GENETIC DIVERSITY ANALYSIS IN TARO [*Colocasia esculenta* (L.) Schott] OF NORTH EAST INDIA

by

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(2009-09-117)

THESIS

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DECLARATION

I hereby declare that the thesis entitled **"Genetic diversity analysis in taro *Colocasia esculenta* (L.) Schott] of North East India"** is a bonafide record of esearch done by me and that the thesis has not previously formed the basis for the ıward of any degree, diploma, fellowship or other similar title, of any other Jniversity or Society.

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LIST OF ABBREVIATIONS

°C	Degree Celsius
%	Percentage
µg	Microgram
µl	Microlitre
µ*M*	Micromolar
A_{260}	Absorbance at 260 nm wavelength
A_{280}	Absorbance at 280 nm wavelength
$AgNO_3$	Silver nitrate
AFLP	Amplified fragment length polymorphism
ANOVA	Analysis of Variance
APS	Ammonium persulfate
Bind silane	3-Methacryloxypropyltrimethoxysilane
bp	Base pair
BSA	Bovine serum albumin
cm	Centimetre
CTAB	Cetyl trimethyl ammonium bromide
CTCRI	Central Tuber Crops Research Institute
DMSO	Dimethyl sulfoxide
DNA	Deoxyribo nucleic acid
dNTPs	Deoxy nucleotide tri phosphates
E	East
EDTA	Ethylene diamine tetra acetic acid
EST	Expressed Sequence Tag
FAOSTAT	Food and Agriculture Organization Statistical Database
g	Gram
GLM	Generalized Linear Model
h	Hour

ha	Hectare
IPC	Integral plate chamber
IPGRI	International Plant Genetic Resources Institute
ISSR	Inter simple sequence repeat
KAU	Kerala Agricultural University
kb	Kilo basepair
kg	Kilogram
M	Molar
MDS	Multidimensional scaling
mg	Milligram
$MgCl_2$	Magnesium chloride
min	Minute
ml	Millilitre
mm	Millimeter
m*M*	Millimolar
N	North
NaCl	Sodium chloride
NAGS	National active germplasm site
NBPGR	National Bureau of Plant Genetic Resources
ng	Nanogram
nm	Nanometre
NPGRL	National Plant Genetic Resources Laboratory
OD	Optical density
pg	Picogram
PAGE	Polyacrylamide gel electrophoresis
PC	Principal component
PCA	Principal component analysis
PCoA	Principal co-ordinate analysis
PCR	Polymerase chain reaction

PEG	Polyethylene glycol
PNG	Papua New Guinea
PVP	Polyvinyl pyrrolidone
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolution per minute
s	Second
SAS	Statistical analysis software
SSLP	Simple sequence length polymorphism
SSR	Simple sequence repeat
STR	Short tandem repeat
t	Tonne
Taq	*Thermus aquaticus*
TBE	Tris-borate EDTA buffer
TE	Tris-EDTA buffer
TEMED	N,N,N',N' –tetramethylethylenediamine
T_m	Melting temperature
Tris HCl	Tris (Hydroxy Methyl) aminomethane hydrochloride
U	Enzyme unit
UV	Ultraviolet
V	Volt
v/v	volume/volume
W	Watt
w/v	weight/volume

INTRODUCTION

1. INTRODUCTION

Colocasia esculenta (L.) Schott, a member of the family Araceae and commonly known as taro, ranks fifth among root crops. It serves as a staple source of diet for people around the world and it is the fourteenth most consumed vegetable worldwide (Rao, 2010). It has a valuable place in the world agricultural scenario because of its important role in subsistence economics and crop diversification. Taro is a widely sought after vegetable owing to its wide range of adaptability. Grown in tropical and subtropical climates, the plant can develop under adverse conditions such as poor soils, excess water, shade and extreme climatic stress (Nunes *et al.*, 2012). In addition, it is easy to conserve, possess high nutritional value and gives high yield per unit area (Wang, 1983; Zarate *et al.*, 2006a, 2006b).

Nevertheless, taro is facing widespread genetic erosion due to introduction of new pests, intensified pressure on land and promotion of commercial cash crops. This can have serious implications in terms of losing valuable allelic diversity, which could otherwise be exploited in taro breeding programs for disease resistance and crop improvement. As the center of origin is considered to be a hotspot of diversity, the accessions from the North Eastern states of India are expected to be more genetically diverse. Furthermore, previous studies based on this region have not been reported. Therefore, characterization of taro accessions from this region for diversity assessment becomes imperative.

Accessions of neglected minor crops are being added worldwide in gene banks and institutes conserving germplasm. However, the introduction of these accessions without their characterization, limits the maximum preservation of their genetic diversity (Padulosi *et al.*, 1999). The concept of "core collection", which represents genetic diversity with a minimum of repetitiveness in a crop species and its relatives, was introduced to counter the problems faced in the management of germplasm (Frankel, 1984; Yonezawa, 1985; Brown, 1989). The assessment of

genetic diversity of accessions is now a pivotal strategy for their successful and efficient preservation (Van Tienderen *et al.*, 2002). The Central Tuber Crops Research Institute (CTCRI) has been identified as the National Active Germplasm Site (NAGS) for tuber crops and is maintaining around 590 accessions of taro collected from different locations in India as well as a few collections from abroad.

Among the available markers for diversity assessment, morphological markers are more preferred as they do not require the use of sophisticated technology and some of them are of clear agronomic importance. However, since they are strongly influenced by environment, it is important to use multiple marker systems for germplasm characterization. Microsatellites are emerging as the marker of choice owing to their advantages over other molecular markers. The molecular marker data can be used in association with morphological analysis to accurately assess the variation and reduce the redundancy in germplasm collection.

Standardized national (National Bureau Plant Genetic Resources) and international (International Plant Genetic Resources Institute) descriptors are available for taro morphological characterization. Similarly, several microsatellite markers have been developed and used for the analysis of genetic diversity in taro. The conservation of the regions flanking the microsatellites allows the transferability of these markers to study the accessions within the same species. This study was undertaken to analyze the genetic diversity of taro collections from North Eastern states of India using morphological and SSR (Simple Sequence Repeat) markers. Standardization of DNA extraction from taro leaves was attempted here. Denaturing PAGE using sequencing gel apparatus followed by silver staining was performed for identification and analysis of SSR markers.

Various measures of genetic diversity have been computed to quantify the variability among the accessions and identify the traits contributing to the variability. Evaluation and visualization of the relationship among the accessions was done using

cluster analysis. In order to investigate the congruence between molecular and morphological data, a cophenetic correlation study was also carried out.

The study can enable the selection of accessions from divergent clusters as prospective parents in taro hybridization programs. By exploiting heterosis, taro breeders can develop cultivars to satisfy market needs and to respond to diverse biotic (e.g. taro leaf blight) and abiotic (e.g. drought) challenges. The study can also facilitate taro germplasm conservation and maintenance in an effective manner by elimination of duplicates, if present, in the vast germplasm collection.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Taro [*Colocasia esculenta* (L.) Schott], a member of the Araceae family, is a traditional root crop of the tropics grown for its edible corms and leaves. It is believed to be one of the earliest cultivated root crops in the world (Plucknett, 1976; Kuruvilla and Singh, 1981). It is a highly polymorphic species, cultivated from the equator to Japan (45°N) with more than 10,000 existing landraces (Ivancic and Lebot, 2000). The common (local) name of taro in different parts of the country are: arvi (Hindi), chēmpu (Malayalam), seppan kizhangu (Tamil), kachchi (Kannada), chamadumpa (Telugu), alu (Marathi) and kachu (Bengali) (Edison *et al.*, 2003)

Worldwide, taro ranks fourteenth among staple vegetable crops with about 12 million tonnes produced globally from about 2 million hectares with an average yield of 6.5 t ha^{-1} (FAOSTAT, 2010). Taro is a major crop grown in an area of 51,724 hectares producing 8,10,995 metric t with a yield of 15.68 t ha^{-1} in India (Srinivas *et al.*, 2012).

There are two major taxonomic varieties; one is the dasheen type (*Colocasia esculenta* var. *esculenta*), which has a large central corm with suckers and stolons; and the second is the eddoe type (*Colocasia esculenta* var. *antiquorom*), which has a small central corm and a large number of smaller cormels (Purseglove, 1972). In India including Andaman Islands, var. *esculenta* (Dasheen types) is the predominant variety of taro (Bose *et al.*, 2003). The species *Colocasia* exists in two cytotypes – as diploids with 2n = 28 and triploids with 2n = 42 chromosomes (Yen and Wheeler, 1968; Ramachandran, 1978; Coates *et al.*, 1988). The basic chromosome number is considered to be x = 14 (Yen and Wheeler, 1968; Kuruvilla and Singh, 1981; Matthews, 1990). Cultivated types are mostly diploid ($2n = 2x = 28$), although some triploids are also found ($2n = 3x = 42$) (Mace and Godwin, 2002). Taro is an annual crop propagated asexually (Quero-Garcia *et al.*, 2004).

Major constraints for existing taro breeding programmes are narrow genetic bases (Lebot and Aradhya, 1991) and the lack of knowledge of the genetic diversity in the species. For the successful breeding of novel taro varieties with new combinations of desired characters, a high genetic diversity between the parents is desirable (Kreike *et al.*, 2004). Breeders face the difficult choice of selecting the right parents in the absence of an accurate assessment of the genetic distances existing between local varieties (Quero-Garcia *et al.*, 2004).

Leaf blight has become a major limiting factor for taro production in all taro growing countries including India causing yield loss of 25–30 per cent (Jackson *et al.*, 1980; Thankappan, 1985; Misra and Chowdhury, 1997). Efficient breeding of taro for leaf blight resistance must aim for multigenic resistance. Practically, the strategy should be based on recurrent selection using a wide genetic base composed of parents from diverse geographic origins (Lebot *et al.*, 2004).

For conservation, evaluation, and utilization of genetic resources, different types of characters are frequently measured in each genotype: (i) quantitative characters (morpho-agronomical), (ii) qualitative characters (these are usually multi-state variables) and (iii) discrete genetic marker characteristics using for example SSR, RFLP or AFLP data (Franco *et al.*, 1997). From this perspective, the morphological and molecular characterization data of North East Indian lines of taro can facilitate the exploitation of the genetic diversity present in the center of origin as carried out in this study.

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

2.1 CENTER OF ORIGIN OF TARO

Taro [*Colocasia esculenta* (L.) Schott] is an ancient crop, originated in the Indo-Malayan region probably in eastern India and Bangladesh (Yen and Wheeler, 1968) and is being grown throughout the humid tropics. From the Indo-Malayan center of origin, it is thought to have spread eastward into Southeast Asia, eastern Asia, and the Pacific Islands; westward to Egypt and the eastern Mediterranean; and finally southward and westward from there into Africa, where it moved into the Carribbean and the Americas (Chang, 1958; Yen and Wheeler, 1968). *Colocasia esculenta* has great genetic diversity spreading over tropical regions (Coates *et al.*, 1988). Various studies support two centers of origin for taro – one in South-east Asia and another in Melanesia (Lebot and Aradhya, 1991; Lebot *et al.*, 2004; Kreike *et al.*, 2004; Sardos *et al.*, 2012).

2.2 NUTRITIONAL VALUE

C. esculenta serves as a staple source of diet for people around the world and it is the fourteenth most consumed vegetable worldwide (Rao, 2010). All parts of the plant including corm, cormels, rhizome, stalk, leaves and flowers are edible and contain abundant starch (Bose *et al.*, 2003). Fresh corms are composed of moisture (~ 69%), starch (25%), dietary fiber (1.5%), protein (1.1%) and sugar (1%). *C. esculenta* leaves contain higher levels of protein and are also excellent source of carotene, potassium, calcium, phosphorous, iron, riboflavin, thiamine, niacin, vitamin A, vitamin C and dietary fiber (Bradburry and Holloway, 1998). *C. esculenta* corms and leaves are also credited with having medicinal values and are used to reduce tuberculosis, ulcers, pulmonary congestion and fungal infection (Singh *et al.*, 2011).

2.3 GENERAL PLANT MORPHOLOGY

Taro is a herbaceous plant which grows to a height of 1-2 m. The plant consists of a central corm (lying just below the soil surface) from which the leaves

grow upwards; roots grow downwards, while the cormels and runners (stolons) grow laterally. The root system is fibrous and lies mainly in the top one meter of soil. In the dasheen type of taro, the corm, which constitutes the main edible part of the plant, grows up to 30 cm long and 15 cm in diameter. The cormels alone constitute a significant proportion of the edible harvest in eddoe taro. The surface of each corm is marked with rings showing the points of attachment of scale leaves or senesced leaves. Axillary buds are present at the nodal positions on the corm.

Corms and cormels are quite similar in their internal structure. The outmost layer is a thick brownish periderm, which covers the starch-filled ground parenchyma. Vascular bundles and laticifers ramify throughout the ground parenchyma. Idioblasts (cells which contain raphides or bundles of calcium oxalate crystals associated with acridity of taro) also occur in the ground tissue and in nearly all other parts of the plant.

The apex of the corm represents the plant's growing point and is usually located close to the ground level. The actively growing leaves arise in a whorl from the corm apex and they determine the plant's height in the field. Each leaf is made up of an erect petiole and a large lamina. The petiole is 0.5-2 m long and is flared out at its base where it effectively clasps around the apex of the corm. The petiole is thickest at its base, and thinner towards its point of attachment to the lamina. Internally, the petiole is spongy in texture and has numerous air spaces which presumably facilitate gaseous exchange when the plant is grown in swampy or flooded conditions. For most taro types, the attachment of the petiole to the lamina is peltate which means that the petiole is attached not to the edge of the lamina, but to some point in the middle. This peltate leaf attachment generally distinguishes taro from tannia which has a hastate leaf i.e. the petiole is attached to the basal edge of the lamina. An important exception to this rule is the "piko" group of taro found in Hawaii which, quite uncharacteristically, has hastate leaves. The lamina of taro is 20-

50 cm long, oblong-ovate, with the basal lobes rounded. It is mostly entire, glabrous and thick. Three main veins radiate from the point of attachment of the petiole, one going to the apex, and one to each of the two basal lamina lobes. Some prominent veins arise from the three main veins, but the overall leaf venation is reticulate.

Occasionally in the field, some taro plants are observed to produce stolons. These structures grow horizontally along the surface of the soil for some distance, rooting down at intervals to give rise to new erect plants. Natural flowering occurs only occasionally in taro, but flowering can be artificially promoted by the application of gibberellic acid. The inflorescence arises from the leaf axils or from the centre of the cluster of unexpanded leaves. Each plant may bear more than one inflorescence. The inflorescence is made up of a short peduncle, a spadix and spathe. The spadix is 6-14 cm long with female flowers at the base, male flowers towards the tip and sterile flowers in between, in the region compressed by the neck of the spathe. The extreme tip of the spadix has no flowers at all and is called as the sterile appendage. The sterile appendage is a distinguishing taxonomic characteristic between dasheen and eddoe types of taro. In eddoe types, the sterile appendage is longer than the male section of the spadix; in dasheen types, the appendage is shorter than the male section. The spathe is a large yellowish bract, about 20 cm long, which sheathes the spadix. The lower part of the spathe wraps tightly around the spadix and completely occludes the female flowers from view. The top portion of the spadix is rolled inward at the apex, but is open on one side to reveal the male flowers on the spadix. The top and bottom portions of the spadix are separated by a narrow neck region, corresponding to the region of the sterile flowers on the spadix. Pollination in taro is probably accomplished by flies. Fruit set and seed production occur only occasionally under natural conditions. Fruits, when produced, occur at the lower part of the spadix. Each fruit is a berry measuring 3-5 mm in diameter and containing numerous seeds. Each seed has a hard testa and contains endosperm in addition to the embryo (Onwueme, 1999).

2.4 MORPHOLOGICAL CHARACTERIZATION

Morphological traits have been successfully used for estimation of genetic diversity and cultivar development since they provide a simple way of quantifying genetic variation (Fufa *et al.*, 2005). Taro exhibits a wide range of agro-morphological polymorphism. Numerous variable but clonally stable traits are being used as markers for varietal identification and assessment of genetic diversity (Mathews, 2004). In India, the indigenous taro germplasm has exhibited considerable morphological variability in over 400 accessions assembled from different regions of the country. These have been classified into subjective morphotypes supported by variation in nearly 40 morpho-agronomic traits (Velayudhan *et al.*, 1993).

Many reports are available on the morphological characterization in taro; however, none of the studies have included germplasm from the North East Indian center of origin. Trimanto *et al.* (2010) characterized 18 taro accessions from Central Java using morphological markers. Two thousand two hundred and ninety eight taro accessions from national germplasm collections were characterized by Lebot *et al.* (2004) using 23 standardized morphological descriptors. However, cluster analysis conducted directly on the morphological data did not produce meaningful clusters. Another study based on 452 accessions from Vanuatu involving 19 agro-morphological characters, revealed the narrow genetic base of the Vanuatu taro germplasm (Quero-Garcia *et al.*, 2004). Preliminary assessment of genetic diversity of taro landraces from South Africa including 17 plant characteristics showed great morphological variation (Mabhaudhi and Modi, 2013). Singh *et al.* (2008) used morphological traits to enable initial selection of 859 accessions from Papua New Guinea for core collection development.

Prana (2000) used 19 botanical and agronomical traits to unravel the genetic variability in 335 taro accessions from West Java. The study successfully identified *C. esculenta* var. *esculenta* as the dominant species being used as a carbohydrate

source in West Java. *Colocasia gigantea* and other wildtypes were furthermore identified. Mbouobda *et al.* (2007) found significant variability in Cameroonian cocoyam germplasm using agro-morphological traits. The principal component analysis (PCA) suggested that this represented 70.7 per cent of the total variability. Jianchu *et al.* (2001) used ethno-botany data to elucidate cocoyam genetic diversity that exists in China. International Plant Genetic Resources Institute (IPGRI) descriptors are also being utilized in many cocoyam studies (IBPGR, 1989; IPGRI, 1999). Opoku-Agyeman *et al.* (2004) used cocoyam agro-morphological descriptors developed by IPGRI to characterize germplasm in Ghana.

2.5 MOLECULAR CHARACTERIZATION

Significant progress has been made in the recent years in the application of molecular markers to plant genetic resources characterization and evaluation (Gupta and Varshney, 2000). They offer many advantages for the determination of genetic diversity and the identification of cultivars (such as not being influenced by the environment and detection directly at the DNA level) over the morphological traits which are governed by complex genetic interactions (Karp *et al.*, 1998; Jiang and Liu, 2011). Molecular marker data can be used in association with passport data and morphological analysis to reduce the redundancy in the collection and consequently, the maintenance cost (Pissard *et al.*, 2008). The genetic variation obtained through molecular marker is directly related to the number of polymorphisms detected and their reproducibility (Nguyen *et al.*, 2004). A range of molecular markers have been used to study genetic variation in the major crop species such as rice (Ni *et al.*, 2002), sorghum (Dahlberg *et al.*, 2002), maize (Warburton *et al.*, 2002) and wheat (Mukhtar *et al.*, 2002).

2.5.1 DNA Marker Studies in Taro

Characterization of taro germplasm using molecular markers like RFLP, RAPD, AFLP and ISSR was attempted by earlier workers. Matthews *et al.* (1992) examined the Restriction Fragment Length Polymorphism (RFLP) in the rDNA and mitochondrial DNA (mtDNA) of Japanese taro cultivars. The AFLP was used for genetic diversity studies in taro by few workers (Kreike *et al.*, 2004; Lebot *et al.*, 2004). Quero-Garcia *et al.* (2004) attempted to validate the germplasm stratification of more than 450 taro accessions collected from Vanuatu using AFLP. Lakhanpaul *et al.* (2003) used RAPD to assess the genetic diversity in Indian taro. The study showed the presence of high genetic diversity and distinctness in Indian taro germplasm. Taro accessions from different parts of the Andaman Islands in addition to three commercial varieties as reference genotypes was assessed by Singh *et al.* (2011) using both RAPD and ISSR. Nusaifa Beevi *et al.* (2011) used RAPDs to reveal the genetic diversity of 60 lines of South Indian taro.

2.5.2 Simple Sequence Repeats (SSR)

Microsatellites (Litt and Luty, 1989), also known as simple sequence repeats (SSR) (Tautz *et al.*, 1986), short tandem repeats (STR) or simple sequence length polymorphisms (SSLP) (McDonald and Potts, 1997), are the smallest class of simple repetitive DNA sequences. Chambers and MacAvoy (2000) suggested following a strict definition of 2–6 bp repeats. Mahalakshmi *et al.* (2002) reported that they are ubiquitously distributed throughout the genome of eukaryotes and abundant in genomes of plants where they are thought to be a source of genetic variation.

Microsatellites have emerged as the marker of choice for plant genetic resources applications owing to their codominant nature, high levels of polymorphism, abundance and uniform distribution throughout the genome, simplicity of detection through PCR and their ease of transferability and

reproducibility (Mace and Godwin, 2002). This is possible because the flanking regions complementary to the primers are frequently conserved within the same species or among species and correlated genera, although the microsatellite regions are subject to high mutation rates (Hopkins *et al.*, 1999). The size of the repeat unit, the number of repeats, the presence of variant repeats and the frequency of transcription in the area of the DNA repeat are the factors responsible for generating polymorphism (Somasundaram and Kalaiselvam, 2011)

PCR reactions for SSR is done with the forward and reverse primer which anneals at the 5` and 3` ends of the template DNA, respectively. Differences in SSR allele size is often difficult to resolve on agarose gel and high resolution can be achieved through the use of polyacrylamide gels in combination with $AgNO_3$ staining. Di-nucleotide repeat arrays occur much more frequently than tri- or tetra-nucleotide repeat arrays and are easier to run combinatorial screens for them (Semagn *et al.*, 2006). EST-SSR markers have been reported to have lower rate of polymorphism compared to the SSR markers derived from genomic libraries (Cho *et al.*, 2000; Scott *et al.*, 2000; Eujayl *et al.*, 2002; Chabane *et al.*, 2005).

Microsatellites have proven to be particularly useful for inbreeding crops with low levels of intraspecific diversity (Roder *et al.*, 1995) and are increasingly useful for root crops that are mostly vegetatively propagated such as cassava (Chavarriaga-Aguirre *et al.*, 1998; Roa *et al.*, 2000), sweet potato (Buteler *et al.*, 1999), yam (Terauchi and Konuma, 1994) and taro (Singh *et al.*, 2008).

2.5.2.1 SSR in Diversity Analysis

SSRs are the most suitable markers for the genetic assessment of germplasm because of their hypervariability, attributable to allelic variations (Ma *et al.*, 2009; Ellwood *et al.*, 2006; Zhao *et al.*, 2011). Previously, other researchers had used DNA fingerprinting techniques such as Amplified Fragment Length Polymorphism (AFLP)

primers (Quero-Garcia *et al.*, 2004) and Random Amplified Polymorphic DNA (RAPD) primers (Singh *et al.*, 2011) to characterize taro. However, SSR primers were regarded to be advantageous over AFLP and RAPD (Hamza *et al.*, 2004).

Mace and Godwin (2002) identified around 16 polymorphic microsatellite markers (SSRs), which gave good polymorphism when screened against a restricted set of taro genotypes from South-east Asia and Oceania, showing an average of 3.2 alleles detected on each locus. Seven polymorphic primers from this set were utilized by Godwin *et al.* (2003) to evaluate genetic diversity of 511 taro genotypes and rationalize ten national collections from the Pacific Island Countries. In total, 38 alleles were amplified from the seven SSR loci. The SSRs were informative in revealing the genetic differences within and among the different countries.

Another microsatellite enriched library was constructed by Bastide (2000) and using these, Noyer *et al.* (2003) analyzed a subset of samples earlier characterized by Kreike *et al.* (2004). A differentiation between South-east Asian and Melanesian taros was observed in this study, confirming AFLP and isozyme results of Kreike *et al.* (2004). Mace *et al.* (2006) developed a regional core collection for taro germplasm 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, Niue, Tonga, Cook Islands and Samoa. A total of 515 accessions were genotyped (23.4% overall) using specific SSR markers. DNA fingerprint data showed that great allelic diversity existed in Papua New Guinea and the Solomon Islands. Interestingly, rare alleles were identified in taro from the Solomon Islands province of Choiseul which were not observed in any of the other collections.

The first national core collection of any species established in Papua New Guinea (PNG) based on molecular markers was that of taro through SSR markers by

Singh *et al.* (2008). Around 859 accessions of taro collected from 15 provinces of Papua New Guinea were morphologically characterized using 30 agro morphological descriptors initially and a core of 20 per cent was selected, which was further reduced to 10 per cent based on SSR data using seven SSR markers. Recently, Sardos *et al.* (2012) analyzed the genetic diversity of the National Sample of taro using a set of nine SSR markers. The samples were collected from ten villages of Vanuatu, an archipelago located in the South-west Pacific, each of which was located on a different island. The nine SSR markers used to study 344 landraces revealed a total of 89 alleles, ranging from 6 to 17 alleles per locus with an average of 9.89. Rare alleles could also be detected (57.3%). 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 the seven regional core collections of Brazilian taro from the State of Espirito Santo using seven microsatellite loci. Molecular data was useful in demonstrating the primitiveness of the clones, which were cultivated in Brazil. It also showed that SSR markers could be used for allelic identification.

2.5.2.1.1 DNA Isolation

Molecular marker studies require large amount of high quality genomic DNA, emphasizing screening of inexpensive, rapid and simple DNA extraction methods (Narayanan *et al.*, 2006). Numerous protocols for DNA extraction from plants have been published (e.g., Doyle and Doyle, 1990; Sharma *et al.*, 2000; Li *et al.*, 2001; Pirttilä *et al.*, 2001; Drabkova *et al.*, 2002; Shepherd *et al.*, 2002; Mogg and Bond, 2003; Haymes *et al.*, 2004; Ribeiro and Lovato, 2007; Ivanova *et al.*, 2008; Sharma *et al.*, 2013).

The isolation of good quality DNA from leaves of tropical tuber crops is complicated due to presence of phenolic compounds, highly viscous polysaccharides and DNA degrading endonucleases. During tissue homogenization, phenolics become

oxidized and irreversibly bind to protein and nucleic acid (Loomis, 1974; Aljanabi *et al.*, 1999). This irreversible binding produces a gelatinous material, which is hard to separate from organelles and the DNA becomes unsuitable for PCR amplification and restriction enzyme digestion analyses (Porebski *et al.*, 1997). These metabolites have also been reported to interfere with the activity of several biological enzymes like polymerases, ligases and restriction endonucleases (Pirttila *et al.*, 2001; Diadema *et al.*, 2003; Karaca *et al.*, 2005; Varma *et al.*, 2007; Moyo *et al.*, 2008; Singh and Kumar, 2010; Sahu *et al.*, 2012).

In addition, polysaccharides can cause anomalous reassociation kinetics of DNA sample (Merlo and Kemp, 1976). The DNA tends to stick to the wells of the gel during electrophoretic analysis. NaCl at concentration of more than 0.5 *M* together with CTAB is known to remove polysaccharides (Murray and Thompson, 1980; Paterson *et al.*, 1993). Adding high concentrations of PVP and ß-mercaptoethanol is helpful to remove tannins and other polyphenolics from the tissues (Warude *et al.*, 2003). The problem of polyphenols is exacerbated if green, over matured tissue is taken rather than etiolated leaves (Sharma *et al.*, 2000).

2.5.2.1.2 Polymerase Chain Reaction (PCR)

The use of PCR-based markers has been exponential, following the development by Mullis *et al.* (1986) of the Polymerase Chain Reaction (PCR). This technique consists in the amplification of several discrete DNA products, deriving from regions which are flanked by sequences of high homology with the primers (Mondini *et al.*, 2009).

The three major steps, *viz.* denaturation, annealing and extension are involved in the PCR technique. DNA is initially denatured at high temperatures (from 90-97°C). The annealing phase is the most important step and occurs at 50-60°C for 1-2 min. The extension of the primers by DNA polymerase occurs at approximately

72 °C for 2-5 min. The time for the last step depends both on the DNA polymerase itself and on the length of the DNA fragment to be amplified. As a rule of-thumb, 1 min is allowed for the synthesis of 1 kb fragment. After the last cycle, samples are usually incubated at 72 °C for 5 min to fill in the protruding ends of the newly synthesized PCR products. The three-stages are repeated 25–40 times in a typical PCR procedure (Joshi and Deshpande, 2010). Ideally, specific primers should be 15 to 25 bases long, contain 40-60 per cent G-C and anneal to the template at about 55°C, slightly below the melting temperature (T_m) (Weising *et al.*, 2005).

Optimizing PCR can be laborious, because numerous parameters influence the outcome. These include the architecture of the primer(s), the activity and amount of the polymerase, the temperature profile, concentrations of primers, template DNA and $MgCl_2$, template quality, the A-T content of the template DNA, and the presence of certain additives (Chevet *et al.*, 1995; Hung *et al.*, 1990; Roux, 1995; Sharma *et al.*, 1992; Su *et al.*, 1996). Template DNA with high level of contaminants can adversely affect the reaction. The easiest strategy to overcome this is by dilution of the template DNA (Pandey *et al*, 1996; Savolainen *et al.*, 1995). This may reduce the concentration of contaminants below a critical threshold, whereas the DNA can often still be amplified. Inhibition by acidic polysaccharides can be counteracted to a certain extent by including Tween 20, dimethylsulfoxide (DMSO) or PEG 400 at various concentrations in the PCR (Demeke and Adams, 1992).

Numerous chemicals were reported to enhance PCR specificity and/or yield if added at certain concentrations (Levi *et al.*, 1993; Roux, 1995; Stommel *et al.*, 1997; Wan and Wilkins, 1993). Suggested additives include DMSO (2 to 10%), PEG 6000 (5 to 15%), glycerol (5 to 20%), formamide (5%), nonionic detergents such as Tween and Triton X-100 (0.1 to 1%), BSA (0.1%), spermidine (1 m*M*) and gelatin (0.1%).

2.5.2.1.3 Polyacrylamide Gel Electrophoresis (PAGE)

Polyacrylamide gels are generally preferred for achieving high resolution in the low molecular weight range. Gels of different concentrations can be prepared covering a wide range of molecular weights, from 3.5 per cent gels (suitable for 100-2000 bp molecules) up to 12 per cent gels (40-200 bp molecules). Diffusion of small molecules is less pronounced than in agarose gels, and size differences of a single base pair may be scored (Weising *et al.*, 2005).

Highly resolving, denaturing polyacrylamide gels (also called sequencing gels) are used for separating sets of single-stranded DNA fragments resulting from DNA sequencing. The same kind of gel is also routinely used for AFLP and microsatellite analysis, as well as for some variants of RAPD (Welsh and McClelland, 1990; Caetano-Anollés and Gresshoff, 1998; Caetano-Anollés *et al.*, 1991a and b) and microsatellite-primed PCR (Zietkiewicz *et al.*, 1994). Samples are heat-denatured in a buffer containing formamide prior to loading. Including urea at high concentrations in the gel and running the gel at high temperatures (50 to 55°C) prevent the DNA fragments from renaturing, and thus DNA mobility is not influenced by base composition (Weising *et al.*, 2005).

SSR marker studies are mostly based on analysis via polyacrylamide gel electrophoresis, largely differing in the gel concentration. In taro, SSR characterization has been performed using 10 per cent acrylamide/bis-acrylamide (19:1) (Mace and Godwin, 2002; Singh *et al.*, 2008; Nunes *et al.*, 2012; Mabhaudhi and Modi, 2013), 6 per cent denaturing polyacrylamide gel (Hu *et al.*, 2009) and 4 per cent polyacrylamide gel (Sardos *et al.*, 2012). Okpul *et al.* (2005) used 10 per cent polyacrylamide gel followed by silver staining for ISSR analysis in taro.

PAGE has been the technique of choice for microsatellite studies in other crops also. SSR based genetic diversity assessment among Tunisian winter barley has

been carried out using 6 per cent polyacrylamide gel and silver staining (Hamza *et al.*, 2004). Zheng *et al.* (2013) used the above technique for microsatellite development in *Amorphophallus*. In some instances, PAGE has also been tried out in combination with ethidium bromide staining (Das *et al.*, 2012).

2.5.2.1.4 Silver Staining

Silver staining was originally described for the ultrasensitive detection of polypeptides separated by polyacrylamide gel electrophoresis (Merril *et al.*, 1981) and later adapted for nucleic acid detection (Sommerville and Wang, 1981; Herring *et al.*, 1982; Blum *et al.*, 1987).

Beidler *et al.* (1982) devised a photochemical silver staining method for nucleic acid detection with increased sensitivity (5- 7.5 pg DNA mm^{-2}). Since then, various small adaptations of these original silver staining methods have been proposed to increase sensitivity, to reduce steps of the procedure, to eliminate toxic staining components and to better suit the DNA assay or method of fragment separation (Bassam *et al.*, 1991; Santos *et al.*, 1993; Sanguinetti *et al.*, 1994). Bassam *et al.* (1991) enhanced the sensitivity (as low as 1 pg mm^{-2}) by including a gel pre-exposure with formaldehyde during silver nitrate impregnation and by lowering the concentration of silver nitrate. Backgrounds were reduced by inclusion of sodium thiosulfate and by eliminating an oxidation pretreatment with potassium dichromate and nitric acid.

Several other protocols for silver staining have been described (Briard *et al.*, 2000; Ude *et al.*, 2002; Pillen *et al.*, 2000; Creste *et al.*, 2001; Han *et al.*, 2008), most of which take approximately 2 h. One should be aware that silver not only stains DNA but also RNA and proteins. The presence of restriction enzymes, polymerase, and BSA should therefore be minimized (Weising *et al.*, 2005).

Detection of nucleic acids using silver stain has been demonstrated to be highly sensitive, with results similar to autoradiography and fluorescence labeling and detection (Comincini *et al.*, 1995; Christensen *et al.*, 1999). However, with agarose gels, silver does not give better results than ethidium bromide; therefore, it is not generally used (Tegelström, 1992). With polyacrylamide gels, conversely, staining with silver enhances sensitivity by about two orders of magnitude, thus enabling the detection of bands containing only 10 to 30 pg of DNA (Weising *et al.*, 2005).

2.6 STATISTICAL ANALYSIS

2.6.1 Diversity Index

Diversity indices are used in several studies to assess the level of diversity present in the population studied. A high index value indicates a high level of diversity. Shannon-Weaver's diversity index is the most commonly used one in genetic diversity studies. In a comparative study for genetic diversity of four major crops in Ethiopia, Tanto and Demissie (2005) found out that for sorghum the Shannon-Weaver total diversity was 0.74±0.11, while for teff it was 0.78±0.09. This shows that sorghum and teff crops for which Ethiopia is a center of origin has higher diversity compared to barley and Durum wheat crops (0.61±0.18 and 0.71±0.11 respectively), for which Ethiopia is a secondary gene center.

Jain *et al.* (1975) used Shannon-Weaver diversity index to examine the overall genetic divergence for studying the geographical patterns of phenotypic diversity in a world collection of Durum wheat. The study revealed that Ethiopia and Portugal had the greatest diversity (H' = 0.87 and 0.86) and Turkey, Bulgaria, and the United States had significantly less diversity (H' = 0.50, 0.42, and 0.39). H' computed in the morpho-agronomic diversity analysis of 20 lines of thermo-sensitive genetic male sterile rice (Alcasid *et al.*, 2008) was found to be 0.44. The mean index value was

calculated by excluding all the monomorphic traits (H'=0.00). Siopongco *et al.* (1999) used Shannon-Weavers diversity index to assess the morphological diversity in NPGRL's local corn collection. The study revealed that quantitative traits (mean value 0.82) contributed more to the diversity than the qualitative traits (mean value 0.54).

Additionally, the Simpson's diversity index may also be used for comparative analysis. Tanto and Demissie (2005) obtained results similar to Shannon-Weaver index when the Simpson's diversity index was used for diversity analysis in four major crops of Ethiopia.

2.6.2 Measures of Genetic Distance

Genetic distance, similarity between two genotypes, populations, or individuals may be calculated by various statistical measures depending on the data set. Discussions on various distance measures are available in the literature (Felsenstein, 1984; Nei, 1987; Weir, 1990, 1996; Beaumont *et al.*, 1998). Euclidean or straight-line measure of distance is the most commonly used statistic for estimating genetic distance between individuals (genotypes or populations) by morphological data (Kaufman and Rousseeuw, 1990).

2.6.3 Multivariate Analysis

Multivariate analytical techniques simultaneously analyze multiple measurements on each individual under investigation. The use of multivariate statistical algorithms is an important strategy for classification of germplasm and analysis of genetic relationships among breeding material (Mohammadi and Prasanna, 2003). Fufa *et al.* (2005) clustered hard red winter wheat on the basis of pedigree information, morphological characters, end-use quality traits and molecular markers to estimate genetic diversity.

Among the various algorithms, cluster analysis, principal component analysis PCA), principal coordinate analysis (PCoA) and multidimensional scaling (MDS) ire, at present, most commonly employed and appear particularly useful (Melchinger, 1993; Johns *et al.*, 1997; Thompson *et al.*, 1998; Brown-Guedira *et al.*, 2000).

2.6.3.1 Principal Component Analysis (PCA)

PCA can be utilized to derive a 2- or 3-dimensional scatter plot of individuals, such that the geometrical distances among individuals in the plot reflect the genetic distances among them with minimal distortion. Aggregations of individuals in such a plot will reveal sets of genetically similar individuals (Melchinger, 1993; Karp *et al.*, 1997; Warburton and Crossa, 2002).

PCA is defined as "a method of data reduction to clarify the relationships between two or more characters and to divide the total variance of the original characters into a limited number of uncorrelated new variables" (Wiley, 1981). This would allow visualization of the differences among the individuals and identify possible groups. The reduction was achieved by linear transformation of the original variables into a new set of uncorrelated variables known as principal components (PCs). The first step in PCA is to calculate eigen values, which define the amount of total variation that is displayed on the PC axes. The first PC summarizes most of the variability present in the original data relative to all remaining PCs. The second PC explains most of the variability not summarized by the first PC and uncorrelated with the first, and so on (Jolliffe, 1986). In the multivariate analysis of wheat germplasm, Ajmal *et al.* (2013) found out that the first three components with eigen values >1 accounted for 70.59 per cent of the total variation of grain yield. Because PCs are orthogonal and independent of each other, each PC reveals different properties of the original data and may be interpreted independently. In this way, the total variation in the original data set may be broken down into components that are cumulative. The proportion of variation accounted for by each PC is expressed as the eigen value

divided by the sum of the eigen values. The eigen vector defines the relation of the PC axes to the original data axes. In the analysis of genetic diversity of Kenyan cowpea accessions based on morphological and microsatellite markers, Kuruma *et al.* (2008) recorded high PC loadings for vegetative attributes with the first three components explaining 65 per cent of the variation.

To extract maximum information from the molecular marker data, PCA or PCoA can be used in combination with cluster analysis, particularly when the first two or three PCs explain > 25 per cent of the original variation (Messmer *et al.*, 1992).

2.6.3.2 Cluster Analysis

Cluster analysis refers to "a group of multivariate techniques whose primary purpose is to group individuals or objects based on the characteristics they possess, so that individuals with similar descriptions are mathematically gathered into the same cluster" (Hair *et al.*, 1995). The cluster analysis sequestrates genotypes into clusters which exhibit high homogeneity within a cluster and high heterogeneity between clusters (Jaynes *et al.*, 2003). Thus, if the classification is successful, individuals within a cluster shall be closer when plotted geometrically and different clusters shall be farther apart (Hair *et al.*, 1995).

There are broadly two types of clustering methods: (i) distance-based methods, in which a pair-wise distance matrix is used as an input for analysis by a specific clustering algorithm (Johnson and Wichern, 1992), leading to a graphical representation (such a tree or dendrogram) in which clusters may be visually identified; and (ii) model-based methods, in which observations from each cluster are assumed to be random draws from some parametric model. Distance-based clustering methods can be categorized into two groups: hierarchical and nonhierarchical. Hierarchical clustering methods are more commonly employed in analysis of genetic

diversity in crop species. These methods proceed either by a series of successive mergers or by a series of successive divisions of group of individuals. The former, known as "agglomerative hierarchical" methods, start with a single individual. Thus, there are initially as many clusters as individuals. The most similar individuals are first grouped and these initial groups are merged according to their similarities.

Another important aspect in cluster analysis is determining the optimal number of clusters or number of acceptable clusters. In essence, this involves deciding where to "cut" a dendrogram to find the true or natural groups. An "acceptable cluster" is defined as "a group of two or more genotypes with a within-cluster genetic distance less than the overall mean genetic distance and between cluster distances greater than their within cluster distance of the two clusters involved" (Brown-Guedira *et al.*, 2000).

Aharizad *et al.* (2012) applied cluster analysis using Ward's algorithm and squared Euclidean distances and assigned 94 bread wheat inbred lines into three groups. Dendrogram constructed using five morphological traits divided 11 oilseed rape cultivars into two clusters with one stand-alone cultivar 'DK Secure'. No significant correlation of genetic diversity with morphological traits was obtained in this study based at Lithuania by Paulauskas *et al.* (2013). Dendrogram analysis performed on 200 corn accessions based on morphological markers from NPGRL formed 32 distinct clusters which included 16 single accession clusters and a single large cluster with 78 accessions (Siopongco *et al.*, 1999)

2.6.4 Analysis of Variance

Analysis of variance (ANOVA) is a collection of statistical models to analyze the differences between group means. It provides a statistical test of whether or not the means of several groups are equal. ANOVA has been widely used in morphological analysis of genetic diversity. The groups, variables or their mean

values can be tested for statistical significance at 1 per cent or 5 per cent probability. In the morpho-agronomical characterization of Ethiopian taro accessions (Beyene, 2013), ANOVA was carried out to examine the presence of statistically significant differences among the genotypes for the quantitative characters. The characters that manifested significant differences are petiole length and maximum horizontal distance ($P<0.01$). Anil *et al.* (2011) performed ANOVA to assess the morphological variability in 17 wild elephant foot yam collections from South West India and the results showed significant variation among the accessions ($P<0.05$). However, the values of the coefficient of variation indicated that leaf characters are least important in distinguishing the accessions.

2.6.5 Correlation of Morphological and Molecular Data

Relationships between phenotypic and molecular marker data of genotypes may provide useful information in order to determine the most promising entries for future breeding programmes. A few studies on the relationship between phenotypic and molecular data performance have been published in sorghum (Anas and Yoshida, 2004; Geleta *et al.*, 2006), in which low correlations between molecular marker and phenotypic data in sorghum were reported. Molecular markers have the ability to cover the entire genome (coding as well as non-coding regions), while phenotypic differences are connected to specific genes or coding regions. Therefore, differences revealed by molecular markers are not necessarily associated with phenotypic variation. Hence, to express accurately the relationships among genotypes, a combination of morphological and molecular information is required. Burstin and Charcosset (1997) investigated the relationship between molecular and phenotypic distances computed from quantitative traits. Their results revealed that the relationship is most likely triangular, which means close genetic distances are associated with close phenotypic distances, whereas distant genetic relationships can correspond with both close and distant phenotypic relationships.

2.6.5.1 Mantel's Test

In case the researcher is interested to make use of more than one measure of genetic distance to analyze a given data set or different data sets, it is important to ascertain the correspondence between matrices derived from different distance measures. The test of matrix correspondence, popularly known as Mantel's test (Mantel, 1967), analyzes matrix correspondence on the basis of the assumption of asymptotic normality for a particular test criterion. Mantel's test is a regression in which the variables are themselves distances or dissimilarity matrices summarizing pair-wise similarities–dissimilarities between units of study. Mantel's test has been used in the analysis of genetic diversity in crop plants, particularly in ascertaining the correspondence of matrices derived by means of different marker systems over the same set of genotypes (van Bueningen and Busch, 1997; Bohn *et al.*, 1999; Lombard *et al.*, 2000; Lubberstedt *et al.*, 2000; Virk *et al.*, 2000; Vuylsteke *et al.*, 2000). Mantel's test is used to examine the relationship between matrices derived from different distance measures. Mantel's test of significance is evaluated via permutation procedures to overcome the problem of dependent elements (Manly, 1991).

Mantel's test is important in the analysis of genetic diversity, where various data sets may be used to assess the relationships among different individuals or populations. Mantel's test has been used in the analysis of genetic diversity in many different crop plants (Kumar *et al.*, 2008; Sarıkamış *et al.*, 2009; Priolli *et al.*, 2010).

Sl. No.	Acc. No.	IC No.	Place of collection	Type/Botanical variety
6	C-9	IC-204240		*Colocasia esculenta*
7	C-14	IC-204267	-	*Colocasia esculenta*
8	E-8	IC-089813	-	*Colocasia esculenta*
9	F-9	IC-545379	-	*Colocasia esculenta*
10	G-14	IC-410320		*Colocasia esculenta*
11	H-2	IC-545308		*Colocasia esculenta*
12	H-9	IC-211388	Unknown	*Colocasia esculenta*
13	I-13	IC-089140		*Colocasia esculenta*
14	I-14	IC-208982	-	*Colocasia esculenta*
15	I-15	IC-032986	-	*Colocasia esculenta*
16	J-8	IC-012486	-	*Colocasia esculenta*
17	J-13	IC-039813	-	*Colocasia esculenta*
18	K-15	IC-020428	-	*Colocasia esculenta*
19	L-8	IC-330438	Ukhrul, Manipur	*Colocasia esculenta*
20	L-12	IC-012593	-	*Colocasia esculenta*
21	L-14	IC-012601	-	*Colocasia esculenta*
22	717	-	Jowai, Meghalaya	*Colocasia esculenta*
23	741	-	Agarthala, Gulbazar,Tripura	*Colocasia esculenta*
24	724	-	Marngar, Meghalaya	*Colocasia esculenta*
25	719	-	Pnonpen village, Meghalaya	*Colocasia esculenta*

3.2 MORPHOLOGICAL DATA COLLECTION

The National Bureau of Plant Genetic Resources (Unnikrishnan *et al.*, 1987) descriptors for taro were used for the morphological characterization. Certain modifications were made to incorporate additional traits using the International Plant

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The study entitled "Genetic diversity analysis in taro [*Colocasia esculenta* (L.) Schott] of North East India" was carried out at the Division of Crop Improvement, Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram during 2013-2014. In this chapter, details pertaining to the experimental material and procedures used in the study are elaborated.

3.1 GERMPLASM COLLECTION

Taro accessions collected from the various North East Indian states are conserved *ex situ* at the CTCRI germplasm repository, of which 25 accessions were selected for the present study. The geographical origin of the selected accessions is represented in Table 1 and the characteristics of the germplasm location are represented in Table 2. The plants were raised in the field with five plants per accession spaced at 60 cm between the ridges and 45 cm between plants within a ridge. Planting was carried out as per the Package of Practices (KAU, 2011). The corms/cormels were planted on the ridges during the onset of rainy season. Mulching and weeding were carried out as and when necessary.

Table 1. Geographical locations of the taro accessions

Sl. No.	Acc. No.	IC No.	Place of collection	Type/Botanical variety
1	A-6	IC-521313	Others	*C. esculenta* var. *antiquorum*
2	A-12	IC-416937	Upper Siang, Arunachal Pradesh	*Colocasia* sp.
3	B-2	IC-394312	-	*Colocasia esculenta*
4	B-3	IC-089582	-	*Colocasia esculenta*
5	B-4	IC-310104	-	*Colocasia esculenta*

Genetic Resources Institute (IPGRI, 1999) descriptors for taro. Twenty nine above ground characters comprising of five quantitative traits were selected and the plants were scored at the maximum growth stage i.e. 4-5 months. Nine characters were selected for characterization of tubers after harvest, five of which were quantitative traits. An average value from three plants was used to record all the quantitative traits.

Table 2. Characteristics of the location

Characteristic	
Latitude	8°40' N
Longitude	77°0' E
Minimum night temperature	19°C
Maximum day temperature	33.4°C
Rainfall	1400-1500 mm
Day length	11 h 23 min (shortest in December) to 12 h 39 min (longest in June)
Crop season	June to December

3.3 MORPHOLOGICAL DATA ANALYSIS

The morphological data was recorded in the form of numerical scores assigned to each trait as shown in Table 3. The numerical data was tabulated in an excel worksheet and subjected to analysis using the R statistical package.

Table 3. List of morphological characters and their descriptor states

Sl. No.	Character	Descriptor states
1	Germplasm type	Cultivated – 1, Wild – 2, Weedy – 3
2	Growth habit	Erect – 1, Spreading – 2
3	Type of stem	Stolon – 1, Sucker – 2, Rhizome – 3
4	Tillering	Low (1-3) – 1, Medium (4-6) – 2, High (>6) – 3, Others – 4
5	Plant size	Dwarf (<50 cm) – 1, Medium (50-100 cm) – 2, Tall (>100 cm) – 3
6	Leaf arrangement	Clockwise – 1, Anticlockwise – 2
7	Lamina orientation	Drooping – 1, Horizontal – 2, Cup shaped – 3, Erect (apex up) – 4, Erect (apex down) – 5
8	Leaf margin	Entire – 1, Undulate – 2
9	Leaf margin colour	Green – 1, Light green – 2, Dark green – 3, Bluish green – 4, Glaucous green – 5, Grey – 6, Cream – 7, Purple – 8, Purplish green – 9, Dark purple – 10, Whitish green – 11, Purple shaded cream – 12
10	Leaf colour (upper)	Green – 1, Light green – 2, Dark green – 3, Bluish green – 4, Glaucous green – 5, Grey – 6, Cream – 7, Purple – 8, Purplish green – 9, Dark purple – 10, Whitish green – 11, Purple shaded cream – 12
11	Leaf colour (lower)	Green – 1, Light green – 2, Dark green – 3, Bluish green – 4, Glaucous green – 5, Grey – 6, Cream – 7, Purple – 8, Purplish green – 9, Dark purple – 10, Whitish green – 11, Purple shaded cream – 12

Sl. No.	Character	Descriptor states
12	Sinus colour	Green – 1, Light green – 2, Dark green – 3, Bluish green – 4, Glaucous green – 5, Grey – 6, Cream – 7, Purple – 8, Purplish green – 9, Dark purple – 10, Whitish green – 11, Purple shaded cream – 12
13	Leaf variegation	Present – 1, Absent – 2
14	Leaf type	Narrow (>1.3:1) – 1, Medium (1.2-1.3:1) – 2, Broad (<1.2:1) – 3
15	Petiole junction pattern and colour (upper)	Yellow spotted – 1, Purple spotted – 2, Yellow asterisk – 3, Purple asterisk – 4, Solid (no pattern) – 5
16	Petiole junction pattern and colour (lower)	Semilunar – 1, Colour spread to veins – 2, Colour not spread to veins – 3, No pattern – 4
17	Vein colour (upper)	Green – 1, Light green – 2, Dark green – 3, Bluish green – 4, Glaucous green – 5, Grey – 6, Cream – 7, Purple – 8, Purplish green – 9, Dark purple – 10, Whitish green – 11, Purple shaded cream – 12
18	Vein colour (lower)	Green – 1, Light green – 2, Dark green – 3, Bluish green – 4, Glaucous green – 5, Grey – 6, Cream – 7, Purple – 8, Purplish green – 9, Dark purple – 10, Whitish green – 11, Purple shaded cream.– 12
19	Petiole colour- top 1/3rd	Green – 1, Light green – 2, Dark green – 3, Bluish green – 4, Glaucous green – 5, Grey – 6, Cream – 7, Purple – 8, Purplish green – 9, Dark purple – 10, Whitish green – 11, Purple shaded cream – 12

Sl. No.	Character	Descriptor states
20	Petiole colour- middle	Green – 1, Light green – 2, Dark green – 3, Bluish green – 4, Glaucous green – 5, Grey – 6, Cream – 7, Purple – 8, Purplish green – 9, Dark purple – 10, Whitish green – 11, Purple shaded cream – 12
21	Petiole colour- base	Green – 1, Light green – 2, Dark green – 3, Bluish green – 4, Glaucous green – 5, Grey – 6, Cream – 7, Purple – 8, Purplish green – 9, Dark purple – 10, Whitish green – 11, Purple shaded cream – 12
22	Petiole type	Short (<1.5:1) – 1, Medium (1.5-2:1) – 2, Long (>2:1) – 3
23	Leaf sheath pattern	Closed – 1, Open – 2
24	Leaf sheath colour	Green – 1, Light green – 2, Dark green – 3, Bluish green – 4, Glaucous green – 5, Grey – 6, Cream – 7, Purple – 8, Purplish green – 9, Dark purple – 10, Whitish green – 11, Purple shaded cream – 12
25	Sheath type	Short (>2:1) – 1, Medium (1.5-2:1) – 2, Long (<1.5:1)
26	Flower formation	Flowering – 1, Non flowering – 2
27	Seed formation	Present – 1, Absent – 2
28	Corm shape	Cylindrical – 1, Conical – 2, Elliptical – 3, Round – 4
29	Cormel shape	Cylindrical – 1, Conical – 2, Round – 3, Stoloniferous cylindrical – 4, Elongated – 5
30	Number of cormels	< 5 – 1, 5-10 – 2, > 10 – 3
31	Weight of corms	< 0.5 kg – 1, 0.5-4 kg – 2, > 4 kg – 3
32	Weight of cormels	< 0.1 kg – 1, 0.1-0.5 kg – 2, > 0.5 kg – 3

Sl. No.	Character	Descriptor states
33	Corm length	< 8 cm – 1, 8-18 cm – 2, > 18 cm – 3
34	Cormel length	< 8 cm – 1, 8-18 cm – 2, > 18 cm – 3
35	Flesh colour	White – 1, Off white – 2, White with light pink tinge – 3
36	Cooked cormel edibility	Non acrid – 1, Slightly acrid – 2, Medium acrid – 3, Highly acrid – 4
37	Disease incidence	Resistant – 1, Moderately resistant – 2, Tolerant – 3, Moderately susceptible – 4, Susceptible – 5, Incidence not recorded – 6
38	Maturity	6-7 months – 1

3.3.1 Diversity Indices

The percentage distribution of traits, Shannon Weaver's diversity index and Simpson's diversity index were calculated for the various traits studied. The percentage distribution of the various traits across the 25 accessions was computed by calculating the proportion of accessions expressing a particular trait as percentage. The Shannon Weaver's diversity index (H) was calculated based on the following formula:

$$H = -\Sigma [p_i \times \ln (p_i)]$$

where,

p_i = proportion of a particular character i.e. number of individuals with a character/ total number of individuals

The Simpson's diversity index (D) was calculated based on the formula given below:

$$D = 1 - \frac{\Sigma n (n-1)}{N (N-1)}$$

where,

n = number of accessions with a particular trait

N = total number of accessions

3.3.2 Principal Component Analysis

Principal component analysis was done using the R statistical package to study the contribution of each character in the overall variation. The cluster plot representation of the PCA was done. The box plot and biplot analyses were also carried out to represent the distribution of the various traits under different accessions.

3.3.3 Analysis of Variance (ANOVA)

ANOVA was performed for the ten quantitative characters using SAS (Statistical Analysis System) software based on GLM procedure. Duncan's multiple range test for the analysis of mean values of each character was also done.

3.3.4 Cluster Analysis

Hierarchial cluster analysis was performed using the hclust function of the R package based on Euclidean distance. Complete linkage method using Ward's genetic distance was used to construct the dendrogram.

3.4 STANDARDIZATION OF DNA ISOLATION PROTOCOL

The extraction of good quality DNA from taro was difficult owing to the presence of high amounts of polyphenol and mucilage. Therefore, several extraction procedures were tried out to identify a protocol that yielded good DNA in terms of quality and quantity.

3.4.1 DNA Extraction Using Amnion AMpreP Plant gDNA Mini kit

Freshly collected young leaves weighed up to 100-200 mg were powdered in liquid nitrogen using sterile mortar and pestle. The powdered samples were homogenized in 750 µl of 1X gDNA homogenizer buffer to make a fine paste and transferred into sterile two ml microfuge tubes. In order to ensure the transfer of the entire ground tissue, the mortar and pestle was rinsed with 250 µl 1X gDNA homogenizer buffer and collected in a microfuge tube. Five µl of the RNase solution was added and mixed 5-6 times by inversion. The homogenate was then incubated in a water bath (Memmert, Germany) set at 65°C for ten min with intermittent mixing. After adding one ml of 1X gDNA extraction buffer, the tubes were mixed 5-6 times by inversion and incubated at 65°C for 15 min with intermittent mixing. The tubes were allowed to cool down to room temperature before centrifuging (Spectrafuge 7M, Labnet International, US) at 10,000 rpm for two min. The supernatant was collected into fresh sterile two ml microfuge tubes and equal volume of isopropanol was added. The tubes were gently inverted and allowed to stand at room temperature for 2-5 min to precipitate out the DNA. The DNA precipitate was collected after spinning the tubes at 10,000 rpm for 15 min and discarding the supernatant. The pellet so obtained was washed in 70 per cent ice cold ethanol to remove any residual salts by spinning at 10,000 rpm for five min. The tubes were then air dried at room temperature to remove traces of ethanol completely. The DNA extract was suspended in 100 µl of DNA recovery solution and stored at -20°C.

3.4.2 DNA Extraction Using Modified Kit Method

The above extraction procedure was modified with the following steps to enhance the yield and purity of DNA. A pinch of PVP was added to the ground samples before adding the 1X gDNA homogenizer buffer. The supernatant obtained before isopropanol precipitation was treated with equal volume of 24:1 (v/v) chloroform/isoamyl alcohol and mixed thoroughly by inversion for 2-5 min. The

mixture was centrifuged at 10,000 rpm for ten min and the upper aqueous phase was collected in fresh sterile tubes. The same protocol was tried using a higher amount of sample i.e. 300 mg.

3.4.3 CTAB Method I

Modified Lodhi *et al.* (1994) method of DNA extraction based on CTAB was used.

Freshly collected young leaves weighed up to 150 mg was powdered finely in liquid nitrogen using sterile mortar and pestle. A pinch of PVP and ascorbic acid was added and mixed. Before the sample thawed, 2 ml of extraction buffer (Appendix I) pre-warmed at 65°C was added and gently homogenized. The lysate was transferred to a 2 ml tube, to which 5 µl RNase was added and mixed well by inversion. The sample was incubated in a water bath maintained at 37°C for 30 min with frequent mixing. To this, 5 µl proteinase K was added, mixed and incubated at 37°C for 30 min with intermittent shaking. The supernatant was collected followed by centrifugation at 10,000 rpm for 15 min and mixed with equal volume of chloroform/isoamyl alcohol (24:1) for 3-5 min. The chloroform/isoamyl alcohol treatment was repeated with the aqueous phase obtained after centrifugation. Isopropanol was added to 0.8 volumes for precipitating DNA followed by centrifugation at 10,000 rpm for 10 min. The resultant pellet was washed in 70 per cent ethanol and centrifuged at 10,000 rpm for 5 min. After air-drying the pellet to ensure the complete removal of ethanol traces, 50-100 µl of 1X TE buffer was added to dissolve the pellet. The extracted DNA was stored at -20°C until further use.

3.4.4 CTAB Method II

The CTAB extraction method (Sharma *et al.*, 2008a), developed for tuber crops, was adopted with slight modifications.

The extraction buffer was prepared by adding 2 per cent PVP and 2 per cent β-mercaptoethanol to the freshly prepared buffer (Appendix II) preheated at 65°C. Freshly collected young leaves weighed up to 300 mg was ground to a fine powder in liquid nitrogen using autoclaved mortar and pestle. Before the powdered samples thawed, 2 ml of preheated extraction buffer was added and gently homogenized. The homogenate was transferred to sterile 2 ml microfuge tubes, labeled with the appropriate accession names. Five µl of RNase (10 mg ml^{-1}) was added to the above samples and mixed gently by inversion. The samples were then incubated in a water bath at 37°C for 1 h with intermittent shaking followed by incubation at 65°C for 30 min with frequent mixing. The homogenate was centrifuged at 10,000 rpm for 10 min and the supernatant was treated with equal volume of 24:1 (v/v) chloroform/isoamyl alcohol. The upper aqueous layer was collected after centrifuging at 10,000 rpm for 10 min and treated with $2/3^{rd}$ volume of isopropanol. The precipitated DNA was pelletized by centrifuging at 10,000 rpm for 15 min and washed with 70 per cent ethanol by spinning at 10,000 rpm for 5 min. The DNA pellets were air dried to remove the ethanol traces completely followed by suspension in 50-100 µl of 1X TE buffer (Appendix III). The DNA suspension was stored at -20°C till further use.

3.5 ANALYSIS OF THE EXTRACTED DNA

3.5.1 Agarose Gel Electrophoresis

The integrity and quality of the extracted DNA was checked using agarose (0.8%) gel electrophoresis. Five µl of the DNA sample mixed with 1 µl 6X loading dye (Fermentas) was loaded in the agarose gel prepared in 1X TBE (Appendix IV). The gel was run in a horizontal gel electrophoresis unit for 45 min at 75V. The run

was stopped after the dye front reached 3/4th of the gel length and visualized using the AlphaImager documentation system.

3.5.2 Spectrophotometer

The absorbance of DNA was measured at 260 nm and 280 nm to assess the yield and purity. Sterile distilled water was used to calibrate the spectrophotometer (Systronics, India) to blank i.e. zero absorbance at the above mentioned wavelengths. Ten µl DNA sample was diluted to 2 ml using sterile distilled water and the OD values at 260 nm and 280 nm were recorded. The concentration of DNA present in the sample was quantified using the following formula:

Concentration (µg ml^{-1}) = absorbance at 260 nm x 50 x dilution factor

Where,

the value 50 corresponds to 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 OD at 260 nm / OD at 280 nm ratio.

3.6 PCR STANDARDIZATION

For standardization of PCR conditions, the genomic DNA of few taro accessions was used. The composition of the reaction mixture was as follows:

10X Taq buffer (with 15 m*M* $MgCl_2$)	:	2 µl
dNTPs (10 m*M* each)	:	0.2 µl
Forward primer (10 µ*M*)	:	0.4 µl
Reverse primer (10 µ*M*)	:	0.4 µl
Template DNA (10 ng µl^{-1})	:	2 µl
Taq polymerase (5 U µl^{-1})	:	0.2 µl

Water	:	14.8 µl
Total volume	:	20 µl

PCR was carried out in Biorad C1000^{TM} thermal cycler programmed for an initial denaturation at 94°C for 5 min followed by 35 cycles with denaturation at 95°C for 1 min, annealing at 56°C for 2 min and extension at 72°C for 3 min. The final extension was performed at 72°C for 10 min followed by hold at 4°C. The amplified products were resolved in a 2 per cent agarose gel using 100 bp ladder for checking amplification and visualized under the Alphalmager gel documentation system.

3.6.1 Optimization of PCR Conditions

The conditions of the above PCR programme were modified suitably to yield consistent amplicons under reduced programme duration. The cycle conditions were standardized with denaturation for 45 s, annealing for 60 s and extension for 90 s. The duration of the final extension was also reduced to 8 min.

3.7 PRIMER SCREENING

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

Cel series- Cel A06, CelA08, Cel B03, Cel B12, Cel C03, Cel C06, Cel D12, Cel F04, Cel F12 and Cel H12.

uq series- uq73-164, uq84-207, uq88B-94, uq97-256, uq110-283, uq115-71, uq132-147 and uq201-302.

3.7.1 Gradient PCR for T_a Optimization

Based on the data from preliminary primer screening at an annealing temperature of 56°C, it was found necessary to perform gradient PCR to standardize the annealing temperature for the primers selected from the Cel series. Gradient PCR was carried out with a temperature range of 40-60°C for Cel A06, Cel B03, Cel C03, Cel C06, Cel F04 and Cel H12 to choose the most appropriate annealing temperature giving specific bands with minimum ambiguity.

3.8 SSR ANALYSIS OF TARO ACCESSIONS

After initial screening, 10 primers, the details of which are given in Table 4, were chosen for characterization based on their polymorphism and quality of electrophoretic patterns. Microsatellite analysis of the 25 North East Indian taro lines was performed using the above selected primers at their respective standardized T_a. The amplicons were resolved in 2 per cent agarose gel initially to check for amplification.

Table 4. List of primers selected for characterization

Sl. No.	SSR ID	Primer sequence	Repeat type	Expected product size
1	Cel A06	F- GCTTGTCGGATCTATTGT R- GGAATCAGTAGCCACATC	$(CT)_{31}$	251
2	Cel B03	F- TTGCTTGGTGTGAATG R- CTAGCTGTGTATGCAGTGT	$(GA)_3(GATA)_3(TA)_{12}$	175
3	Cel C03	F- TGTTGGGAAAGAGGG R- GGGGAATAACCAGAGAA	$(CT)_{14}$	117
4	Cel C06	F- CCAGAAGAGACGTTACAGA R- ACGACTTTGGACGGA	$(CT)_{16}$	166

5	Cel F04	F- AGGGAATACAATGGCTC R- ACGAGGGAAGAGTGTAAA	$(CT)_{29}$	-
6	Cel H12	F- TAGTTAGCGTGCCTTTC R- CAACAACTTAATGCTTCAC	$(GA)_{18}$	-
7	uq73-164	F- ATGCCAATGGAGGATGGCAG R- CGTCTAGCTTAGGACAACATG	$(CT)_{15}$	164
8	uq84-207	F- AGGACAAAATAGCATCAGCAC R- CCCATTGGAGAGATAGAGAGA	$(CT)_{18}$	207
9	uq97-256	F- GTAATCTATTCAACCCCCCTT R- TCAACCTTCTCCATCAGTCC	$(CA)_8$	256
10	uq201-302	F- CTAAGGAGAGGAGATCCGAAC R- CAAGACGATGCTGAACCAC	$(C)_{15}$	283

3.9 DENATURING PAGE FOR SSR ANALYSIS

Seven M urea denaturing PAGE (6% polyacrylamide) was performed using Biorad Sequi- Gen® Cell (38 X 50 cm) according to the Biorad manual with heat denatured samples and detected by silver staining (Pillen *et al.*, 2000).

3.9.1 Integral Plate Chamber (IPC) Unit Assembly

The outer glass plate was thoroughly cleaned using liquid detergent (Laboline) and tap water followed by rinsing with distilled water. The IPC was also cleaned using distilled water. After placing the IPC and the glass plate on the work bench, 85 per cent ethanol was used to wipe both the surfaces using lint free tissue. The outer glass plate was silanized by applying bind silane coated on a lint free tissue from top to the bottom of the plate in vertical motions. The IPC was coated with a layer of Laboline, which acts as a repellant, in a similar fashion. The vinyl spacers and combs were wiped using 85 per cent ethanol while allowing the plates to dry. A 0.4 mm spacer was positioned along each long edge of the IPC glass plate. Ensuring that the

siliconized surface faces down, the outer glass plate was positioned on the IPC. The IPC-glass plate sandwich was then set upright vertically on the bench top. The clamps were secured on to the either side of the IPC-glass plate sandwich by moving the levers towards the IPC panel. The IPC assembly was positioned vertically in a precision caster base containing the precision caster gasket and fixed to the base by locking the cam pegs. The entire assembly was laid flat on the bench with the IPC panel (drain port) facing the user.

3.9.2 Gel Preparation and Casting

The acrylamide solution was prepared according to Appendix V and injected into the injection port of the caster base using a syringe. As soon as the gel front reached the other end of the IPC assembly, the vinyl comb was inserted between the plates. The gel was allowed to polymerize for 10-15 min.

3.9.3 Gel Running

The IPC assembly was then dislodged from the precision caster base and fit vertically into the universal base using a stabilizer bar. The temperature indicator was adhered to the surface of the outer plate to monitor the temperature during the run. The upper and the lower buffer chambers were filled with the required volume of 1X TBE buffer. The gel was pre-run for 20 min at 100 W after positioning the top and bottom safety covers. Following completion of the pre-run, the power supply was stopped and the top safety cover removed. The wells were thoroughly rinsed using a pipette to remove any deposited urea. Six µl of samples denatured at 95°C for 5 min were loaded along with 100 bp ladder. After replacing the safety cover, the samples were electrophoresed at 100 W for 35-40 minutes. The power supply was turned off after the completion of the run and the upper buffer chamber was partially emptied by attaching the connector to the drain port on the IPC unit. The clamps were removed and the glass plate containing the gel was separated carefully from the IPC unit. Fragments of gel were removed from the plate by cleaning with lint free tissue.

3.9.4 PCR Product Detection

The glass plate was transferred into a large trough containing the fixer (Appendix VI) and placed on a shaker for 20 min, ensuring that the gel surface faced upwards. Similarly, staining was performed using silver stain (Appendix VII) after the gel was washed in another trough containing purified water for 5 min. Subsequent to a further wash, the stained gel was developed by transferring into a trough containing the developer (Appendix VIII) and gently rocking the trough in a to and fro motion. A white surface was placed under the gel to enable visualization during development. After the bands had visibly developed, the plate was immediately transferred into the fixer. Following a final wash step, the gel was allowed to dry.

3.10 ANALYSIS OF MOLECULAR MARKER DATA

3.10.1 Band Scoring

Clear and reproducible bands were only selected for scoring. Binary scoring was carried out by assigning "1" for presence of band and "0" for absence of band. The data matrix created in excel format was used as the input for cluster analysis.

3.10.2 Quantification of Genetic Variability

Estimation of genetic diversity parameters results in an overview of the genetic variability and can be used as a criterion for comparing both marker efficiency and groups from different studies (Laurentin, 2009).

3.10.2.1 Polymorphic Marker Ratio

A system is polymorphic when its most common stage has a frequency of less than 0.95 (Ott, 1992). If a band occurs in 94% of the individuals, the most common stage will be "presence of the band" and therefore will be considered as polymorphic. Whereas, if the band is present in only 6% of the individuals, the most common stage will be "absence of band", which will occur in 94% of the individuals and therefore

the band will be polymorphic. A simple ratio of the polymorphic loci number to the number of total loci known as polymorphic marker ratio was determined.

3.10.2.2 Average Number of Alleles per Locus

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

$$n = (1/K) \Sigma n_i$$

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

3.10.2.3 Shannon's Diversity Index

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

$$H_i = -\Sigma p_i \log_2 p_i$$

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

3.10.3 Cluster Analysis

The R statistical package was used for hierarchial cluster analysis based on Euclidean distance. A dendrogram grouping the 25 accessions based on SSR marker data was constructed based on complete linkage method using Jaccard's distance.

3.11 CORRELATION OF MORPHOLOGICAL AND MOLECULAR DATA

Mantel's test was used to assess the correlation between the distance measures of the morphological and molecular characterization data.

RESULTS

4. RESULTS

The results of the study entitled "Genetic diversity analysis in taro [*Colocasia esculenta* (L.) Schott] of North East India" carried out at the Division of Crop Improvement, Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram during 2013-2014 are presented in this chapter.

4.1 MORPHOLOGICAL DATA ANALYSIS

Twenty five taro accessions from the North Eastern states of India were used for the study. A wide range of variability was observed among the accessions for some of the characters studied (Plate 1 and 2).

4.1.1 Diversity Indices

The percentage distribution, Shannon's diversity index and Simpson's diversity index computed for the various traits across the twenty five accessions are represented in Table 1. Germplasm type, growth habit, type of stem, leaf sheath pattern, leaf length: breadth ratio, flower formation, seed formation and maturity showed no variation and remained constant for the twenty five accessions studied. The Shannon Weaver's diversity index ranged from 0.17 to 1.58 with a mean value of 0.87 whereas, the Simpson's diversity index ranged from 0.41 to 1.85 with a mean value of 1.00.

4.1.2 Principal Component Analysis

The first four components with eigen values greater than one accounted for 76.59 per cent of the total variation (Table 2). Maximum variation was explained by the first component (34.33%), which included leaf margin colour, leaf colour (lower), sinus colour, petiole colour (top 1/3rd, middle and base) and leaf sheath colour with the highest PC loadings. Cluster plot representation of the PCA (Figure 1) divided the

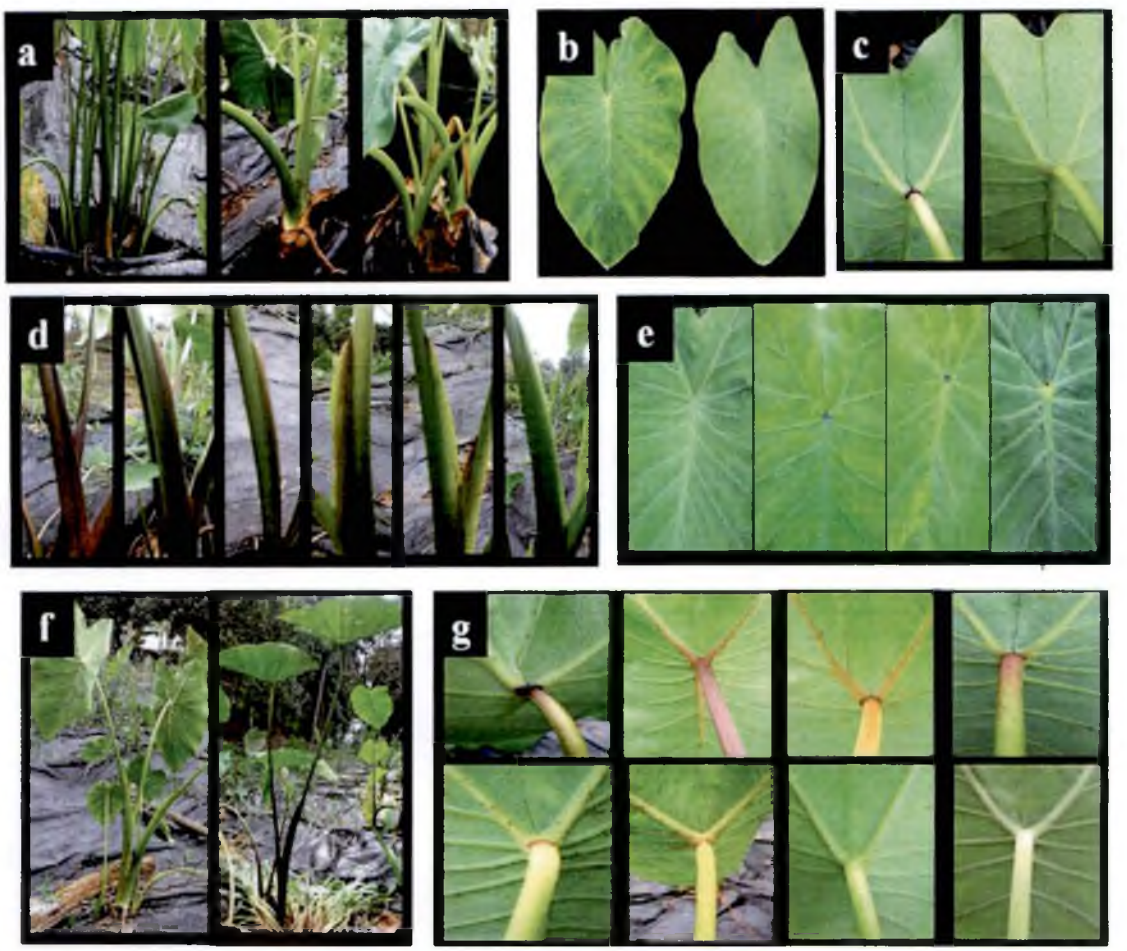

Plate 1(a-g). Comparison of variations in above ground morphological traits. a- Tillering; b- Leaf variegation; c- Sinus colour; d- Sheath colour; e- Petiole junction colour and pattern (U); f- Lamina orientation; g- Petiole junction pattern and colour (L)

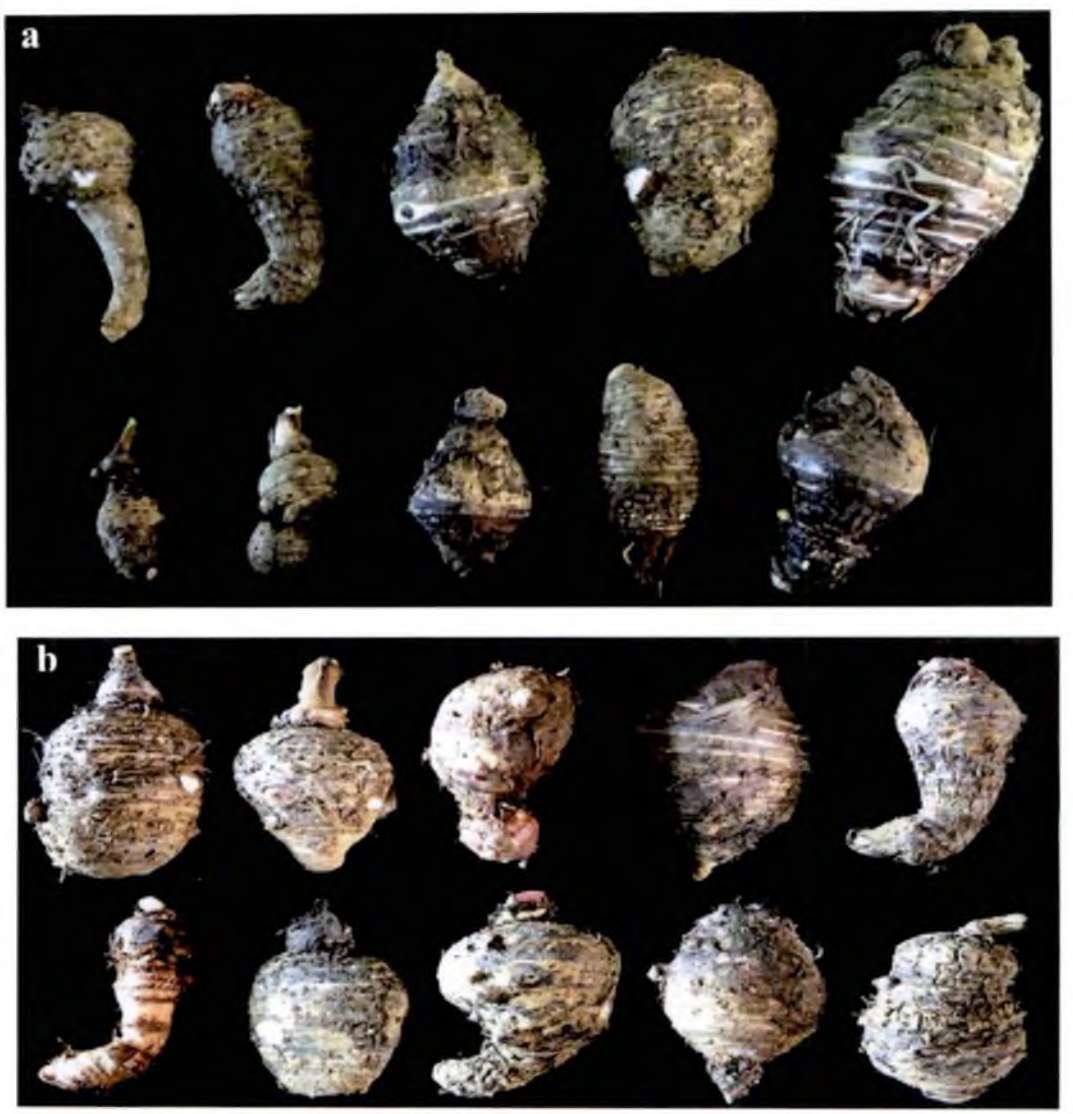

Plate 2 (a and b). Comparison of variations in below ground morphological traits. a- Corm size variations; b- Corm shape variations

Plate 2 (c-g). Comparison of variations in below ground morphological traits.

c – Round; d – Cylindrical; e - Stoloniferous cylindrical; f – Conical;

g – Elongated

accessions into five groups. The biplot representation (Figure 2) was in agreement with the PCA results with leaf margin colour, leaf sheath colour and petiole colour (top 1/3rd, middle and base) contributing more to the overall variability. The range of character distribution represented in the form of box plot (Figure 3) revealed that leaf margin colour, leaf colour (lower), petiole colour (top 1/3rd and middle) accounted for maximum variation.

Table 1. Percentage distribution and diversity indices (H and D) for the morphological traits

Sl. No.	Character	Trait	Percentage (%)	Shannon Weaver's diversity index (H)	Simpson's diversity index (D)
1	Germplasm type	Cultivated	100	0	0
2	Growth habit	Erect	100	0	0
3	Type of stem	Rhizome	100	0	0
4	Leaf arrangement	Clockwise Anticlockwise	76 24	0.55	1.26
5	Lamina orientation	Cup-shaped Erect-apex down	4 96	0.17	1.85
6	Leaf margin	Undulate	100	0	0
7	Leaf margin colour	Green Light green Purplish green Purple Dark purple	28 12 20 20 20	1.58	0.41
8	Leaf colour (upper)	Green Dark green	28 72	0.59	1.19
9	Leaf colour (lower)	Green Light green Glaucous green Purplish green	56 12 24 8	1.12	0.77

Sl. No.	Character	Trait	Percentage (%)	Shannon Weaver's diversity index (H)	Simpson's diversity index (D)
10	Sinus colour	Green Light green Dark green Purplish green Dark purple	56 12 12 12 8	1.29	0.71
11	Leaf variegation	Present Absent	92 8	0.28	1.70
12	Petiole junction pattern and colour (upper)	Spotted (yellow and purple) Asterisk Solid	20 56 24	0.99	0.81
13	Petiole junction pattern and colour (lower)	Semilunar Colour spread to veins Colour not spread to veins No pattern	4 28 40 28	1.21	0.62
14	Vein colour (upper)	Green Dark green	28 72	0.59	1.19
15	Vein colour (lower)	Green Light green Whitish green Purplish green	4 20 4 72	0.82	1.11
16	Petiole colour (top 1/3rd)	Light green Whitish green Purplish green Cream Purple shaded cream	24 24 12 4 36	1.44	0.51
17	Petiole colour (middle)	Green Light green Whitish green Purplish green	8 56 16 20	1.14	0.76

Sl. No.	Character	Trait	Percentage (%)	Shannon Weaver's diversity index (H)	Simpson's diversity index (D)
18	Petiole colour (base)	Green Light green Purplish green Purple Dark green	52 24 12 8 4	1.27	0.69
19	Leaf sheath pattern	Closed	100	0	0
20	Leaf sheath colour	Green Light green Purplish green Purple Dark purple	48 28 8 12 4	1.3	0.65
21	Flower formation	Non-flowering	100	0	0
22	Seed formation	Absent	100	0	0
23	Maturity	6-7 months	100	0	0
24	Corm shape	Cylindrical Conical Elliptical Round	32 48 8 12	1.17	0.69
25	Cormel shape	Cylindrical Conical Round Stoloniferous Cylindrical Elongated	64 12 8 8 8	1.15	0.88
26	Flesh colour	White Off white White with light pink tinge	56 32 12	0.94	0.85
27	Cooked cormel edibility	Non acrid Slightly acrid Medium acrid Highly acrid	60 28 8 4	0.99	0.88

Sl. No.	Character	Trait	Percentage (%)	Shannon Weaver's diversity index (H)	Simpson's diversity index (D)
28	Disease incidence	Moderately resistant Tolerant Moderately susceptible Susceptible Incidence not recorded	4 4 8 20 64	1.1	0.91
29	Tillering*	Low (1-3) Medium (4-6) Other	76 12 12	0.72	1.21
30	Plant size*	Dwarf (<50 cm) Medium (50-100)	12 88	0.37	1.57
31	Leaf L:B ratio* (Leaf type)	Narrow	100	0	0
32	Petiole: leaf length ratio* (Petiole type)	Short Medium Long	4 68 28	0.75	1.08
33	Petiole: sheath ratio* (Sheath type)	Short Medium	52 48	0.69	0.99
34	Number of cormels*	< 5 5-10 >10	8 12 80	0.64	1.32
35	Weight of corm*	< 0.5 kg	100	0	0
36	Weight of cormels*	< 0.1 kg 0.1-0.5 kg	16 84	0.44	1.46
37	Corm length*	< 8 cm 8-18 cm	60 40	0.67	1.03
38	Cormel length*	< 8 cm 8-18 cm	80 20	0.5	1.35
*** Quantitative traits**				**Mean: 0.87**	**Mean: 1.00**

Table 2. Principal component analysis in 25 accessions of *C. esculenta*

Variables	PC1	PC2	PC3	PC4
Tillering	-0.045	0.125	-0.022	0.0005
Plant size	0.019	0.013	-0.007	-0.034
Leaf arrangement	-0.004	-0.004	-0.002	0.040
Leaf margin colour	0.461	0.382	-0.339	-0.095
Leaf colour (upper)	-0.013	-0.013	-0.044	-0.031
Leaf colour (lower)	0.232	-0.062	-0.123	0.230
Sinus colour	0.238	0.133	-0.668	0.221
Petiole junction pattern and colour (upper)	0.012	0.046	0.019	-0.057
Petiole junction pattern and colour (lower)	0.021	0.019	0.049	-0.049
Vein colour (upper)	-0.053	0.029	-0.012	-0.027
Vein colour (lower)	-0.024	0.070	-0.130	-0.878
Petiole colour (top 1/3rd)	0.248	-0.838	-0.267	-0.189
Petiole colour (middle)	0.521	-0.202	0.402	0.134
Petiole colour (base)	0.399	0.077	0.241	-0.106
Petiole type	0.017	0.028	-0.049	-0.021
Leaf sheath colour	0.417	0.230	0.226	-0.134
Sheath type	0.009	-0.016	-0.022	-0.017
Corm shape	0.006	-0.040	-0.178	0.0360
Cormel shape	0.035	0.022	0.122	-0.125
No. of cormels	-0.002	0.049	-0.046	-0.029
Corm length	-0.021	-0.015	0.002	0.060
Flesh colour	-0.049	-0.009	-0.027	0.0008
Cooked cormel edibility	0.023	-0.015	0.048	-0.036
Disease incidence	0.023	-0.020	-0.059	-0.044
Eigen values	**2.428**	**2.035**	**1.945**	**1.721**
Percent variation	**34.33**	**16.94**	**14.13**	**11.19**
Cumulative percentage	**34.33**	**51.27**	**65.40**	**76.59**

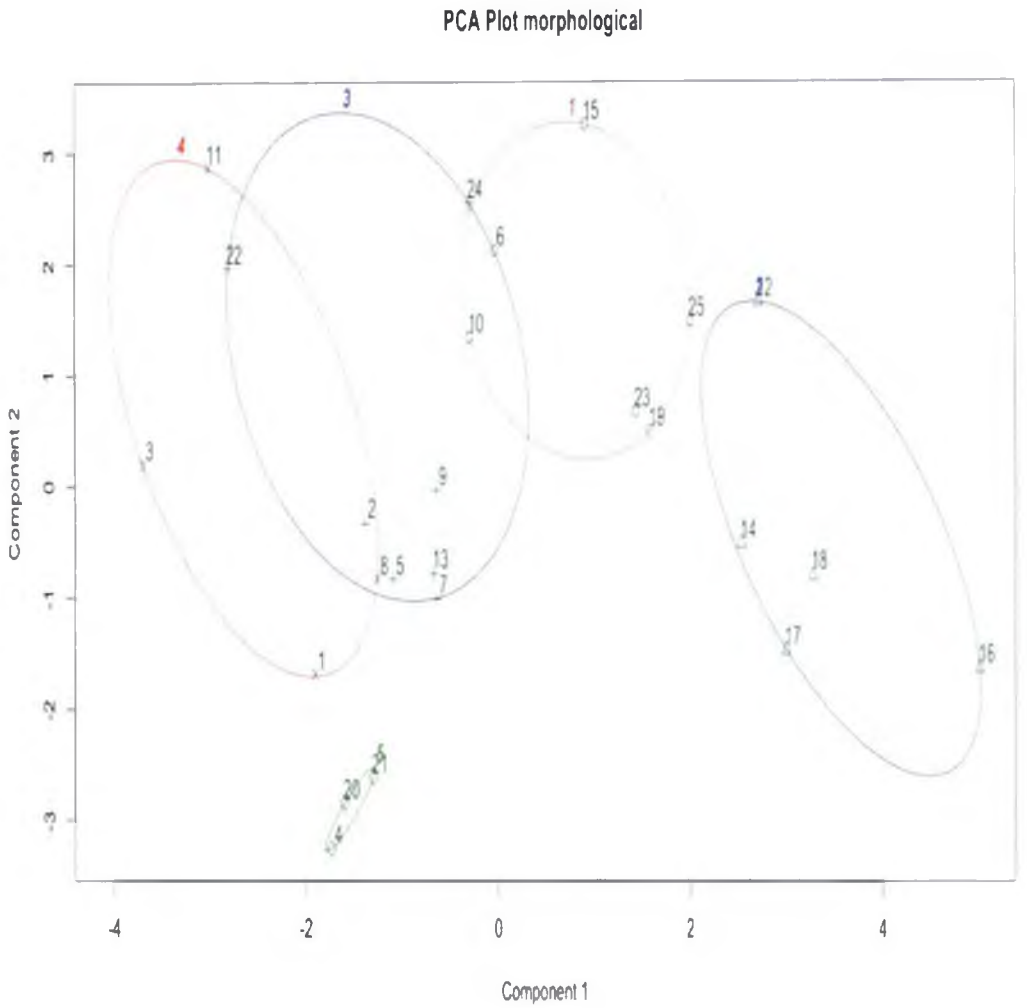

Figure 1. Cluster plot representation of accessions based on PCA

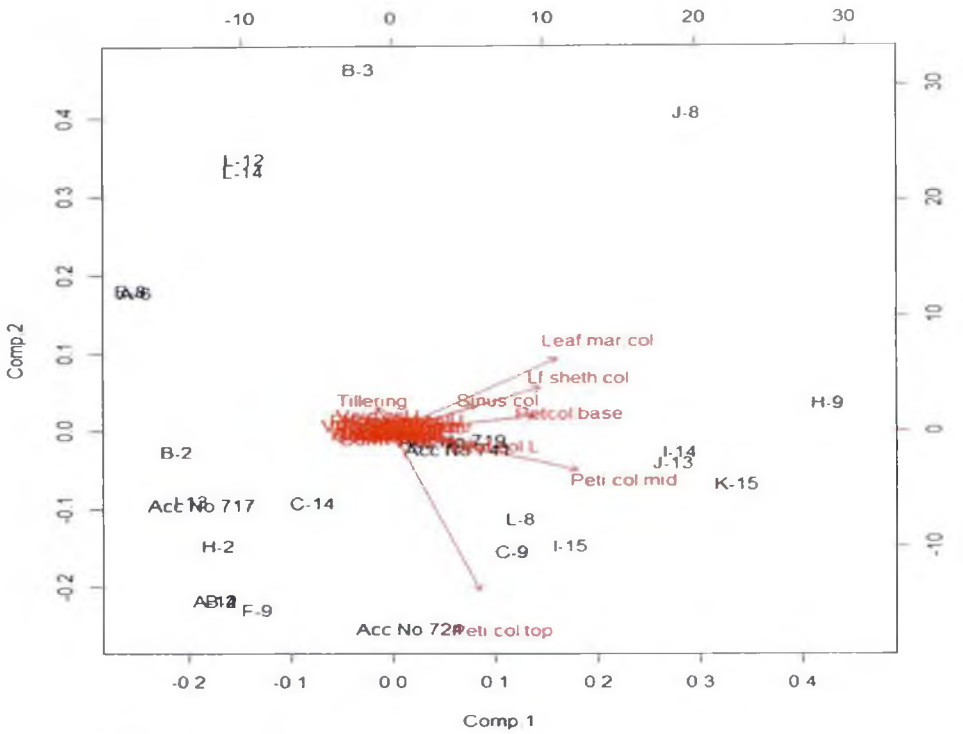

Figure 2. Biplot showing the spatial distribution of the various characters and accessions

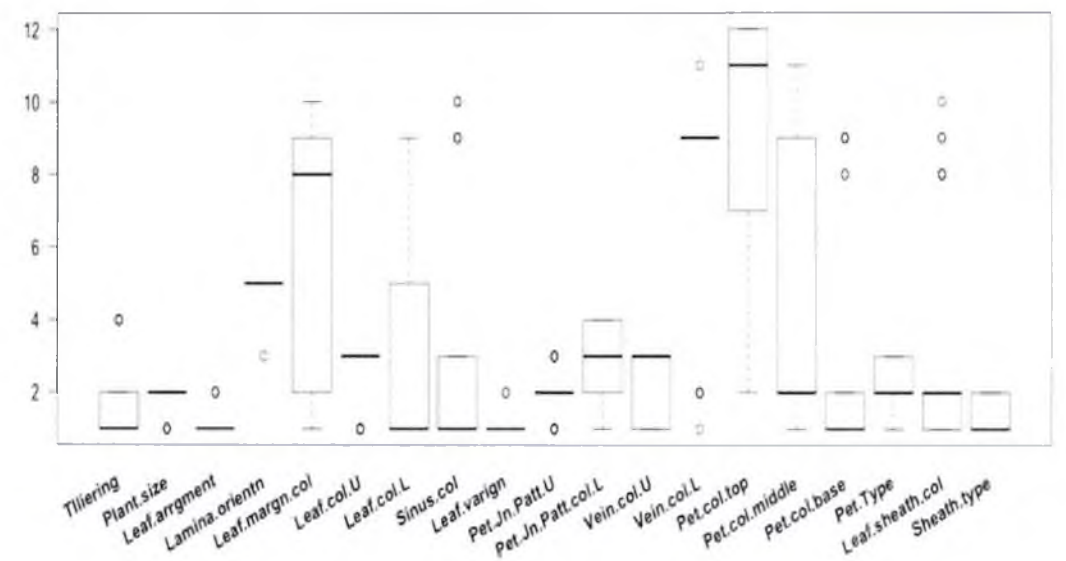

Figure 3. Box plot representing the range of character distribution

4.1.3 Analysis of Variance

Analysis of variance performed on the data of ten quantitative characters showed significant (P<0.01) variation for certain characters (Table 3). Based on the coefficient of variation, the degree of variability was found to be high for tillering, plant size, number of cormels, weight of corms and cormels and cormel length. Duncan's multiple range test (Table 4a and b) was done to compare the mean values in ANOVA.

Table 3. Variation in the quantitative characters for 25 taro accessions

Characters	Mean	Coefficient of variation	F value
Tillering	1.77	49.32	**6.16***
Plant size	66.28	53.61	0.73
Leaf L:B ratio	1.445	3.83	**10.55***
Petiole : leaf ratio	1.85	11.03	3.08
Petiole : sheath ratio	1.96	8.00	**3.88***
No. of cormels	17.65	31.62	**10.14***
Wt. of corms	154.67	28.14	**11.61***
Wt. of cormels	208	20.58	**19.24***
Corm length	11.29	10.10	**22.95***
Cormel length	6.68	23.43	2.74
			* **Significant at P<0.01 level**

Table 4a. Duncan's multiple range test for above ground quantitative characters

(mean values with the same letter do not differ significantly)

Accession ID	Tillering	Plant size	Leaf L:B ratio	Petiole: leaf ratio	Petiole: sheath ratio
A-6	efg(1)	b(58.5)	fgh(1.40333)	cdef(1.7033)	abc(2.0233)
A-12	cdefg(1.6667)	b(61)	fgh(1.40667)	bcdef(1.8333)	abc(2.01)
B-2	g(0)	a(135.5)	ab(1.6333)	ef(1.59)	abc(2.0167)
B-3	fg(0.3333)	b(64.5)	gh(1.37333)	ef(1.58)	abc(2.0533)
B-4	fg(0.6667)	ab(72.83)	fgh(1.39667)	cdef(1.7133)	abc(2.0067)
C-9	cdef(2)	b(60.33)	fgh(1.41)	def(1.6633)	f(1.58)
C-14	ab(4)	b(61.67)	efg(1.42333)	bcdef(1.83)	abc(1.99)
E-8	bcde(2.6667)	ab(67.87)	fgh(1.40)	bcde(1.8833)	abcd(1.9233)
F-9	fg(0.6667)	ab(79)	bcd(1.57333)	abc(2.0867)	abc(2.1033)
G-14	bcd(3.0000)	b(62)	gh(1.36333)	bcde(1.89)	ab(2.1567)
H-2	efg(1)	b(41.67)	gh(1.33)	bcde(1.8833)	abc(2.1333)
H-9	cdefg(1.6667)	b(59.67)	gh(1.38667)	def(1.67)	cdef(1.8233)
I-13	bcde(2.6667)	b(60)	abc(1.61333)	ef(1.5933)	def(1.6467)
I-14	bc(3.3333)	b(57.67)	efg(1.43)	bcde(1.85)	abc(2.11)
I-15	fg(0.6667)	b(54)	fgh(1.41333)	bcde(1.8733)	a(2.1867)
J-8	efg(1)	ab(78)	cde(1.51667)	abc(2.1)	a(2.21)
J-13	fg(0.6667)	ab(71.83)	edf(1.49667)	ab(2.14)	ab(2.14)
K-15	bcde(2.6667)	b(59)	cde(1.52)	bcdef(1.7367)	abcde(1.9033)
L-8	cdefg(1.6667)	ab(71)	hi(1.31)	abcd(2.0067)	abc(1.9933)
L-12	fg(0.3333)	ab(73.83)	gh(1.36667)	bcde(1.8633)	abcd(1.9333)
L-14	ab(3.6667)	b(66.83)	gh(1.38)	abc(2.0867)	abc(2.0633)
Acc. 717	efg(1)	b(44.83)	gh(1.32667)	bcdef(1.74)	bcdef(1.8667)
Acc. 719	a(5)	b(57.17)	a(1.70667)	abcd(2.04)	ef(1.6167)
Acc. 724	defg(1.3333)	ab(82.5)	gh(1.35667)	f(1.4433)	f(1.6067)
Acc. 741	cdefg(1.6667)	b(55.83)	bcd(1.59667)	a(2.3767)	bcdef(1.8733)

Table 4b. Duncan's multiple range test for below ground quantitative characters

(mean values with the same letter do not differ significantly)

Accession ID	No. of cormels	Wt. of corms	Wt. of cormels	Corm length	Cormel length
A-6	cdefg(17.667)	cdefgh(160)	klm(106.67)	gh(10)	fg(4.5)
A-12	cdefg(18.667)	hij(90)	efghi(213.33)	efg(11.6667)	cdefg(5.333)
B-2	bcd(26.333)	bcd(216.67)	fghij(200)	ab(16.1667)	bcdef(7.167)
B-3	fgh(12)	bc(233.33)	defg(240)	fgh(10.5)	a(10.5)
B-4	cdefg(19.33)	bcde(206.67)	a(406.67)	gh(9.5)	bcdef(6.667)
C-9	h(4.333)	cdef(190)	hijkl(153.33)	def(12.5)	abc(8.333)
C-14	efg(15.333)	cdefgh(151.67)	efghi(220)	i(6.8333)	defg(5.167)
E-8	gh(10)	ij(51.67)	no(23.33)	i(5.6667)	bcdefg(6.167)
F-9	gh(9.333)	fghij(108.33)	lmn(93.33)	abc(15.1667)	bcdef(7.5)
G-14	bcdef(22.333)	fghij(110)	cde(286.67)	gh(9.8333)	cdefg(5.333)
H-2	defg(16.333)	j(40)	ijkl(143.33)	gh(10.1667)	bcdefg(6.167)
H-9	gh(11)	efghi(126.67)	efgh(230)	ab(16.1667)	ab(9.167)
I-13	defg(16)	hij(96.67)	bcd(313.33)	i(5)	g(3.5)
I-14	defg(15.667)	cdefg(186.67)	efghi(218.33)	h(9.1667)	abc(8.333)
I-15	gh(9)	j(30)	mno(40)	h(9.1667)	cdefg(6)
J-8	fgh(12)	hij(100)	jkl(131.67)	efgh(11.1667)	bcdef(7)
J-13	fgh(12.667)	fghij(113.33)	jkl(126.67)	cde(13.1667)	bcdefg(6.333)
K-15	defg(17)	ghij(105)	cdef(260)	h(9.1667)	efg(4.833)
L-8	a(56.333)	b(273.33)	bc(320)	abc(15.1667)	bcdefg(6.333)
L-12	defg(17)	cdefg(186.67)	fghijk(183.33)	bcd(14.1667)	abcd(8)
L-14	efg(15.333)	bcde(203.33)	ghijkl(160)	h(9.1667)	cdefg(5.667)
Acc. 717	bc(28)	bcde(200)	ab(366.67)	abc(15)	abcde(7.833)
Acc. 719	bcde(24.667)	hij(100)	bc(330)	fgh(10.5)	bcdef(7)
Acc. 724	h(3.333)	a(446.67)	o(13.33)	a(17)	bcdef(6.667)
Acc. 741	b(31.667)	defgh(140)	a(420)	gh(10.3333)	abcde(7.667)

4.1.4 Cluster Analysis

Cluster analysis using Wards distance revealed five principal clusters (Figure 4) in the dendrogram with cluster V comprising the maximum number of accessions. The cluster data was found to be similar to the accession grouping based on PCA (Table 5). However, based on PCA, accessions C-9 and Acc. 724, which were expected to be under cluster V, were grouped under cluster I in the dendrogram. This led to the separation of Acc. 724 and Acc. 717 in the dendrogram, though they shared the same geographical origin i.e. Meghalaya. Nevertheless, Acc. 741 (from Tripura) and Acc. 719 (from Meghalaya) were grouped together in both the clusters irrespective of their different geographical origin.

Table 5. Comparison of accession grouping based on dendrogram and PCA

Dendrogram			PCA		
Cluster	**Accessions**	**No. of genotypes**	**Cluster**	**Accessions**	**No. of genotypes**
Cluster I	I-15, L-8, C-9, Acc. 724	4	**Cluster I**	I-15, L-8, G-14, Acc. 719, Acc. 741	5
Cluster III	G-14, Acc. 719, Acc. 741	3			
Cluster II	K-15, I-14, J-13, H-9, J-8	5	**Cluster II**	K-15, I-14, J-13, H-9, J-8	5
Cluster IV	B-3, L-12, L-14	3	**Cluster V**	B-3, L-12, L-14	3
Cluster V	A-6, A-12, B-2, E-8, H-2, B-4, I-13, C-14, F-9, Acc. 717	10	**Cluster IV**	A-6, A-12, B-2, E-8, H-2	5
			Cluster III	B-4, I-13, C-14, F-9, C-9, Acc. 724, Acc. 717	7

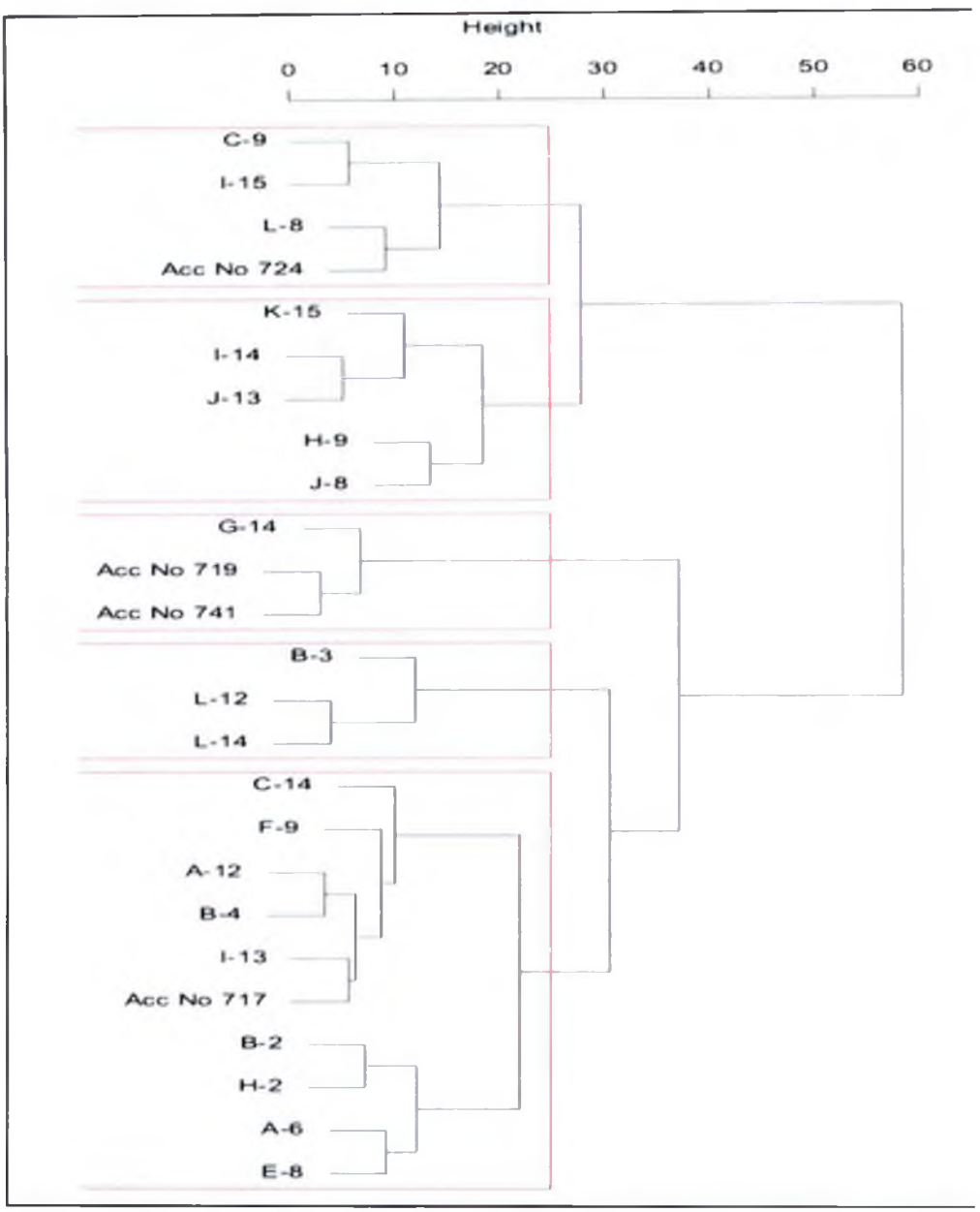

Figure 4. Dendrogram grouping of accessions based on morphological characters

4.2 STANDARDIZATION OF DNA ISOLATION PROTOCOL

Kit based and manual methods of DNA isolation were tried out in taro to select the optimum method for genomic DNA extraction from 25 accessions. Plate 3 shows the DNA profile on agarose gel (0.8%) and Table 6 shows the spectrophotometric readings of the DNA isolated from the various methods tried. DNA isolated using kit method (Amnion AMpreP Plant gDNA Mini kit) was sheared and gave aberrant spectrophotometer readings. Kit based method when combined with chloroform: isoamyl alcohol treatment yielded unsheared DNA. However, the quantity and purity of the DNA was poor. Upon increasing the quantity of tissue from 100 mg to 300 mg, the extracted DNA was found to be highly viscous and rendered unsuitable for quantity and quality check. CTAB method I (Lodhi *et al.,* 1994) resulted in poor quality and quantity of DNA in terms of sheared DNA and abnormally low spectrophotometric readings. The CTAB method II adopted from Sharma *et al.* (2008a) gave intact and unsheared DNA in the gel profile. The absorbance values from spectrophotometer revealed higher quantity of DNA with purity more or less between the range of 1.8-2.0.

The agarose gel profile and the spectrophotometer readings of the DNA extracted from 25 taro accessions using the above method is represented as Plate 4 and Table 7, respectively.

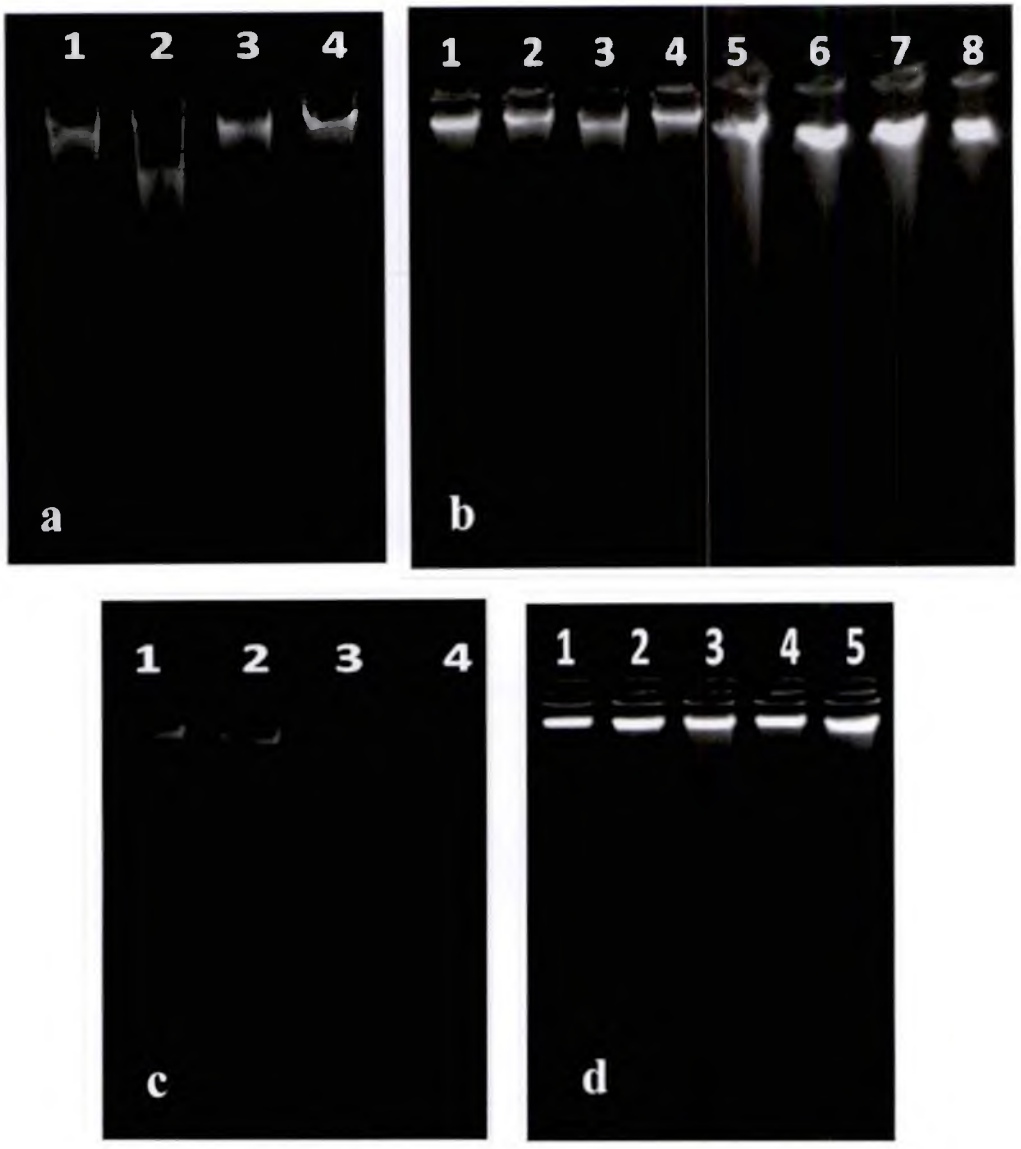

Plate 3 (a-d). Agarose gel profile of DNA extracted using various methods. a- Kit method (Amnion AMpreP Plant gDNA Mini kit); b- Modified kit method (1-4 treated with chloroform: isoamyl alcohol; 5-8- higher tissue quantity); c- CTAB method I (Lodhi *et al.* (1994)); d- CTAB method II (Sharma *et al.* (2008))

Table 6. Quantity and purity of the DNA extracted through various methods

Method	Sample	Absorbance (A_{260} nm)	Absorbance (A_{280} nm)	A_{260}/A_{280}	DNA Yield (ng μl^{-1})
Amnion AMpreP Plant gDNA Mini kit	1	0.021	0.0090	2.33	210
	2	0.080	0.0360	2.22	800
	3	0.014	0.0010	14.00	140
	4	0.025	0.0080	3.13	250
Modified kit method (with chloroform isoamyl alcohol treatment)	1	-0.001	0.0077	0.13	-10
	2	-0.002	0.0090	0.22	-20
	3	0.031	0.0070	4.43	310
	4	0.054	0.0250	2.16	540
Modified kit method (with higher sample quantity; 300 mg)	5*	-	-	-	-
	6*	-	-	-	-
	7	0.076	0.0430	1.77	760
	8*	-	-	-	-
CTAB method I: Lodhi *et al.* (1994)	1	0.008	0.0000	-	80
	2	0.006	-0.0020	-3.00	60
	3	0.008	-0.0010	-8.00	80
	4	0.012	0.0020	6.00	120
CTAB method II: Sharma *et al.* (2008a)	1	0.056	0.0270	2.07	560
	2	0.022	0.0110	2.00	220
	3	0.119	0.0590	2.02	1190
	4	0.100	0.0500	2.00	1000
	5	0.098	0.0490	2.00	980

* - *highly mucilaginous DNA; difficult to quantify*

Plate 4. Agarose gel profile (0.8%) of DNA extracted from 25 taro accessions.

(1: A-6, 2: A-12, 3: B-2, 4: B-3, 5: B-4, 6: C-9, 7: C-14, 8: E-8, 9: F-9, 10: G-14, 11: H-2, 12: H-9, 13: I-13, 14: I-14, 15: I-15, 16: J-8, 17: J-13, 18: K-15, 19: L-8, 20: L-12, 21: L-14, 22: Acc. 717, 23: Acc. 719, 24: Acc. 724, 25: Acc. 741)

Table 7. Quality and yield of DNA from 25 taro accessions using CTAB method II (Sharma *et al.* (2008a))

Sl. No.	Sample	Absorbance (A_{260} nm)	Absorbance (A_{280} nm)	A_{260}/A_{280}	DNA Yield (ng μl^{-1})
1	A-6	0.081	0.040	2.03	810
2	A-12	0.049	0.025	1.96	490
3	B-2	0.092	0.046	2.00	920
4	B-3	0.041	0.019	2.16	410
5	B-4	0.082	0.040	2.05	820
6	C-9	0.166	0.084	1.98	1660
7	C-14	0.072	0.036	2.00	720
8	E-8	0.105	0.050	2.10	1050
9	F-9	0.250	0.123	2.03	2500
10	G-14	0.108	0.059	1.82	1080
11	H-2	0.093	0.052	1.80	930
12	H-9	0.053	0.027	1.96	530
13	I-13	0.012	0.006	2.00	120
14	I-14	0.055	0.026	2.12	550
15	I-15	0.088	0.044	2.00	880
16	J-8	0.040	0.018	2.22	400
17	J-13	0.030	0.015	2.00	300
18	K-15	0.034	0.018	1.89	340
19	L-8	0.053	0.027	1.96	530
20	L-12	0.098	0.048	2.04	980
21	L-14	0.056	0.026	2.15	560
22	Acc. 717	0.022	0.011	2.00	220
23	Acc. 719	0.119	0.059	2.02	1190
24	Acc. 724	0.100	0.050	2.00	1000
25	Acc. 741	0.098	0.049	2.00	980

4.3 PCR STANDARDIZATION

Standardization of PCR conditions was attempted to obtain clear and consistent amplicons under reduced cycle duration. PCR profile obtained using SSR primers with the original reaction conditions is represented in Plate 5a. The modified PCR conditions gave a clearer profile under reduced reaction duration as shown in Plate 5b.

4.4 PRIMER SCREENING

For preliminary screening, 18 SSR primers were used of which, only ten were selected based on their polymorphism and repeatability. Plates 6 and 7 show the gel profile of the 18 SSR primers used at an annealing temperature of 56°C. Ce1 D12, Ce1 B12, Ce1 F12, Ce1 A08 and uq88B-94 were eliminated after the preliminary screening of taro accessions due to poor amplification profile. Though uq110-283, uq115-71 and uq132-147 gave a good amplification pattern, these primers were not selected for SSR analysis since they could not detect any polymorphism.

The annealing temperature for the selected Ce1 primers was standardized by gradient PCR (Plate 8) and the annealing temperatures used for the various primers is represented in Table 8.

4.5 SSR ANALYSIS OF TARO ACCESSIONS

The amplicons obtained after the SSR analysis of 25 taro accessions using the selected primers were initially resolved in a two per cent agarose gel (Plates 9a and b) and bands were obtained in the expected product size as mentioned in Table 4 of Chapter 2.

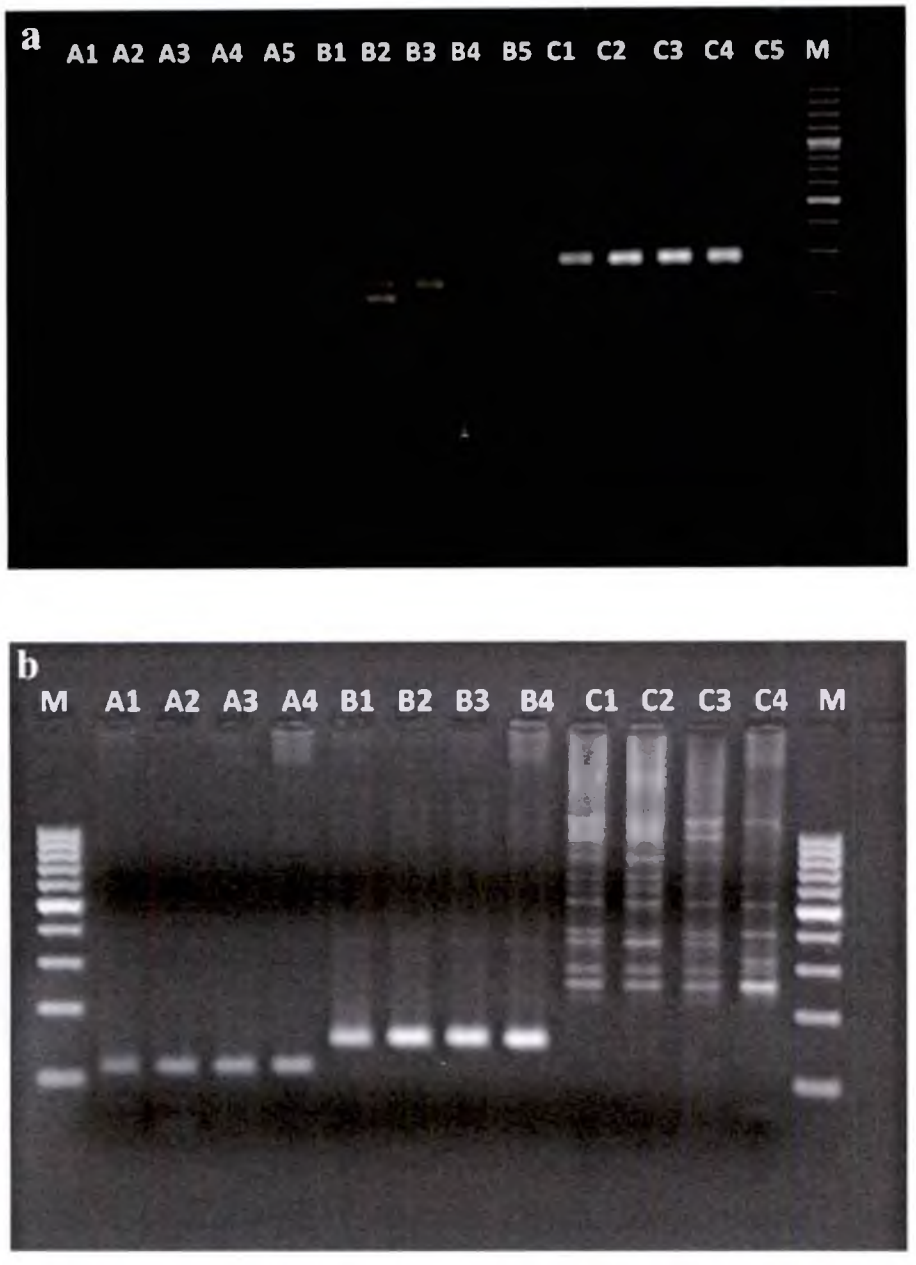

Plate 5 (a and b). Gel profile obtained under various PCR conditions.

a- Original reaction conditions; b- Modified reaction conditions

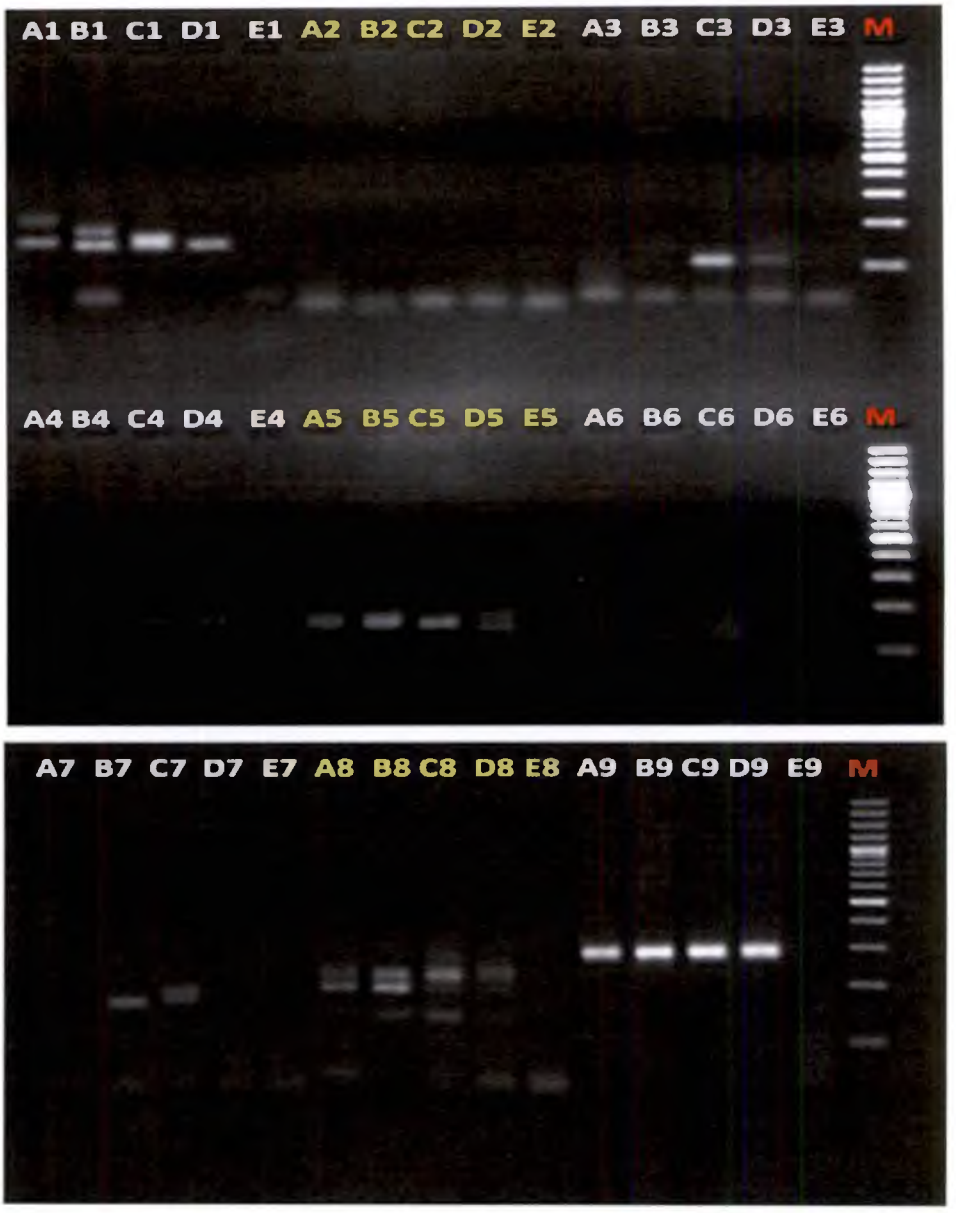

Plate 6. Agarose gel (2%) profile of the SSR primers screened. 1-9 and A-E represents the 9 SSR primers and DNA from 5 taro accessions respectively. (1- Ce1 B03; 2- Ce1 D12; 3- Ce1 H12; 4- Ce1 B12; 5- Ce1 F04; 6- Ce1 F12; 7- uq73-164; 8- uq84-207; 9- uq110-283)

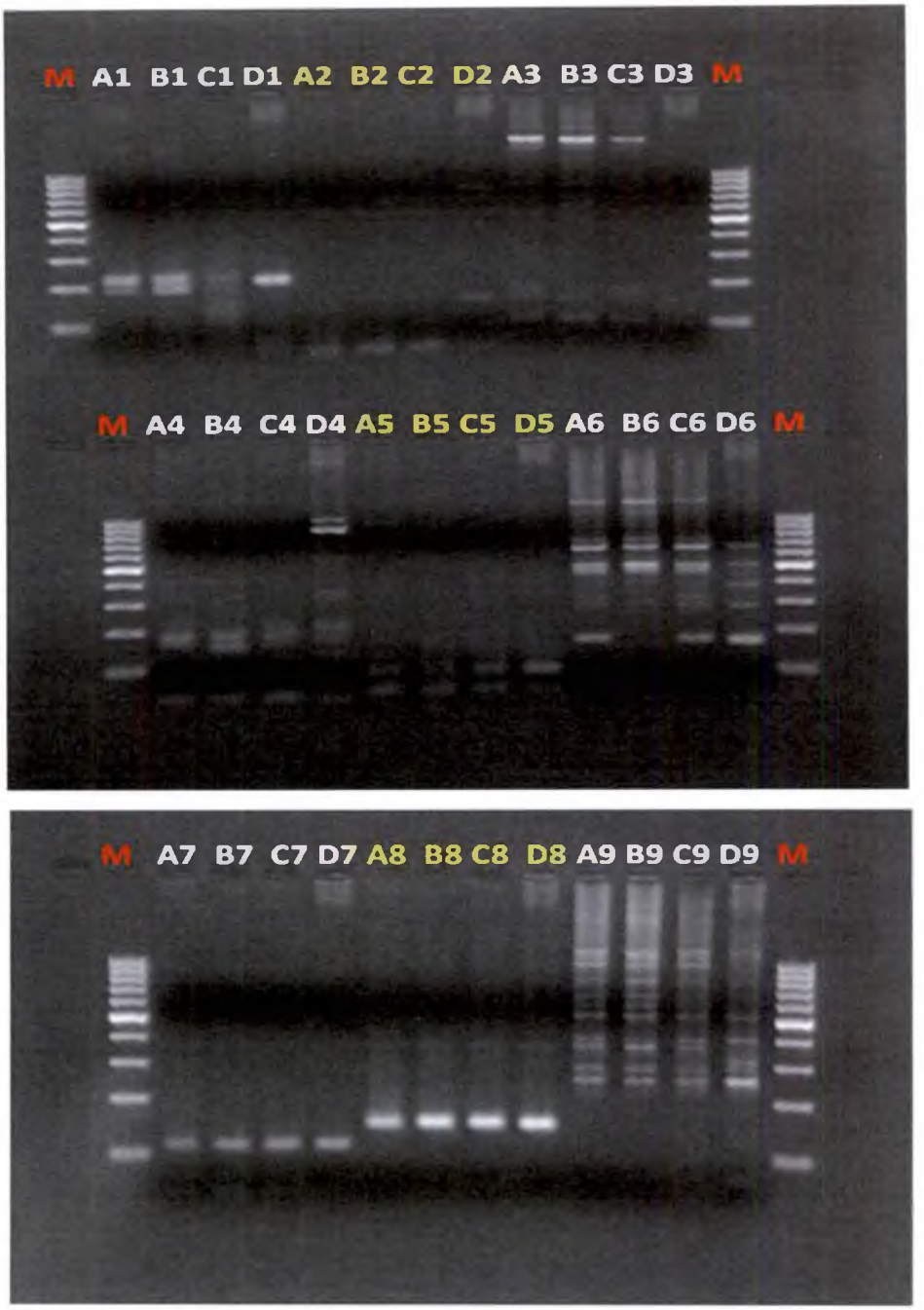

Plate 7. PCR profile (2% agarose) of the SSR primers screened. 1-9 and A-D represents the 9 SSR primers and DNA from 4 taro accessions respectively. (1- Ce1 A06; 2- Ce1 A08; 3- Ce1 C03; 4- Ce1 C06; 5- uq88B-94; 6- uq97-256; 7- uq115-71; 8- uq132-147; 9- uq201-302)

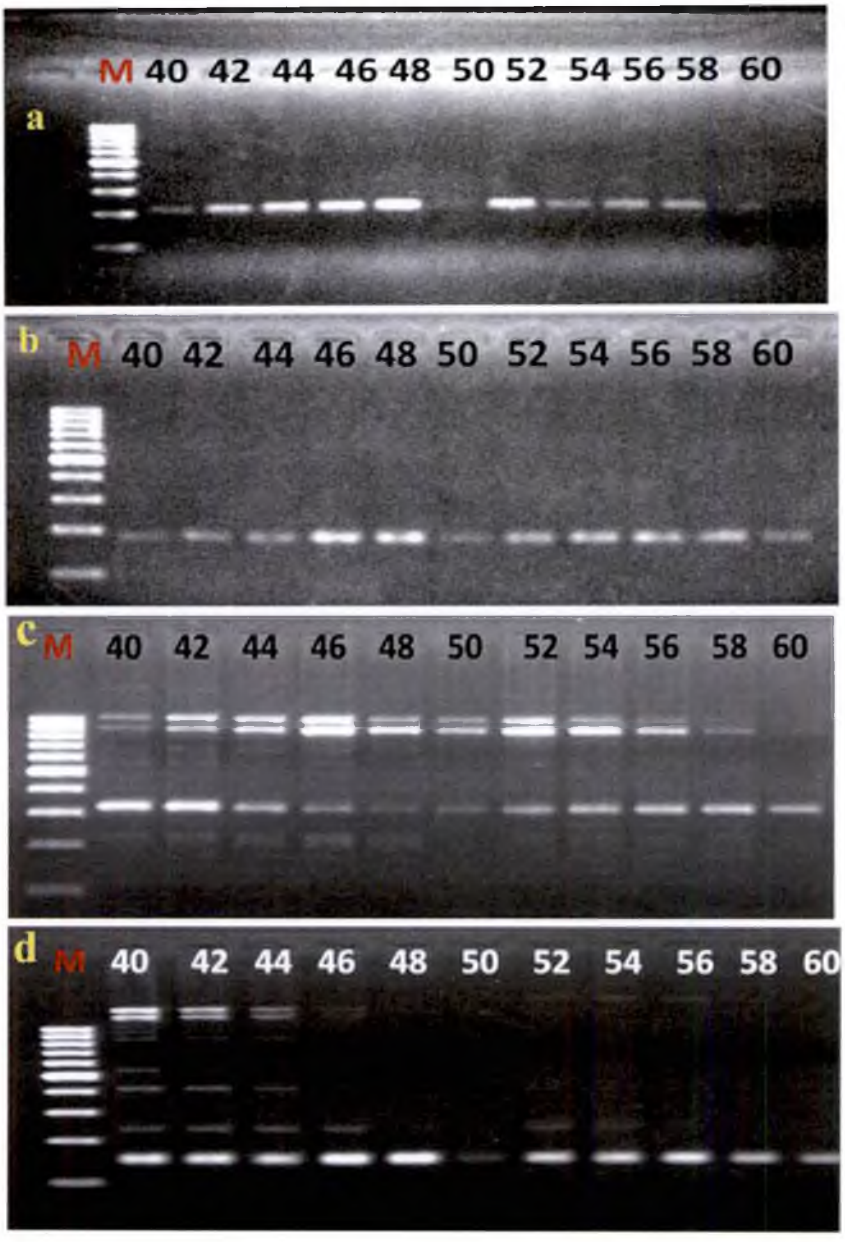

Plate 8 (a-d). Gradient PCR profiles for Ce1 primers. a- Ce1 A06; b- Ce1 B03; c- Ce1 C06; d- Ce1 F04 (40-60 represents the range of annealing temperature in °C)

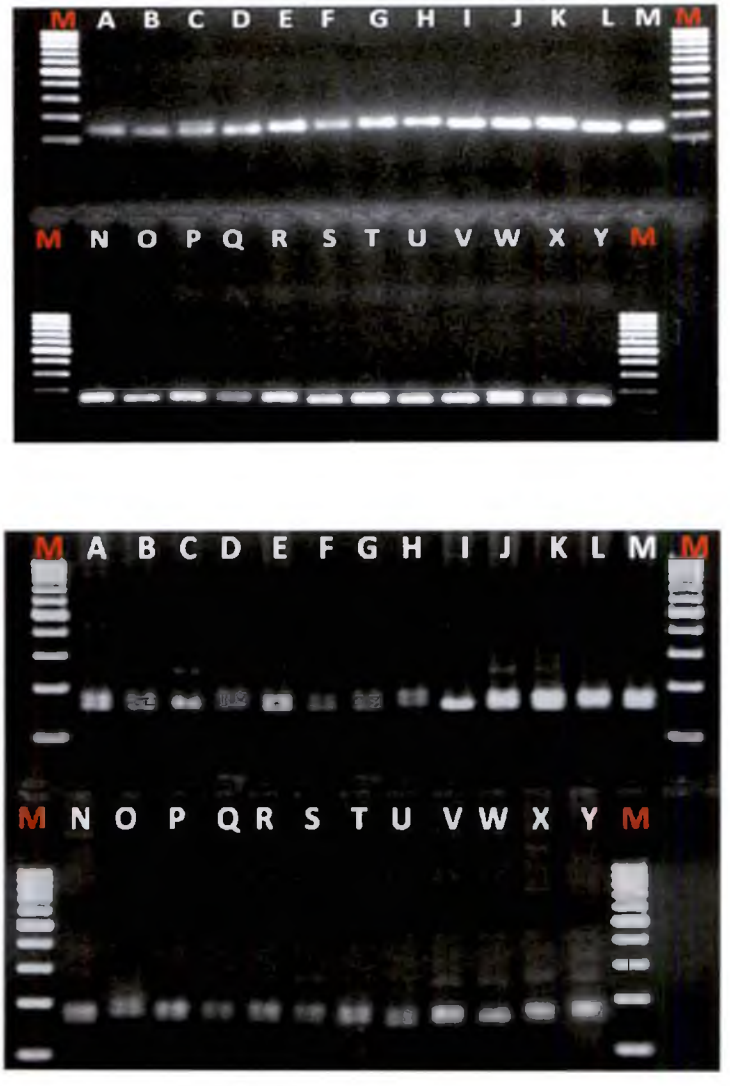

Plate 9a. PCR profile (2% agarose) of 25 taro accessions using Ce1 F04 and uq73-164 respectively

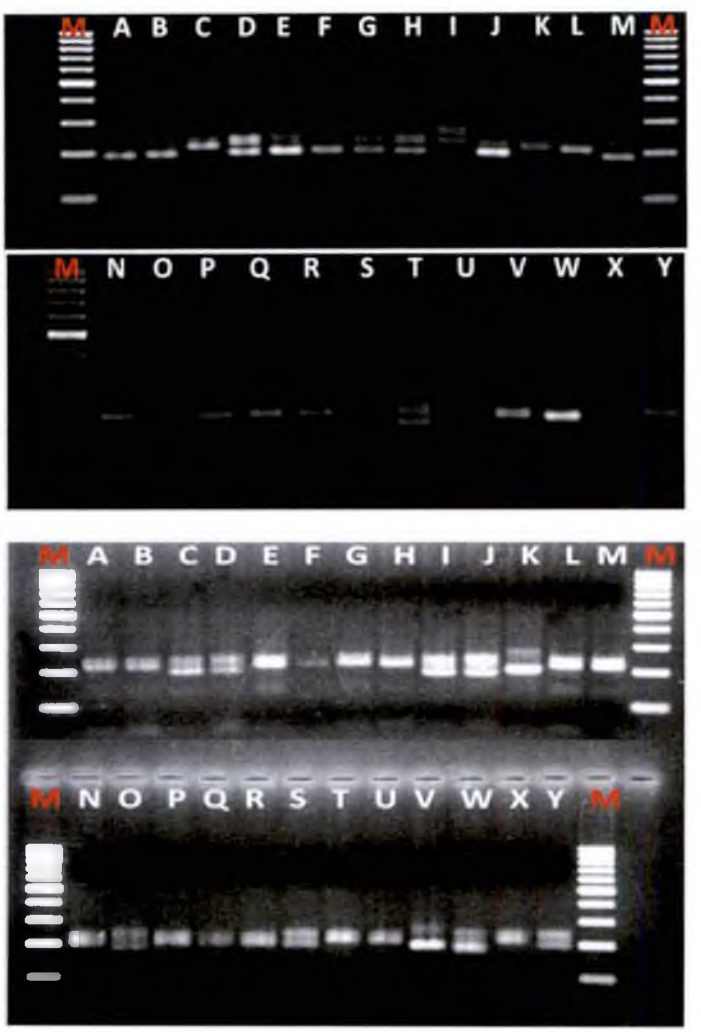

Plate 9b. PCR profile (2% agarose) of 25 taro accessions using Ce1 A06 and uq84-207 respectively

4.6 DENATURING PAGE FOR SSR ANALYSIS

For obtaining higher resolution in the separation of the PCR products, denaturing PAGE was done. Plates 10 (a-e) represent the denaturing PAGE profiles of the amplicons analyzed by silver staining. The number of bands obtained was higher compared to two per cent agarose gel analysis. This was also evident in the average number of alleles computed for each primer from the PAGE data. None of the ten selected primers exhibited monomorphism and null alleles were also not detected for any primer.

4.7 ANALYSIS OF MOLECULAR MARKER DATA

4.7.1 Quantification of Genetic Variability

The various parameters estimated for the quantification of genetic variability using ten SSR markers is represented in Table 8. The average number of alleles per locus varied from 6.0-12.57, with the highest number of alleles per locus shown by the primer Cel B03. Cel C06 gave the highest value for Shannon's diversity index (2.37), which ranged from 1.59 to 2.37. The polymorphic marker ratio was found to be high for all the primers (0.76-1.0). However, maximum ratio of one was given by Cel A06, Cel B03, Cel C06, Cel F04 and uq201-302.

4.7.2 Cluster Analysis

Cluster analysis done based on Euclidean distance grouped the twenty five accessions in to five major clusters in the dendrogram (Figure 5). Cluster II comprised the maximum number of accessions (9).

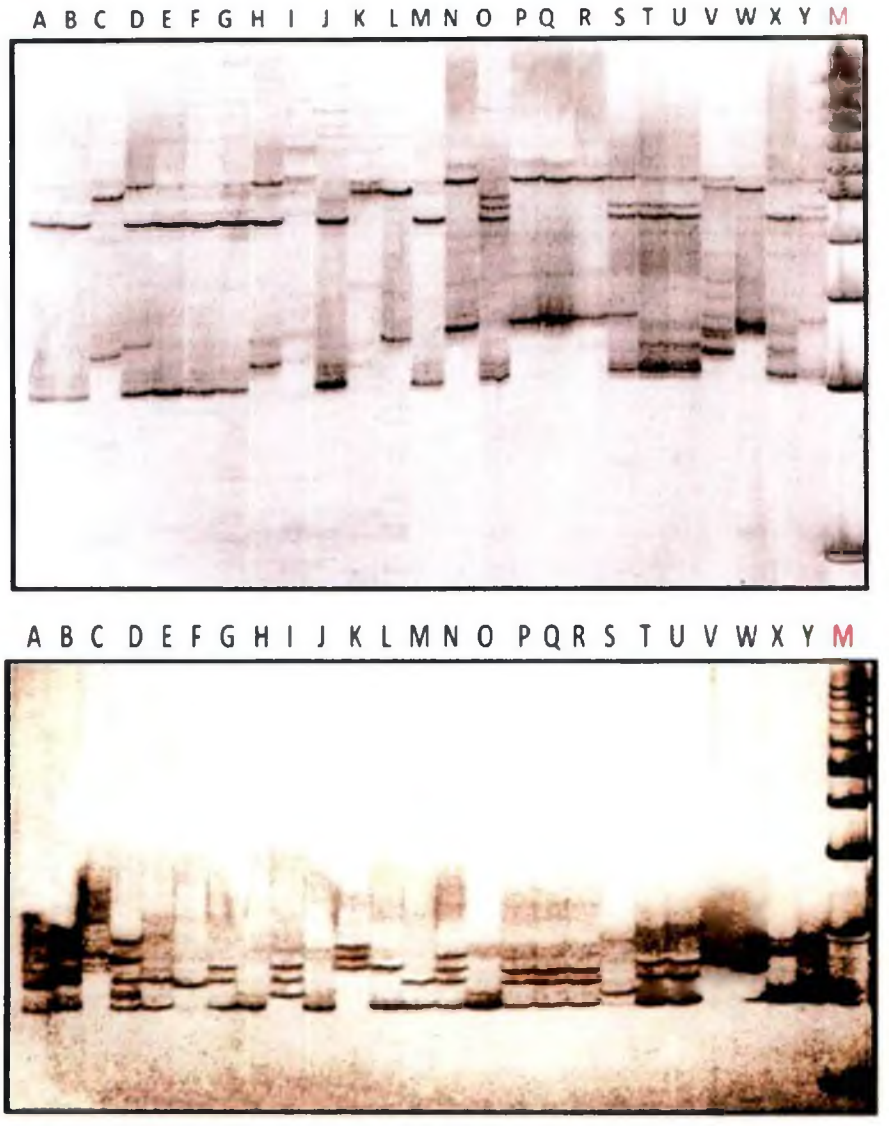

Plate 10a. Denaturing PAGE (6%) profile of 25 taro accessions using Ce1 A06 and Ce1 B03 respectively

A: A-6, B: A-12, C: B-2, D: B-3, E: B-4, F: C-9, G: C-14, H: E-8, I: F-9, J: G-14, K: H-2, L: H-9, M: I-13, N: I-14, O: I-15, P: J-8, Q: J-13, R: K-15, S: L-8, T: L-12, U: L-14, V: Acc. 717, W: Acc. 719, X: Acc. 724, Y: Acc. 741)

Plate 10b. Denaturing PAGE (6%) profile of 25 taro accessions using Ce1 C03 and Ce1 C06 respectively

(A: A-6, B: A-12, C: B-2, D: B-3, E: B-4, F: C-9, G: C-14, H: E-8, I: F-9, J: G-14, K: H-2, L: H-9, M: I-13, N: I-14, O: I-15, P: J-8, Q: J-13, R: K-15, S: L-8, T: L-12, U: L-14, V: Acc. 717, W: Acc. 719, X: Acc. 724, Y: Acc. 741)

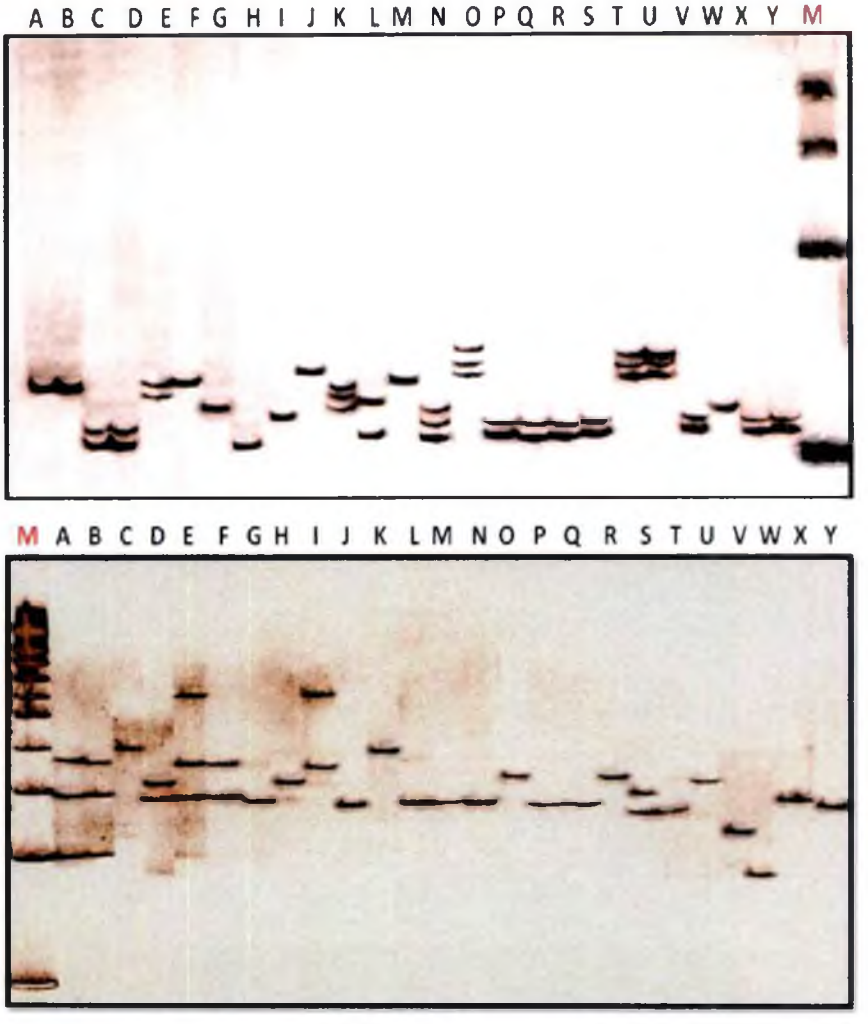

Plate 10c. Denaturing PAGE (6%) profile of 25 taro accessions using Ce1 F04 and Ce1 H12 respectively

(A: A-6, B: A-12, C: B-2, D: B-3, E: B-4, F: C-9, G: C-14, H: E-8, I: F-9, J: G-14, K: H-2, L: H-9, M: I-13, N: I-14, O: I-15, P: J-8, Q: J-13, R: K-15, S: L-8, T: L-12, U: L-14, V: Acc. 717, W: Acc. 719, X: Acc. 724, Y: Acc. 741)

Plate 10d. Denaturing PAGE (6%) profile of 25 taro accessions using uq73-164 and uq84-207 respectively

(A: A-6, B: A-12, C: B-2, D: B-3, E: B-4, F: C-9, G: C-14, H: E-8, I: F-9, J: G-14, K: H-2, L: H-9, M: I-13, N: I-14, O: I-15, P: J-8, Q: J-13, R: K-15, S: L-8, T: L-12, U: L-14, V: Acc. 717, W: Acc. 719, X: Acc. 724, Y: Acc. 741)

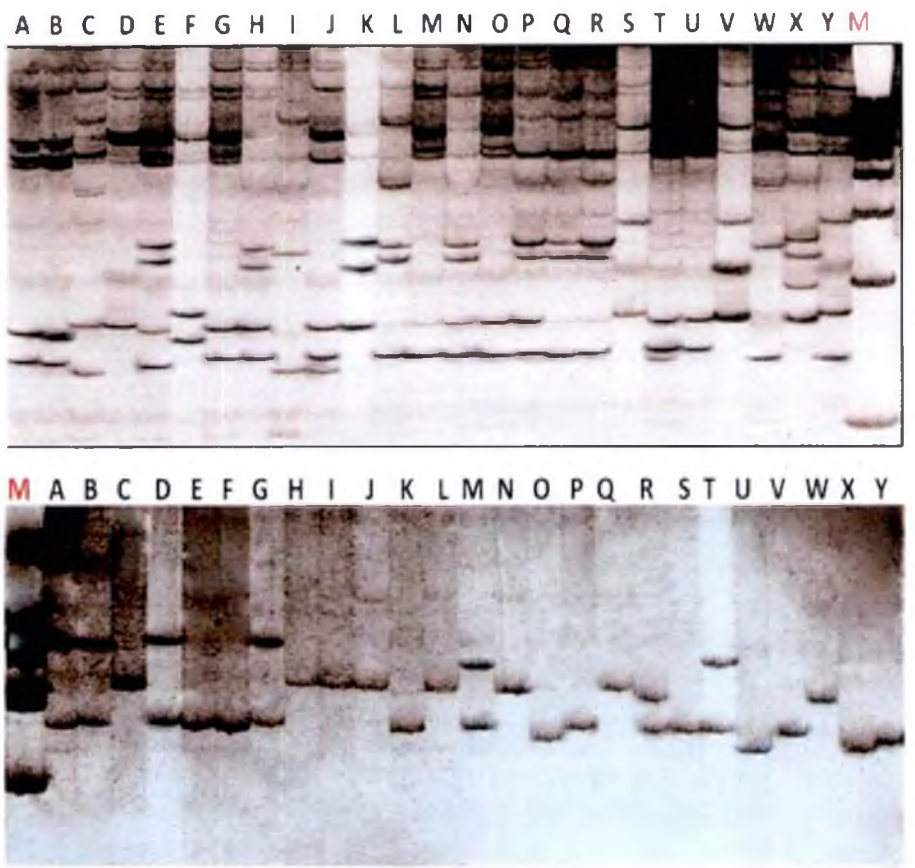

Plate 10e. Denaturing PAGE (6%) profile of 25 taro accessions using uq97-256 and uq201-302 respectively

(A: A-6, B: A-12, C: B-2, D: B-3, E: B-4, F: C-9, G: C-14, H: E-8, I: F-9, J: G-14, K: H-2, L: H-9, M: I-13, N: I-14, O: I-15, P: J-8, Q: J-13, R: K-15, S: L-8, T: L-12, U: L-14, V: Acc. 717, W: Acc. 719, X: Acc. 724, Y: Acc. 741)

Table 8. Measures of diversity estimated for the SSR primers

Primer	T_m (°C)	Average no. of alleles per locus	Polymorphic marker ratio	Shannon's diversity index (H)
Ce1 A06	52	7.67	1.00	2.24
Ce1 B03	56	12.57	1.00	1.73
Ce1 C03	54	7.08	0.92	2.17
Ce1 C06	58	8.50	1.00	2.37
Ce1 F04	56	6.88	1.00	1.99
Ce1 H12	54	6.00	0.78	1.68
uq73-164	56	11.00	0.90	2.08
uq84-207	56	8.71	0.76	2.33
uq97-256	56	7.92	0.85	2.25
uq201-302	56	9.71	1.00	1.59

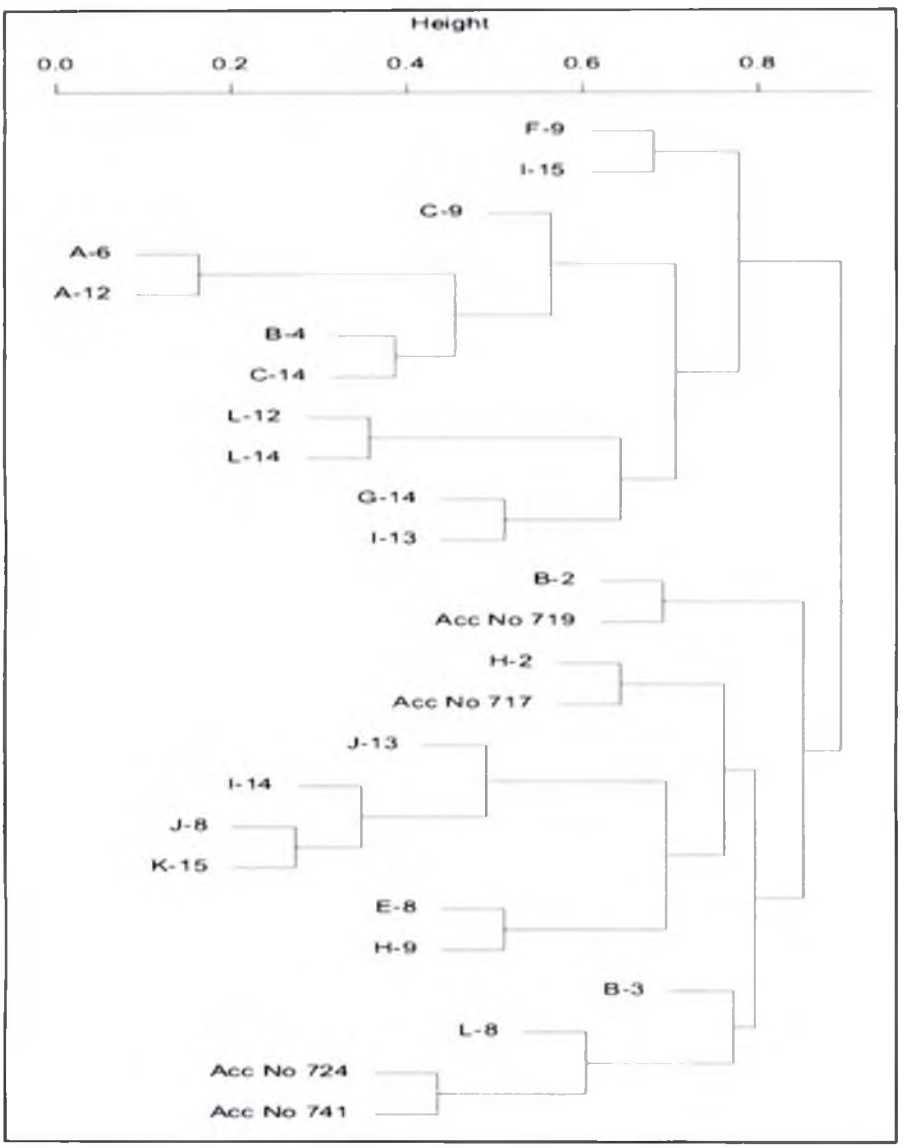

Figure 5. Dendrogram analysis of 25 taro accessions using molecular data

4.8 CORRELATION OF MORPHOLOGICAL AND MOLECULAR DATA

Mantel's test ($r = 0.1432$; $p = 0.0648$) was done to assess if any correlation existed between the morphological and molecular data. The analysis established that no correlation existed between theses data. However, the accession-wise comparison of the dendrogram data (Table 9) revealed that accessions K-15, I-14, J-13, H-9 and J-8 were grouped together both in molecular and morphological clustering. Similarly, A-6, A-12, B-4, C-14 and I-13 belonged to the same cluster in dendrograms from morphological and molecular data.

Table 9. Accession-wise comparison of morphological and molecular clustering

(Common accessions have been italicized)

Molecular analysis			**Morphological analysis**		
Cluster	**Accessions**	**No. of genotypes**	**Cluster**	**Accessions**	**No. of genotypes**
Cluster I	F-9, I-15	2	Cluster I	C-9, I-15, L-8, Acc. 724	4
Cluster II	C-9, *A-6, A-12, B-4, C-14*, L-12, L-14, G-14, *I-13*	9	Cluster V	*C-14*, F-9, *A-12, B-4, I-13*, Acc. 717, B-2, H-2, *A-6*, E-8	10
Cluster III	B-2, Acc. 719	2	Cluster III	G-14, Acc. 719, Acc. 741	3
Cluster IV	H-2, Acc. 717, *J-13, I-14, J-8, K-15*, E-8, *H-9*	8	Cluster II	*K-15, I-14, J-13, H-9, J-8*	5
Cluster V	B-3, L-8, Acc. 724, Acc. 741	4	Cluster IV	B-3, L-12, L-14	3

Discussion

5. DISCUSSION

The North Eastern region of India, believed to be one of the centers of origin of taro [*Colocasia esculenta* (L.) Schott] is blessed with immense genetic wealth of the crop. A high level of genetic variation expected among taro accessions belonging to this diversity hotspot is supported by the pantropical distribution of the crop. Inhabitation of geographically diverse environments adds to the morphological variability observed in this highly polymorphic species. Intense ultraviolet radiation characteristic to regions close to the equator can also influence variations through mutations (Singh *et al.*, 2011).

Numerous studies characterizing the genetic variability of *C. esculenta* from different geographical locations using molecular and morphological markers have been reported. However, none of these reports have documented the diversity prevailing in this center of origin. The prerequisite for any crop improvement initiative is the availability of data on the characterization of existing genetic resources. Such initiatives become imperative for taro; being a crop facing intensive biotic stress in addition to the technical constraints associated with its production. Genetic diversity analysis can benefit taro breeders in making an informed choice for parental selection in hybridization programs. The characterization data can facilitate the collection, conservation and evaluation of germplasm. In this context, the present study was undertaken as a preliminary approach to analyze the genetic diversity of taro germplasm from North East India using morphological and microsatellite (SSR) markers.

In this study, the genetic diversity among 25 taro accessions from North East India was determined using 38 morphological descriptors and ten SSR markers. The study has also attempted to standardize a genomic DNA extraction protocol suitable for taro in addition to standardizing the PCR conditions for the SSR primers. Denaturing PAGE followed by silver staining was used for microsatellite analysis.

Cluster analysis was performed to visualize the accession grouping and a correlation study investigating the congruence between the morphological and molecular data was also carried out.

5.1 MORPHOLOGICAL DATA ANALYSIS

A wide range of morphological variability was observed among the accessions especially in the above ground qualitative characters. This is exemplified by the fact that a combination of two standard taro descriptors had to be used for the characterization since variations in addition to the prescribed traits had to be categorized. Somatic mutations accumulated through vegetative multiplication could modify one gene or a complex of them responsible for the modification of a visible morphological character (mainly colours of stem and corm). Since they have been propagated asexually for thousands of years, morphotypes can be quite distinct as a result of fixing somatic mutations (Kuruvilla and Singh, 1981), even when they share the same genetic background. Though a rare phenomenon, flowering and seed setting is naturally seen in taro. Protogyny and self-incompatibility systems in the inflorescence facilitate cross-fertilization, which usually results in variable progenies (Shaw, 1975; Okpul and Ivancic, 1996; Johnston and Gendua, 1998).

In some situations morphological data alone can facilitate the selection of genotypes with desirable traits. For instance, it has been reported that the presence of stolons, common in wild types, was found to be often associated with undesirable traits such as poor corm shape, poor taste quality and acridity (Lebot *et al.*, 2004). *C. esculenta* inhabiting wild environment might require a higher proportion of calcium oxalate crystals as pest repellants in order to survive in a hostile environment compared to cultivars for which farmers have developed practices to protect them (Sardos *et al.*, 2012).

5.1.1 Diversity Indices

The percentage distribution of various traits suggests that several traits were not uniformly distributed among the accessions studied. With the exception of germplasm type, growth habit, type of stem, leaf margin, leaf sheath pattern, flower formation, seed formation, maturity, leaf L:B ratio and weight of corms, all other traits exhibited variable distribution pattern. The study could highlight the extent of diversity present even with a limited sample size using the diversity indices (H = 0.87, D = 1.00). This can be compared to a study based on the morpho-agro characterization of 100 accessions of taro collected from the different parts of Ethiopia (Beyene, 2013) which gave a low diversity index (H = 0.27). However, morphological analysis of North East Indian taro accessions using above ground traits alone revealed a higher diversity index (H = 0.87) (Vinutha *et al.*, unpublished data, 2014; unreferenced). This suggests that subterranean traits do not contribute much to the variability. Sullivan *et al.* (1998) has used H and D to examine changes in the diversity of plants and mammals over years. The ability to quantify diversity in this way is an important tool for biologists to understand community structures. This measure has been used to describe variation in wheat (Pecetti *et al.*, 1992), barley (Tolbert *et al.*, 1979; Engels, 1994), rice (Holcomb *et al.*, 1977) and chickpea (Upadhyaya, 2003).

5.1.2 Principal Component Analysis

Most often the first three PC's explain more than 70 per cent of the variability. In this study, the first four components had to be considered to explain 76.59 per cent of the variation. However, ordination among 100 Ethiopian accessions (Beyene, 2013) showed that the first seven PCs cumulatively accounted for only 64.59 per cent of variation. This suggests the existence of greater variability in taro in the geographical region considered in the present study. PC analysis revealed that leaf and petiole colour characteristics majorly contributed to the variation explained

among the 25 accessions. Tuber characters were found to be unimportant to explain the diversity. This cannot be interpreted as a general observation since corm and cormel characters and foliar characters especially petiole surface pattern were found to be important in distinguishing the various accessions of *A. paeoniifolius* (Anil *et al.*, 2011).

The biplot provided a visual impression of how the genotypes interrelated with each of the morphological traits. The biplot and boxplot representations were in complete agreement with the PCA data though these displays were two-dimensional compromises of multi-dimensional relationships. The selected morphological traits can be used as a tool for initial field level decision of explorers in tapping *C. esculenta* variability during explorations.

5.1.3 Analysis of Variance

ANOVA identified significant variation ($P<0.01$) for seven out of the ten quantitative characters analyzed, of which four were tuber characters. Similar observation was made by Anil *et al.* (2011) who found out high degree of variability existing for cormel weight per corm, cormel length, cormel number and corm fresh weight in *A. paeoniifolius* based on the coefficient of variation. The coefficient of variation ranged from 8.0-53.61 per cent in this study, which was higher compared to 12.60-39.32 per cent obtained in the study by Beyene (2013). In the present study, Duncan's multiple range test ($\alpha = 0.05$) performed on the quantitative traits, differentiated accessions based on grouping of mean values. For example, an accession with the letter 'g' differs from all other accessions lacking 'g' for that particular trait.

5.1.4 Cluster Analysis

Cluster analysis was done to group the 25 accessions based on complete linkage method and gave five major clusters at a truncate level of 25. The genetic

relationships were nearest between accessions in cluster V and farthest in cluster II. The cluster data from dendrogram was in agreement with data from cluster analysis after PCA suggesting that the accessions have been grouped according to the most variable traits. The analysis also revealed lack of association between morpho-agronomic traits and geographical origin; the observation being consistent with previous reports (Zubair *et al.*, 2007; Ahmad *et al.*, 2008; Ali *et al.*, 2008). It should be noted that taro inhabits diverse environments. Geographically close habitats can be ecologically quite different and conversely, habitats that are geographically distant from one another can be very similar in their environmental conditions. This accounts for the lack of association as morphological characteristics are conditioned by the environment.

5.2 STANDARDIZATION OF DNA ISOLATION PROTOCOL

Commercial kits for genomic DNA extraction was found ineffective for the extraction of high quality genomic DNA from mucilage rich taro. Mucilage, a highly viscous secondary metabolite composed of a polar polymer of glycoprotein, present in tubers, seeds, and stems, co-precipitates with DNA and inhibits the action of Taq polymerase (Jose and Usha, 2000; Ghosh *et al.*, 2009). The presence of mucilage also hinders the accurate pipetting of DNA, leading to volumetric errors (Ghosh *et al.*, 2009); which accounts for the aberrant spectrophotometric readings.

CTAB method of DNA isolation which is mostly accepted in plant species was used in this study. The method by Lodhi *et al.* (1994) was found unsuitable for taro probably because of the specific crop characteristics. However, DNA extraction protocol specific to tuber crops by Sharma *et al.* (2008a) was adopted successfully. An effective method for removal of polysaccharides was obtained by increasing the concentration of CTAB along with NaCl in the extraction buffer (Syamkumar *et al.*, 2005; Abdellaoui *et al.*, 2011; Sahu *et al.*, 2012).

Since taro is rich in secondary metabolites, a careful and quick processing of the sample was required mainly during tissue homogenization. The inclusion of PVP and β-mercaptoethanol in extraction buffer which are known to prevent the oxidation of secondary metabolites in the disrupted plant material (Prittila *et al.*, 2001; Warude *et al.*, 2003), avoided the brown pigmentation in the sample, increasing the yield and quality of DNA. Phenol: chloroform: isoamyl alcohol (25:24:1) has been reported to give high purity DNA in many plant species (Aras *et al.*, 2003; Sablok *et al.*, 2009; Lele, 2011). However, phenol was avoided in this protocol since it hindered the isolation process. To ensure the long term storage of the isolated DNA, TE buffer was used.

The absorbance ratio (A_{260}/A_{280}) in the range of 1.8 to 2.0 indicates a high level of purity of DNA (Weising *et al.*, 2005). The isolated genomic DNA from young taro leaves confirmed high purity with A_{260}/A_{280} value ranging from 1.8-2.22. Generally, mature plant tissues are not preferred for DNA extraction mainly due to the presence of high concentrations of polysaccharides, polyphenols, and other secondary metabolites (Dabo *et al.*, 1993; Zhang and Steward, 2000).

5.3 PCR STANDARDIZATION AND PRIMER SCREENING

Comparative studies in plants have shown that simple sequence repeats (SSR) markers, which are single locus markers with multiple alleles, are more valuable than other markers and provide an effective means for discriminating between genotypes (Powell *et al.*, 1996; Li *et al.*, 2001). An important limitation, however, regarding use of microsatellites for polymorphism studies is the prior need for optimization of PCR conditions for each SSR marker, since primers vary in length and G-C content (Ogliari *et al.*, 2000). In the present study, the duration of each step of the PCR cycle was adjusted to obtain specific and consistent amplicons under less duration; allowing faster analysis of results. The signal strength of the amplicons was also high.

This is probably because being proteinaceous, the activity and efficiency of Taq polymerase gradually decreases with longer cycle durations.

It is essential to select the most informative primer when several microsatellite markers are reported for a crop. Based on the nature of sequence used for microsatellite development, SSR markers can be classified into neutral or non-neutral (functional) markers. Markers derived from genic regions are functional and less subjected to variations since they are under selection pressure. Neutral markers, on the other hand, are derived from genomic regions and are under less selection pressure. Since they are more amenable to mutative variations, neutral markers are generally preferred for diversity studies. Besides, SSRs derived from ESTs or cDNA often fail to produce PCR products if one or both primer binding sites happen to be on the splice sites (Park *et al.*, 2009).

In addition to level of polymorphism, quality of electrophoretic patterns obtained for each primer should be considered. By rating the quality of the SSR-PCR products on a scale of 1 to 5 (good to poor), it is suggested that only those SSR primers with a rating of 1 and 2 be used in genomic analyses (Smulders *et al.*, 1997; Ramsay *et al.*, 2000). Based on these factors, ten primers (Cel A06, Cel B03, Cel C03, Cel C06, Cel F04, Cel H12, uq73-164, uq84-207, uq97-256 and uq201-302) were shortlisted for final SSR analysis.

Most often, recommended reaction conditions for the primers can be obtained from the literature. The reported annealing temperatures for some of the selected primers (uq series) ranged from 65-66°C. However, amplifications may not be obtained for the reported conditions due to involvement of many factors such as different types / brands of thermocyclers, reaction components or even minor differences in thickness of walls of PCR tubes (Dograr and Akkaya, 2001). A generally accepted rule is to use an initial annealing temperature which is 5°C lower than the denaturing temperature. Most of the times this value is not optimal and it is

necessary to empirically determine the annealing temperature (Sisea and Pamfil, 2009).

Interestingly, the annealing temperatures standardized for the selected primers were far lower (52-58°C) than the reported annealing temperatures but still gave the specific product. The reason for this might be that at elevated temperatures the nucleation of the primer hybridization becomes more difficult, thus, the nonspecific target sites with some mismatched base pairs compete with the specific target sites for the primer hybridization. The ratio of primers binding to the correct sites decreases allowing mismatched priming of the polymerization. Once the mismatched priming occurs, extension is also faster at higher temperatures, and thus, there would be promotion of a nonspecific polymerization reaction. However, at lower annealing temperatures, nucleation would take place easily and there would be no or little competition of nonspecific sites with a couple of mismatches for the correct target sites. Thus, a specific amplification would take place. In the optimization of PCR amplification for wheat SSR markers, Dograr and Akkaya (2001) made similar observations.

5.4 SSR ANALYSIS OF TARO ACCESSIONS

Initial resolution of the amplicons using two per cent agarose gave products in the expected product size. This suggested that the standardized PCR conditions worked well for all the selected markers. However, the agarose gel profiles were not useful for scoring of bands as they underrepresented the wide range of polymorphism given by the 25 accessions. Hence, six per cent denaturing polyacrylamide gel followed by silver staining was used for the accurate analysis of molecular genetic diversity, which could detect larger number of alleles comparatively. This method is commonly resorted to when the base pair differences between the alleles is too small (Berindean *et al.*, 2013) as they can resolve fragments differing by a single base pair. In the comparative analysis of silver staining, autoradiography and ABI sequencer

methods of microsatellite analysis, Christensen *et al.* (1999) established that identical overall results were observed for all the methods though there was variability in the interpretation of individual loci.

5.5 ANALYSIS OF MOLECULAR MARKER DATA

5.5.1 Quantification of Genetic variability

SSR polymorphisms are mostly length variations differing in the number of repeat units which is reflected in the average number of alleles per locus. The average number of alleles per locus in the present study ranged from 6.0-12.57, which was comparatively very high compared to the study by Mace and Godwin (2002), which reported an average 3.2 alleles/locus. Nonetheless, the value was comparable to microsatellite analysis in other crops; cassava (Chavarriaga-Aguirre *et al.*, 1998) and poplar (van der Schoot *et al.*, 2000), which revealed on average 7.5 and 13 alleles/locus respectively. Initially, it was thought that the occurrence of unequal crossing-over between repeat units during meiosis accounted for the length variation in minisatellites (Jeffreys *et al.*, 1998) while DNA replication slippage was responsible for the variation in microsatellites (Strand *et al.*, 1993). However, several studies have demonstrated that both types of repeats can be derived by either of the two mechanisms (Richard *et al.*, 2008).

The parameters of diversity estimated for the ten primers in this study gave very high values confirming the informativeness and usefulness of the markers in assessing diversity. The high values also signify the existence of high variability among the taro accessions studied. However, it would be difficult to point out the marker most suitable for assessing the genetic diversity in taro based on these values. In general, primers with polymorphic marker ratio as 1 are the most useful and these include Cel A06, Cel B03, Cel C06, Cel F04 and uq201-302.

Though a robust molecular marker, SSR markers have their own limitations. DNA replication slippage can also occur during *in vitro* amplification of the SSRs to produce 'stutter bands'. In addition, homoplasy can occur if two bands are similar in size but not identical in sequence. The homoplasy can be confirmed by nucleotide sequencing (Angers *et al.*, 2000). Homoplasy can lead to an underestimation of the actual divergence between populations (Estoup *et al.*, 1995; Jarne and Lagoda, 1996; Curtu *et al.*, 2004). However, SSR electromorph homoplasy does not present a significant problem in population genetics analyses since large amounts of variability at SSR loci are often compensated for by their homoplasious evolution (Estoup *et al.*, 2002). PCR detects size variation by the differences in the number of repeat units, which arose by replication slippage or unequal crossing over. If a SSR with 5 repeat units is extended to a SSR with 6 repeat units, the latter can reduce its repeat unit number to 5 by a single 'back-mutation'. If this happens, SSR analysis cannot differentiate between the original SSR locus with 5 repeat units and the back mutated SSR locus with 5 repeat units, even though two steps of mutation were involved. This type of homoplasy cannot be checked, and may also lead to an underestimation of the genetic diversity of a population (Goldstein *et al.*, 1995; Selkoe and Toonen, 2006).

5.5.2 Cluster Analysis

At a truncate level of 0.79, the cluster analysis grouped the 25 accessions into five major clusters with intra and inter-cluster genetic distance higher than morphological cluster analysis. Alike morphological characterization, molecular data analysis also confirmed the lack of congruence between accession grouping and geographical origin. Acc. 741 from Tripura was found to share the same cluster and genetic distance with Acc. 724, from Meghalaya. In the genetic diversity analysis of Indian taro using RAPD by Lakhanpaul *et al.* (2003), clustering pattern did not show any strict relationship between geographical distribution and genotypic diversity as accessions from different geographic regions were placed in nearly all the clusters. Sharma *et al.* (2008b) used isozymes and RAPD to study the genetic structure of taro.

The two dendrograms derived from isozyme and RAPD data showed no correlation between the clusters and the geographical origins of the accessions.

Selection of divergent parents for heterotic breeding should be based on a combined analysis of the morphological and molecular data cluster. Different clusters may have different breeding values thereby enabling breeders to improve different traits. Therefore parental selection should be made based on the relative merits of each cluster for each trait depending on the objective of the breeding program (Mulualem *et al.*, 2013). Dewey and Lu (1959) further showed that while selecting genotypes from a particular cluster, the inter cluster distance and cluster mean performance should be taken into consideration. Hence, inter-mating between genotypes included in these clusters may give high heterotic response and thereby better segregants in view of the genetic diversity. However, low inter cluster distance indicates that genotypes of the two clusters are less divergent. Intensive selection for agronomically important characters and similarity in parentage might be the cause of narrow genetic diversity and uniformity between these clusters (Mulualem *et al.*, 2013).

5.6 CORRELATION OF MORPHOLOGICAL AND MOLECULAR DATA

The results of the Mantel test revealed that the goodness of fit between phenotype and molecular marker analysis was not significant. This is in concordance with the results of previous studies based on morphological and molecular markers. However, accessions wise comparison of the two clusters revealed that certain accessions tend to remain grouped together in both molecular and morphological clustering. K-15, I-14, J-13, H-9 and J-8 formed one group and A-6, A-12, B-4, C-14 and I-13 formed another suggesting that inter-mating of accessions within each group would not be of much use to exploit heterosis owing to the high degree of similarity. The first group could be mostly characterized as accessions with white flesh colour, purple sheath colour and purplish green petiole colour while the second group mostly

comprised of accessions with non-acrid tubers. Nevertheless, hybridizations between accessions of these two groups and those involving accessions from these two groups with other accessions might give a heterotic response. Similarly, in terms of germplasm conservation and maintenance, it would be worthwhile to investigate further the presence of duplicates within each group using other markers.

Quero-Garcia *et al.* (2004) reported similar results in taro using AFLP and agro-morphological markers. In a study based on Indian taro, Lakhanpaul *et al.* (2003) established that the classification of taro germplasm into morphotypes does not seem to be supported by the pattern of RAPD variation. The lack of perfect congruence between morphological and molecular data suggests that the morphological system may be useful for the morphotypes management but is not appropriate to study the genetic structure of the *C. esculenta* (Singh *et al.*, 2011). The discordance between the morphological and molecular structures may result from similar selection pressure at different places leading to similar forms with different genetic background (Pissard *et al.*, 2007).

However, isozyme data was found to support the morphological data in a study on taro by Trimanto *et al.* (2010). This is because isozymes represent variations at the gene expression level and may come under the influence of environmental conditions. Besides, morphological diversity is influenced by the environment; since every environment has different conditions, plants adapt to their home range.

From these observations, it can be concluded that foliar traits are more informative for morphological characterization of taro. The study on standardization of DNA isolation reveals that stronger extraction conditions in terms of high concentration of CTAB, PVP and β-mercaptoethanol are required. The ten selected primers were informative for SSR analysis and revealed high values of polymorphic marker ratio and average number of alleles per locus. Though morphological and molecular characterization could explain a high level of genetic diversity of taro in

this center of origin, the goodness of fit in Mantel's test could not identify any correlation between the two distance measures. However, two groups have been identified to exhibit identical cluster patterns in morphological and molecular characterization.

SUMMARY

6. SUMMARY

The study entitled "Genetic diversity analysis in taro [*Colocasia esculenta* (L.) Schott] of North East India" was carried out at the Division of Crop Improvement, Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram during 2013-2014. The objective of the study was to assess the genetic diversity among taro accessions from North East India, which is considered to be one of the centers of origin of taro, using morphological descriptors and SSR markers. Twenty five accessions of taro from the North East Indian states maintained at CTCRI were selected for morphological characterization.

Scoring of above and below ground morphological traits was done using 38 NBPGR and IPGRI descriptors at the maximum growth stage. For the ten quantitative traits studied, an average value from three plants per accession was considered. The Shannon Weaver's and Simpson's diversity index computed according to percentage distribution of the various traits for the 25 accessions, gave high mean values (H'=0.87; D=1.00). The first four PCs accounted for 76.59 per cent of the total variation. Leaf margin colour, petiole colour (top 1/3rd, middle and base), leaf colour lower, sheath colour and sinus colour, with high PC loadings, contributed maximum to the total variability. These traits were also found to be deviated from the center in the biplot representation. Significant variation ($P<0.01$) was given by tillering, leaf L:B ratio, petiole: sheath ratio, number of cormels, weight of corms and cormels and corm length. The quantitative data subjected to Duncan's multiple range test ($\alpha= 0.05$), indicated significant variations among certain accessions. Hierarchial cluster analysis using R package based on Ward's method gave five broad clusters at a truncate level of 25.

Young leaf samples freshly collected from the field was used for the molecular studies. The presence of high amounts of mucilaginous polysaccharides hindered the isolation of good quality DNA from taro using several protocols tried. However, a

method based on higher concentration of CTAB, PVP and β-mercaptoethanol (Sharma *et al.*, 2008a), gave better quality DNA in terms of both quantity and quality.

Standardization of SSR-PCR conditions was attempted to obtain consistently good amplification under reduced cycle durations. Eighteen SSR primers reported in the literature were used for initial screening. Six Cel (Cel A06, Cel B03, Cel C03, Cel C06, Cel F04 and Cel H12) and four uq (uq73-164, uq84-207, uq97-256 and uq201-302) series primers were selected for the final analysis based on the quality of polymorphic and electrophoretic patterns. It was found necessary to standardize the annealing temperature of the selected Cel series primers using gradient PCR.

Presence of amplicons of the expected size was confirmed initially in agarose gel (2%). Denaturing PAGE (6 %) followed by silver staining was carried out to resolve and detect even single nucleotide variations in the number of sequence repeats. Binary scores (1 for presence and 0 for absence of band) tabulated in the excel format was subjected to statistical analysis using R package and SAS program.

All the selected primers gave high polymorphism across the 25 accessions studied. This was explained by the high values of Shannon's index (1.59-2.37), average number of alleles (6.0-12.57) and polymorphic marker ratio (0.76-1.0). Jaccard's method of hierarchial cluster analysis performed using R package divided the accessions into five broad clusters at a truncate distance of 0.79. However, accessions were not grouped based on their geographical similarities in both molecular and morphological clustering.

Correlation analysis of morphological and molecular data using Mantel's test established the absence of any relation ($r = 0.1432$; $p = 0.0648$). However, by accession wise comparison of the different clusters obtained in both the cases, certain accessions tended to group together in both the clusters: K-15, I-14, J-13, H-9 and J-8 formed one group and A-6, A-12, B-4, C-14 and I-13 formed another. The genetic distance among the accessions within each group is expected to be very low owing to

the high similarity at both molecular and morphological levels. Hence, parental accessions should be avoided from the same group for breeding. However, accessions taken from different groups for hybridization are likely to give a positive heterotic response. The present study also disapproves that geographical divergence contributes to genetic divergence i.e. accessions from geographically dissimilar regions are likely to be genetically related and vice versa. It is also important to analyze the clusters for breeding values specific to the objective of the hybridization program before selection of parents. The morphological characterization data can support the same.

Though the estimates of genetic diversity and cluster analysis reveal high genetic divergence among accessions at the center of origin, more sensitive molecular tools would be helpful to rule out duplicate accessions within individual clusters. This is especially important in the case of the two groups exhibiting identical cluster patterns for both morphological and molecular characterizations.

Comparative analysis of diversity levels with other centers of origin or geographical regions is restricted by the limited sample size. Investigating a large number of accessions using more number of polymorphic markers provide much reliable data which can also contribute towards the development of a regional core collection.

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* Originals not seen

APPENDICES

APPENDIX I

CTAB Extraction Buffer (CTAB method I)

Tris- HCl (pH 8.0)	100 m*M*	
EDTA	25 m*M*	
NaCl	1.5 *M*	
CTAB	2.5 %	
β-mercaptoethanol	0.2 % (v/v)	freshly added prior to DNA
PVP	1 % (w/v)	extraction

APPENDIX II

CTAB Extraction Buffer (CTAB method II)

Tris- HCl (pH 8.0)	100 m*M*	
EDTA	20 m*M*	
NaCl	2 *M*	
CTAB	2 %	
β-mercaptoethanol	2 % (v/v)	freshly added prior to DNA
PVP	2 % (w/v)	extraction

APPENDIX III

TE buffer (10X)

Tris- HCl (pH 8.0)	10 m*M*
EDTA	1 m*M*

APPENDIX IV

TBE Buffer (10X)

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

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

APPENDIX V

Polyacrylamide gel (6%) containing 7 M urea

42 g urea was dissolved in a beaker containing 10 ml TBE buffer (10X) and 15 ml distilled water by heating in a microwave oven for 30-40 s. 15 ml acrylamide solution (19:1) was filtered and added to a measuring cylinder followed by the urea solution. The final volume was made up to 100 ml using distilled water and stored in dark till use. 60 µl TEMED and 600 µl APS (100 mg/ml) was added and mixed just before casting the gel.

APPENDIX VI

Fixer

Acetic acid	200 ml
Distilled water	1800 ml

APPENDIX VII

Silver stain

2 g silver nitrate dissolved in distilled water to a final volume of 2000 ml and 3 ml formaldehyde added.

APPENDIX VIII

Developer

60 g sodium carbonate dissolved in distilled water to a final volume of 2000 ml and stored at -20°C. 3 ml formaldehyde and 4 ml sodium thiosulphate (10 mg/ml) was added and mixed thoroughly before use.

ABSTRACT

GENETIC DIVERSITY ANALYSIS IN TARO [*Colocasia esculenta* (L.) Schott] OF NORTH EAST INDIA

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COLLEGE OF AGRICULTURE

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ABSTRACT

The study entitled "Genetic diversity analysis in taro [*Colocasia esculenta* (L.) Schott] of North East India" was carried out at the Division of Crop Improvement, Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram during 2013-2014. The objective of the study was to assess the genetic diversity among taro accessions from North East India, which is considered to be one of the centers of origin of taro, using morphological descriptors and SSR markers. The knowledge can be exploited in the heterotic breeding of taro to develop improved varieties suiting various needs. National and international germplasm repositories conserving root and tuber crops can also use the data to maintain taro germplasm efficiently.

Twenty five accessions of taro collected from the various North Eastern states of India and maintained at CTCRI were selected for the study. Morphological characterization was performed at the maximum vegetative stage and tuber characters at harvest using ten quantitative and 28 qualitative traits. A combination of NBPGR and IPGRI descriptors were used to explain the wide range of morphological variability. The data was statistically interpreted in terms of diversity indices, PCA, ANOVA and cluster analysis using R statistical package and SAS program.

The diversity indices (H'=0.87; D=1.00) revealed a high level of morphological diversity among the taro accessions. The first four components explained 76.59 per cent of the total variation with leaf margin colour, petiole colour (top 1/3rd, middle and base), leaf colour lower, sheath colour and sinus colour contributing maximum to the variability. ANOVA showed significant ($P<0.01$) variation for 7 out of the 10 quantitative traits studied. Duncan's multiple range test gave a grouping based on the mean values of quantitative traits. Five major groups were revealed after hierarchical cluster analysis based on Euclidean distance, which did not bear any relation to the geographical origins of the accessions.

A protocol was developed for the isolation of good quality DNA overcoming the high levels of secondary metabolites in taro. PCR conditions for SSR detection in taro were also optimized successfully.

Ten out of 18 SSR primers were selected for the study after screening. Denaturing PAGE followed by silver staining was performed to analyze the variability among accessions at the molecular level. The average number of alleles and Shannon's diversity index ranged from 6.0-12.57 and 1.59-2.37, respectively. The polymorphic marker ratio was found to be high for all the primers (0.76-1.0); however, Cel A06, Cel B03, Cel C06, Cel F04 and uq201-302 gave the maximum ratio of one. Cluster analysis based on Jaccard's distance revealed five broad clusters which could not be correlated to the geographical similarities among the accessions.

The parameters estimated from molecular and morphological characterization data established a high level of genetic diversity prevalent in the center of origin. The study revealed the absence of congruence between the clustering pattern and geographical origin suggesting that geographically diverse regions share ecologically similar characteristics and vice versa. Differences in morphological and molecular clustering patterns indicate the wide range of adaptations of the crop to the diverse environments inhabited. Though the Mantel's test established no correlation ($r = 0.1432$; $p = 0.0648$) between the molecular and morphological distance measures, the study could identify two groups of accessions that clustered together in both the methods.