.

Olsen, M., Hood, L., Cantor, C., and Botstein, D. 1989. A common language for physical mapping of the human genome. *Science* 245: 1434-1435.

Paran, I. and Michelmore, R. W. 1993. Development of reliable PCR-based markers linked to downy mildew resistance genes in lettuce. *Theor. Appl. Genet.* 85: 985-993.

Park, Y. J., Lee, J. K., and Kim, N. S., 2009. Simple sequence repeat polymorphisms (SSRPs) for evaluation of molecular diversity and germplasm classification of minor crops. *Molecules* 14(11): 4546-4569.

Pissard, A., Arbizu, C., Ghislain, M., Faux, A. M., Paulet, S., and Bertin, P. 2008. Congruence between morphological and molecular markers inferred from the analysis of the intra-morphotype genetic diversity and the spatial structure of *Oxalis tuberosa*. *Mol. Genetica* 132(1): 71-85.

Plucknett, D. L. 1976. Edible aroids: *Alocasia, Colocasia, Cyrtosperma, Xanthosoma.* In: Simmonds, N. W. (ed.), *Evolution of Crop Plants.* Longman Press, London, UK, pp. 10-12.

Powell, W., Morgante, M., Andre, C., Hanafey, M., Vogel, M. J., Tingey, S. V., and Rafalski, A. 1996. The comparison of RFLP, RAPD, AFLP and SSR (microsatellites) markers for germplasm analysis. *Mol. Breed.* 2: 225-235.

Prana, M. S., Hartati, N. S., Prana, T. K., and Luswara, T. 2000. Evaluation on genetic variation in taro (*C. esculenta* (L.) Schott.) collected from West Java using isozymic markers. *Ann. Bogorienses* 6(2): 80-87.

Prittila, A. M., Hirsicorpi, M., Kamarainen, T., Jakkola, L., and Hohtala, A. 2001. DNA isolation method for medicinal and aromatic plants. *Pl. Mol. Biol. Rep.* 19: 273a-273f.

Purseglove, J. W. 1972. *Tropical Crops: Monocotyledons.* Longman Press, London, UK, 607p.

Quero-Garcia, J., Noyer, J. L., Perrier, X., Marchand, J. L., and Lebot, V. 2004. A germplasm stratification of taro (*Colocasia esculenta*) based on agro-morphological descriptors, validation by AFLP markers. *Euphytica* 137: 387-395.

Rajaasekharan, P. E. and Sahijram, L. 2015. *In vitro* conservation of plant germplasm. In: Bahadur, B., Rajam, V., Sahijram, L., and Krishnamurty, K. V. (eds), *Plant Biology and Biotechnology: Volume II, Plant genomics and Biotechnology.* Springer, India, 768p.

Rallo, P., Dorado, G., and Martín, A. 2000. Development of simple sequence repeats (SSRs) in olive tree (*Olea europaea* L.). *Theor. Appl. Genet.* 101: 984-989.

Ramachandran, K. 1978. Cytological studies in South Indian Araceae. *Cytologia* 43: 289-303.

Ramsay, L., Macaulay, M., Degli Ivanissevich, S., MacLean, K., Cardle, L., Fuller, J., Edwards, K. J., Tuvesson, S., Morgante, M., Massari, A., and Maestri, E., 2000. A simple sequence repeat-based linkage map of barley. *Genetics* 156(4): 1997-2005.

Rao, R. V. 2010. Taro genetic diversity and its use in taro improvement. In: Rao, R. V., Matthews, P. J., Eyzaguirre, P. B., and Hunter, D. (eds), *The Global Diversity of Taro – Ethnobotany and Conservation.* Biodiversity International, Rome, Italy, pp. 121-136.

Rattenbury, J. A. 1956. Cytotaxonomy and the migration of primitive people. *Nature* 178: 545-546.

Richard, G. F., Kerrest, A., and Dujon, B. 2008. Comparitive genomics and molecular dynamics of DNA repeats in eukaryotes. *Microbiol. Mol. Biol. Rev.* 72: 686-727.

Roa, A. C., Chavarriaga-Aguirre, P., Duque, M. C., Maya, M. M., Bonierbale, M. W., Iglesias, C., and Tohme, J. 2000. Cross species amplification of cassava (*Mannihot esculenta*) (Euporbiaceae) microsatellites: allelic polymorphism and degree of relationship. *Am. J. Bot.* 87: 1647-1655.

Roca, W. M. 1985. Cassava tissue culture. In: Cock, J. H. and Reyes, J. A. (eds), *Cassava: Research, Production and Utilization.* United Nations Development Program, Cali, pp 173-204.

Roder, M. S., Plaschke, J., Konig, S. U., Borner, A., Sorrells, M. E., Tanksley, S. D., and Canal, M. W. 1995. Abundance, variability and chromosomal location of microsatellites in wheat. *Mol. Gen. Genet.* 246: 327-333.

Sablok, G., Gahlot, P., Gupta, A. K., Pareek, K., and Shekhawat, N, S. 2009. Extraction of PCR usable DNA from trees of arid environment. *Pl. Omics J.* 2: 103-109.

Salisbury, F. B. and Ross, C. W. 1992. *Plant physiology* (4th Ed.). Wadsworth Publishing Company, California, United States of America, 682p.

Sant, R., Taylor, M., and Tyagi, A. 2006. Cryopreservation of *in vitro* grown shoot tips of tropical taro (*Colocasia esculenta* var. *esculenta*) by vitrification. *CryoLetters* 27(3): 133-142.

Sardos, J., Noyer, J. L., Malapa, R., Bouchet, S., and Lebot, V. 2012. Genetic diversity of taro (*Colocasia esculenta* (L.) Schott) in Vanuatu (Oceania): an appraisal of the distribution of allelic diversity (DAD) with SSR markers. *Genet. Resour. Crop Evol.* 59(5): 805-820.

Schnell, R. J., Goenaga, R., and Olano, C. T. 1999. Genetic similarities among cocoyam cultivars based on randomly amplified polymorphic DNA (RAPD) analysis. *Scientia Hortic.* 80: 267-276.

Schott, K. D., Eggler, P., Seaton, G., Rosetto, M., Ablett, E. M., Lee, L. S., and Henry, R. J. 2000. Analysis of SSRs derived from grape ESTs. *Theor. Appl. Genet.* 100: 723-726.

Schulmann, A. H. 2007. Molecular markers to assess genetic diversity. *Euphytica* 158: 313-321.

Sharma, K., Mishra, A. K., and Misra, R. S. 2008a. A simple and efficient method for the extraction of DNA from tropical tuber crops. *Afr. J. Biotechnol.* 7(8): 1018-1022.

Sharma, K., Mishra, A. K., and Misra, R. S. 2008b. The genetic structure of taro: a comparison of RAPD and isozyme markers. *Pl. Biotechnol. Rep.* 2: 191-198.

Shibli, R. A., Smith, M. A. L., and Spomer, L. A. 1992. Osmotic adjustment and growth responses of three Crysanthemum (*Crysanthemum morifolium* Ramat.) cultivars to osmotic stress induced *in vitro*. *J. Pl. Nutr.* 15: 1373-1381.

Sibli, R. A. 1990. Physiological studies of *in vivo* and *in vitro* water requirements and water relations of Crysanthemum (*Crysanthemum morifolium* Ramat.), PhD Dissertation, University of Illinois, Illinois, United States of America, 50p.

Singh, D., Mace, E. S., Godwin, I. D., Mathur, P. N., Okpul, T., Taylor, M., Hunter, d., Kambuou, R., Rao, R. V., and Jackson, G. 2008. Assessment and rationalization of genetic diversity of Papua New Guinea taro (*Colocasia esculenta*) using SSR DNA fingerprinting. *Genet. Resour. Crop Evol.* 55: 811-822.

Singh, S., Singh, D. R., Faseela, F., Kumar, N., Damodaran, V., and Srivastava, R. C. 2011. Diversity of 21 taro (*Colocasia esculenta* (L.) Schott) accessions of Andaman Islands. *Genet. Resour. Crop Evol.* 59(5): 821-829.

Smulders, M. J. M., Bredemeijer, G., Rus-Kortekaas, W., Arens, P., and Vosman, B., 1997. Use of short microsatellites from database sequences to generate polymorphisms among Lycopersicon esculentum cultivars and accessions of other Lycopersicon species. *TAG Theor. Appl. Genet.* 94(2): 264-272.

Somasundaram, S. T. and Kalaiselvam, M. 2011. Molecular tools for assessing genetic diversity. *International Training Course on Mangroves and Biodiversity*. Annamalai University, India, pp. 82-91.

Song, M., Wang, X., and Chen, S. 1994. *In vitro* preservation and propagation of taro. *J. Southwest Agric. Univ.* 5: 108-110.

Sreekumari, M. T. 1992. Cytomorphological and cytogenetic studies in edible aroids. *Ph.D. Thesis*. University of Kerala, Thiruvananthapuram, India. 260p.

Sreekumari, M. T. 1997. Cytological studies in taro – a review. *J. Root Crops*, 23(1): 1 – 7.

Srinivas, T., Nedunchezhiyan, M., Misra, S. R. 2012. *Marketing System of Aroids and Yams in India.* Central Tuber Crops Research Institute, Sreekariyam, Kerala, India, 80p.

Staritsky, G., Dekkers, A. J., Louwaars, N. P., and Zandvoort, E. A. 1986. *In vitro* conservation of aroid germplasm at reduced temperatures and under osmotic stress. In: Withers, L. A. and Alderson, P. G. (eds), *Plant Tissue Culture and its Agricultural applications,* Butterwort, London, pp. 277-284.

Stebbins, G. L. 1957. Self-fertilization and population in the higher plants. *Am. Nat.*, 91: 337–354.

Strand, M., Prolla, T. A., Liskay, R. M., and Petes, T. D. 1993. Destabilization of tracts of simple repetitive DNA in yeast by mutations affecting DNA mismatch repair. *Nature* 365: 274-276.

Taylor, M., Tuia, V., Sant, R., Lesione, E., Prasad, R., Prasad, R. L., and Vasaki, A. 2003. Using *in vitro* techniques for the conservation and utilization of *Colocasia esculenta* var. *esculenta* (taro) in a regional genebank. In: Guarino, L. Taylor, M., and Osborn, T. (eds), *Third Taro Symposium*. Proceedings of an International Scientific Meeting, Nadi, Fiji Islands. Secretariat of the Pacific Community and International Plant Genetic Resources Institute, pp. 69-73.

Terauchi, R. and Konuma, A. 1994. Microsatellite polymorphism in *Dioscorea tokoro*, a wild yam species. *Genome* 37: 794-801.

Trimanto, Sajidan, and Sugiyarto. 2010. Characterization of taro (*Colocasia esculenta*) based on morphological and isozymic patterns markers. *Nus. Biosci.* 2: 7-14.

Unnikrishnan, M. and Sheela, M. N. 2000. Biotechnology in conservation and improvement of tuber crops. In: Chada, K. L., Ravindran, P. L., and Sahijram, L. (eds), *Biotechnology in Horticultural and Plantation Crops,* Malhotra Publishing House, New Delhi, India, 836p.

Unnikrishnan, M., Velayudan, K. C., Hariprakash, B., Ramadasan, N. P., and Naskar, S. K. 2013. Biodiversity of edible aroids in India. In: Misra, R. S. and Nedunchezhiyan, M. (eds), *Aroids: Opportunities and Challenges.* Regional Centre, Indian Council of Agricultural Research – Central Tuber Crops Research Institute, Bhubaneswar, Odisha, India, 364p.

van der Schoot, J., Pospiskova, M., Vosman, B., Smulders, M. J. M. 2000. Development and characterization of microsatellite markers in black poplar (*Populus nigra* L.). *Theor. Appl. Genet.* 101: 317-322.

Vinutha, K. B. 2014. Genetic diversity analysis of North-east Indian taro (*Colocasia esculenta* (L.) Schott). M.Sc. (Integrated) Biotechnology thesis, Kerala Agricultural University, Thrissur, 110p.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., van der Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M., and Zabeau, M. 1995. AFLP: a new technique for DNA fingerprinting. *Nucl. Acids Res.* 23: 4407-4414.

Warude, D., Chavan, P., Joshi, K., and Patwardhan, B. 2003. DNA isolation from fresh, dry, plant samples with highly acidic tissue extracts. *Pl. Mol. Biol. Rep.* 21: 467a-467f.

Weising, K., Nybom, H., Wolff, K., and Kahl, G. 2005. *DNA fingerprinting in plants: Principles, Methods and Applications.* CRC Press, Taylor and Francis roup, 6000Broken Sound Parkway, New York, 444p.

Welsh, J. and McClelland, M. 1991. Genomic fingerprinting using arbitrarily primed PCR and a matrix of pairwise combinations of primers combinations of primers. *Nucleic Acid Res.* 19: 5275-5279.

Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalski, J. A., and Tingey, S. V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucl. Acids Res.* 18: 651-655.

Withers, L. A. 1980. Tissue storage for genetic conservation. *Pl. Gen. Res.* 11: 93-109.

Withers, L. A. 1987. Long-term preservation of plant cells, tissues and organs. *Mol. Cell. Biol.* 4: 221-272.

Withers, L. A. 1991. *In vitro* conservation. *Biol. J. Linnean Soc.* 43: 31-42.

Yam, T. W., Webb, E. L., Arditti, J. 1990. Callus formation and plantlet regeneration from axillary buds of *Colocasia esculenta* var *esculenta* (Araceae). *Planta* 180: 458-460.

Yen, D. E. 1991. Domestication: the lessons from New Guinea. In: Pawley, A. (ed.), *Man and a Half.* Polynesian Society, Auckland, pp. 558-570.

Yen, D. E. 1993. The origins and subsistence of agriculture in Oceania and the potentials for future tropical food crops. *Economic Bot.* 47: 3-14.

Yen, D. E. and Wheeler, J. M. 1968. Introduction of taro into the Pacific: the indications of the chromosome numbers. *Ethnology* 7: 259-267.

Zhang, J. and Steward, J. M. 2000. Economical and rapid method for extracting cotton genomic DNA. *J. Cotton Sci.* 4(3): 193-201.

Zhonglei, L., Wansha, L., Yongping, Y., and Xiangyang, H. 2011. Isolation and characterization of 19 new microsatellite loci in *Colocasia esculenta* (Araceae). *Am. J. Bot.* 98(9): e239-e241.

Zietkiewicz, E., Refalski, A., and Labuda, D. 1994. Genomic fingerprinting by simple sequence repeat (SSR) – anchored polymerase chain reaction amplification. *Genomics* 20: 176-183.

APPENDICES

APPENDIX I

Extraction buffer (CTAB method I and CTAB method II)

Tris HCl (pH 8.0)	- 100mM	
EDTA (pH 8.0)	- 20mM	
NaCl	- 2M	
CTAB	- 2%	
PVP	- 2%	freshly added prior to
β – mercaptoethanol	- 2%	extraction procedure

2% CTAB was weighed, dissolved in 100mM Tris HCl, 20mM EDTA and 2M NaCl and was made to the required volume. The mixture was heated for the components to dissolve. 2% PVP and 2% β – mercaptoethanol was added prior to the extraction procedure.

APPENDIX II

Extraction buffer (CTAB method III)

Tris HCl (pH 8.0)	- 100mM	
EDTA (pH 8.0)	- 20mM	
NaCl	- 2M	
CTAB	- 2%	
PVP	- 2%	freshly added prior to
β – mercaptoethanol	- 0.2%	extraction procedure

2% CTAB was weighed, dissolved in 100mM Tris HCl, 20mM EDTA and 2M NaCl and was made to the required volume. The mixture was heated for the

components to dissolve. 2% PVP and 0.2% β – mercaptoethanol was added prior to the extraction procedure.

APPENDIX III

TE buffer (10X)

Tris HCl (pH 8.0) - 10mM

EDTA (pH 8.0) - 1Mm

Tris HCl and EDTA was measured and was dissolved in required amount of water using a magnetic stirrer.

APPENDIX IV

TBE buffer (10X)

Tris base - 108g

Boric acid - 55g

0.5M EDTA (pH 8.0) - 40ml

Dissolve in 800ml distilled water using a magnetic stirrer. Make up to 1000ml and autoclave before use.

APPENDIX V

40% acrylamide solution

Acrylamide - 38g

Bis – acrylamide - 2g

Distilled water - 100ml

Acrylamide and Bis-acrylamide was measured and was dissolved in distilled water. The final volume was made upto 100ml.

APPENDIX VI

Coating on the outer glass plate and IPC unit

Bind silane (for outer glass plate)

99.5% ethanol	- 497.5ml
0.5% acetic acid	- 2.5ml
Bind silane	- 1µl

99.5% ethanol, 0.5% acetic acid and bind silane was mixed properly in a reagent bottle

Repellant (for IPC unit)

Labolene	- 1ml

APPENDIX VII

6% Polyacrylamide gel containing 7M urea

Urea	- 42g
10X TBE buffer	- 10ml
Distilled water	- 15ml
40% acrylamide solution	- 15ml
TEMED	- 60µl
APS (100mg/ml)	- 600µl

42g urea was dissolved in a beaker containing 10ml 10X TBE buffer and 15ml distilled water by heating in the microwave oven for 1 min. 15ml 40% acrylamide solution was filtered and added to a measuring cylinder followed by urea solution. The final volume was made up to 100ml using distilled water and stored in dark till use. TEMED and APS was added in 1:10 ratio and mixed just before casting the gel.

APPENDIX VIII

PAGE dye

Formamide	- 50ml
Xylene cyanol	- 50mg
Bromophenol blue	- 50mg
0.5M EDTA	- 1ml

To 50ml formamide, 50mg xylene cyanol, 50mg bromophenol blue and 1ml 0.5M EDTA was added and dissolved

APPENDIX IX

Silver staining components

Fixer:

Acetic acid	- 200ml
Distilled water	- 1800ml

200ml acetic acid was taken in a measuring cylinder and was made upto 2000ml using distilled water.

Silver stain

$AgNO_3$	- 2g
Distilled water	- 2000ml
Formaldehyde	- 3ml

2g $AgNO_3$ was dissolved in distilled water and made up to a final volume of 2000ml. 3ml formaldehyde was added to it.

Developer

Na_2CO_3	- 60g
Distilled water	- 2000ml
Formaldehyde	- 3ml

Sodium thiosulfate (10mg/ml) - 400µl

60g Na_2CO_3 was dissolved in distilled water, made up to 2000ml and was stored in -20°C. 3ml formaldehyde and 400µl sodium thiosulfate was added to it and mixed thoroughly before use.

APPENDIX X

Basal MS

MS salt	- 4.41g
Sucrose	- 3%

Agar - 0.8%

Distilled water - 1000ml

MS salt and sucrose was dissolved in 1000ml distilled water. pH 5.7l was obtained with the help of 1N HCl and 1N NaOH. Agar was added and the media was dissolved by boiling.

APPENDIX XI

Shoot Multiplication Media

MS+TDZ (0.5mg/l) (250ml)

MS salt - 4.41g/l

Sucrose - 3%

TDZ - 0.5 mg/l

Agar - 0.8%

pH 5.7 was obtained with the help of 1N HCl and 1N NaOH. Agar was added and the media was dissolved by boiling.

MS+TDZ (1mg/l) (250ml)

MS salt - 4.41g/l

Sucrose - 3%

TDZ - 1.0 mg/l

Agar - 0.8%

pH 5.7 was obtained with the help of 1N HCl and 1N NaOH. Agar was added and the media was dissolved by boiling.

MS+TDZ (0.2mg/l) (250ml)

MS salt	- 4.41g/l
Sucrose	- 3%
TDZ	- 0.2 mg/l
Agar	- 0.8%

pH 5.7 was obtained with the help of 1N HCl and 1N NaOH. Agar was added and the media was dissolved by boiling.

MS+TDZ (0.1mg/l) (250ml)

MS salt	- 4.41g/l
Sucrose	- 3%
TDZ	- 0.1 mg/l
Agar	- 0.8%

pH 5.7 was obtained with the help of 1N HCl and 1N NaOH. Agar was added and the media was dissolved by boiling.

MS+B (5mg/l) (250ml)

MS salt	- 4.41g/l
Sucrose	- 3%
B	- 5 mg/l
Agar	- 0.8%

pH 5.7 was obtained with the help of 1N HCl and 1N NaOH. Agar was added and the media was dissolved by boiling.

MS+B (1mg/l) (250ml)

MS salt	- 4.41g/l
Sucrose	- 3%
B	- 1 mg/l
Agar	- 0.8%

pH 5.7 was obtained with the help of 1N HCl and 1N NaOH. Agar was added and the media was dissolved by boiling.

MS+K (1mg/l) (250ml)

MS salt	- 4.41g/l
Sucrose	- 3%
K	- 1 mg/l
Agar	- 0.8%

pH 5.7 was obtained with the help of 1N HCl and 1N NaOH. Agar was added and the media was dissolved by boiling.

APPENDIX XII

MS+2% mannitol (500ml)

MS salt	- 4.41g/l
Mannitol	- 2%
Agar	- 0.8%

pH 5.7 was obtained with the help of 1N HCl and 1N NaOH. Agar was added and the media was dissolved by boiling.

MS+2% sucrose (500ml)

MS salt	- 4.41g/l
Sucrose	- 2%
Agar	- 0.8%

pH 5.7 was obtained with the help of 1N HCl and 1N NaOH. Agar was added and the media was dissolved by boiling.

ABSTRACT

MOLECULAR CHARACTERIZATION AND *IN VITRO* CONSERVATION OF TARO (*Colocasia esculenta* (L.) Schott)

SREEVIDYA M. R.

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Abstract of the thesis
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Faculty of Agriculture
Kerala Agricultural University, Thrissur

DEPARTMENT OF PLANT BIOTECHNOLOGY
COLLEGE OF AGRICULTURE
VELLAYANI, THIRUVANANTHAPURAM-695 522
KERALA, INDIA
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ABSTRACT

The study entitled "Molecular characterization and *in vitro* conservation of taro (*Colocasia esculenta* (L.) Schott)" was carried out in the Division of Crop Improvement, ICAR – Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram during the period 2016-17. The main objective of the study was to analyze the genetic diversity of taro (*Colocasia esculenta*) using SSR markers and to maintain them in slow growth conditions for medium term conservation. 36 taro accessions comprising germplasm from various parts of the country, varieties and breeding lines were collected from the taro germplasm maintained at ICAR – CTCRI for molecular characterization and *in vitro* studies.

Good quality and pure DNA was isolated from the selected 36 taro accessions by modified Sharma, *et al.* (2008) protocol. The quantity of the DNA obtained ranged from 372ng (Line 11) to 2285 ng/µl (DT 1) and the OD values were also good ranging from 0.050 (C-717) to 0.457 (NEH 21). 10 SSR markers were selected for the study, eight from the Uq series (Mace and Godwin, 2002) and two from the Ce1 series (Noyer *et al.,* 2004). The annealing temperatures of these 10 primers were standardized using gradient PCR and ranged from 60 to 68°C. PCR amplified SSR amplicons were resolved in 6% PAGE and 4% high resolution agarose gel electrophoresis. Since, both methods of electrophoresis gave similar results, 4% agarose gel electrophoresis was adopted for further resolution of amplicons from the rest of the primers as it was less cumbersome. The bands obtained were scored as '0','1' based on the presence (1) or absence (0) of bands for further statistical analysis. The SSR primers were found to be highly polymorphic across the 36 accessions and was explained by parameters like Shannon's diversity index which ranged from 0.88 to 2.09, average number of alleles per locus which ranged from 1.81 to 2.67 and polymorphism information content which ranged from 0.58 to 0.80. The 36 accessions were found to be diverse which was

explained by the heterozygosity value (He) which ranged from 0.65 to 0.82. Jaccard's coefficient was used in NTSys – PC to generate 2 main clusters, I and II. Cluster II had 4 sub clusters. The clustering was not on the basis of geographical similarities as the NEH series which are having geographical similarity were found in different clusters. Common ancestry was also explained when some breeding lines were found in a single cluster. The maximum similarity was found to be 83% between C-557 and NEH 8; NEH 32 and Line 4 as well as Lines 29 and 33. This explained that all the accessions were divergent and there were no duplicates in the set of 36 accessions studied.

The main aim of *in vitro* conservation of taro was to develop an *in vitro* active genebank of taro and to augment the existing IVAG of tuber crops at ICAR – CTCRI. Apart from conservation, a multiple shoot induction step was incorporated so that rare genotypes present in germplasm or mutants, if present, could be multiplied first before being conserved in the slow growth media. The sprouts from the cormels were taken as explants. The explants were sterilized by keeping in bavistin for 25 min, labolene for 10 min and in $HgCl_2$ for 5 min. They were inoculated in basal MS medium for the establishment of contamination free cultures. The shoot multiplication medium was standardized using six sets of media. Two out of these six media were selected. The best media was MS+TDZ (0.1mg/l) which produced an average of 1.96 multiple shoots and the second best was MS+K (1mg/l) which gave on an average, 1.24 multiple shoots. These cultures were further inoculated into slow growth media, full and half strength MS having 2% sucrose and 2% mannitol. Presence of Mannitol though induced slow growth, explants dried after a few days of inoculation. As a result, for slow growth, full and half strength MS having 2% sucrose was used. Of these two media, half strength MS was found to be more effective in achieving slow growth. The cultures were transferred to the IVAG existing in ICAR – CTCRI.

With this study, 36 accessions of taro were found to be diverse with no duplicates being identified and these were kept for medium term conservation in slow growth medium. The study would help the breeders in selecting divergent parents for breeding programs and would help in the conservation of these divergent lines *in vitro*.