CHARACTERIZATION OF SELECTED *CURCUMA* SPECIES GERMPLASM USING MORPHOLOGICAL AND MOLECULAR MARKERS

By

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THESIS

Submitted in partial fulfilment of the

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I, hereby declare that the thesis entitled **"Characterization of selected *Curcuma* species germplasm using morphological and molecular markers"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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Dedicated to my beloved parents

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LIST OF ABBREVATIONS

%	Percentage
A_{260}	Absorbance at 260 nm wavelength
A_{280}	Absorbance at 280 nm wavelength
AFLP	Amplified fragment length polymorphism
bp	Base pair
cm	Centimetre
CTAB	Cetyl Trimethyl Ammonium Bromide
CuMiSat	*Curcuma* Microsatellite marker
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
et al.	et alia
EtBr	Ethidium bromide
F	Forward primer
g	Gram
ICAR-CTCRI	Indian Council of Agricultural Research-Central Tuber Crops Research Institute
ISSR	Inter simple sequence repeat
KAU	Kerala Agricultural University
kb	Kilo bases
kg	Kilogram
L	Litre
m	Meter
M	Molar
mg	milligram
$MgCl_2$	Magnesium Chloride
min	Minute
ml	Millilitre
mm	Millimeter

mM	Millimolar
NaCl	Sodium chloride
ng	Nanogram
nm	Nanometer
˚C	Degree Celsius
OD	Optical density
PAGE	Poly Acrylamide Gel Electrophoresis
PCA	Principal Component Analysis
PCC	Pearson Correlation Coefficient
PCR	Polymerase chain reaction
PGR	Plant Genetic resources
R	Reverse primer
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
RT	Reverse transcriptase
RT-PCR	Reverse transcription-polymerase chain reaction
s	Second
sp.	Species
spp.	Species (plural)
SDW	Sterile distilled water
SSR	Simple sequence repeat
Taq	*Thermus aquaticus*
TBE	Tris-borate EDTA buffer
TE	Tris-EDTA buffer
TM	Trademark

Tm	Melting temperature
Tris HCl	Tris (Hydroxy methyl) aminomethane hydrochloride
U	Enzyme units
UBC	University of British Columbia
UV	Ultra violet
V	Volt
v/v	volume/volume
W	Watt
w/v	weight/volume
µg	Microgram
µl	Microlitre
µM	Micromolar

1. INTRODUCTION

Root and tuber crops are the plants that yield starch enriched roots, tubers, rhizomes, corms and stems. These are key cultivated staple energy sources after cereals for the most part in tropical locales in the world and are rich in dietary fibre, carotenoids and anthocyanin. Apart from supplementing the diet of the people, tuber crops also hold medicinal values to cure numerous sicknesses. Many tropical tuber crops are utilized in the manufacturing of stimulants, expectorants, tonics and carminatives. The comparative importance of these crops is apparent through their annual universal production, which is around 836 million tonnes (Chandrasekara and Kumar, 2016). The energy obtained from tubers is about one-third of that of an equivalent weight of rice or wheat due to the high moisture content of tubers. On a dry weight basis, the amount of protein in roots and tubers is low ranging from 1-2%.

The major tropical root crops are cassava, sweet potato, yams and aroids. In addition to these major crops, there are several other tuberous and rhizomatous minor root crops namely Chinese potato, arrowroot, yam bean, Canna, *Curcuma* etc. which are explored in different parts of the country.

The genus *Curcuma* L. comprises of about 120 species of rhizomatous herbs of which 40 species are reported from India (Skornickova *et al.*, 2007) and the greatest diversity of the genus occurs in India. The rhizome is the vegetative propagating plant part and has been used traditionally due to its great medicinal value from the time immemorial (Srimal, 1997). *Curcuma longa* is one of the most known and important species within the genus which yields turmeric, the colouring and aromatic ingredients of curry powders traditionally used in Asia (Apavatjrut *et al.*, 1999) and being an important medicinal plant, it is also used in the pharmaceutical industries. *Curcuma* starches are believed to have medicinal value and are used in the treatment of gastric ailments. It has been reported to have similar digestive properties as arrowroot starch and hence used as an infant food. Vimala and Nambisan (2005) reported that the starch content in the tubers varies from 10-20% with *C. malabarica* having the highest starch content (21.4%), indicating that these species have immense potential as crops for starch extraction. *C. angustifolia*

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is widely cultivated in the tribal areas of Chhattisgarh and Northeast India as source of arrowroot starch. Also *C. malabarica* and *C. zedoaria* are cultivated in large scale in Kozhikode and Malappuram districts of Kerala for the extraction of starch. The ICAR-Central Tuber Crops Research Institute germplasm holds 45 accessions of *Curcuma* in 8 different species collected from different agro-climatic regions of India and the institute is making an intensive effort to collect, maintain, characterize and conserve the different *Curcuma* species in the field and *in vitro* gene bank.

Characterization of germplasm is very essential in crop plants and it is the basis for selection of accessions for use in crop improvement programmes. A few studies on morphological, biochemical and molecular characterization of *Curcuma* species have been attempted. Morphological characterization of the genus has its limitation as some of the key taxonomic traits are confusing (Santapau, 1945). Currently molecular characterization is being used as an addition to the traditional methods of germplasm characterization for validation in many crop plants (Semagn *et al.*, 2006).

Molecular markers are proved to be valuable tools in the characterization and evaluation of genetic variability within and between species and populations (Syamkumar, 2008). PCR based markers have been used extensively for assessing genetic variation within the species to measure the genetic diversity (Virk *et al.*, 1995). DNA based molecular markers have acted as versatile tool and found their own position in various fields like taxonomy, physiology, embryology and genetic engineering. They offer several advantages over traditional phenotypic markers, as they provide data that can be analyzed objectively (Joshi *et al.*, 1999).

The present study was an attempt to characterize the selected accessions of *Curcuma* species collected from different parts of India and maintained in the field gene bank of ICAR-CTCRI using morphological and molecular markers with the following objectives:

1. Morphological characterization of selected accessions of *Curcuma* species based on aerial morphological features, floral characters and underground rhizome characters.
2. Molecular characterization of selected accessions of *Curcuma* species using ISSR and SSR markers.
3. To understand the intra and inter-specific variation existing within the selected accessions of *Curcuma* species.

2. REVIEW OF LITERATURE

2.1 *CURCUMA* L.

The genus *Curcuma* belongs to the family Zingiberaceae within the sub family Zingiberoideae and tribe Zingiberae (Kress *et al.*, 2002) include about 120 species distributed in tropical and subtropical Asia (Skornickova *et al.*, 2007). The generic name *Curcuma* is the Latinized version derived from the Arabic word *Karkum* meaning yellow (Purseglove *et al.*, 1981). The genus *Curcuma* was first established by Carl Linnaeus (1753) in his book 'Species Plantarum'.

2.1.1 Origin, distribution and diversity

According to Purseglove (1968), the genus *Curcuma* is considered to have originated in the Indo-Malayan region and is broadly distributed in the tropics of Asia to Africa and Australia. It has a wide spread occurrence from sandy coastal habitat to an altitude as high as 2000 m in the Western Ghats and Himalayas. *Curcumas* are found to occur in diverse tropical conditions, margins of forests, secondary forests, plantations, open grasslands, plains in coconut and arecanut groves. The greatest diversity of the genus occurs in India (Ravindran *et al.*, 2007). The other countries rich in *Curcuma* species diversity are Indonesia, Malaysia, China, Thailand, Philippines, Vietnam, Singapore, Brunei, and Papua New Guinea (Sabu, 1991).

The taxonomic hierarchy of the genus *Curcuma* (Kress *et al.*, 2002) is given below:

Kingdom	:	Plantae
Sub kingdom	:	Tracheobionta
Division	:	Magnoliophyta
Class	:	Liliopsida
Subclass	:	Zingiberidae
Order	:	Zingiberales
Family	:	Zingiberaceae

Subfamily : Zingiberoideae

Tribe : Zingibereae

Genus : *Curcuma*

Zingiberaceae family with about 53 genera and more than 1200 species is the largest family in the order Zingiberales (Augsonkitt *et al.*, 2004). Belonging to the monocotyledon family *Curcuma* is known to have about 120 species (Tehrani, 2005) and among these about 40 species are reported in India (Velayudhan *et al.*, 1999) are shown in Table 1.

Table 1 *Curcuma* species occurring in India

Sl. No.	Species	Distribution
1	*C. aeruginosa* Roxb.	West Bengal
2	*C. albiflora* Thw.	Kerala
3	*C. amada* Roxb.	All over India
4	*C. amarissima* Rosc.	West Bengal
5	*C. angustifolia* Roxb.	UP, MP, HP, North East
6	*C. aromatica* Salisb.	Kerala, TN, Karnataka, AP, Orissa and Bihar
7	*C. caesia* Roxb.	Kerala, West Bengal, Karnataka
8	*C. caulina* Grah.	Maharashtra
9	*C. comosa* Roxb.	West Bengal
10	*C. petiolata* Roxb.	West Bengal
11	*C. decipiens* Dalz.	Kerala and Karnataka
12	*C. rubrobracteata* Skornick. *et al.*	West Bengal
13	*C. ferruginea* Roxb.	West Bengal
14	*C. longa* Linn.	All over India
15	*C. montana* Roxb.	South India
16	*C. neilgherrensis* Wight	South India
17	*C. oligantha* Trim.	Kerala
18	*C. pseudomontana* Grah.	South India
19	*C. reclinata* Roxb.	Madhya Pradesh
20	*C. xanthorrhiza* Roxb.	West Bengal
21	*C. zedoaria* Rosc.	All over India
22	*C. sylvatica* Val.	Kerala
23	*C. aurantiaca van* Zijp.	South India
24	*C. sulcata* Haines	Maharashtra

25	*C. inodora* Blatt.	Gujarat, Maharashtra and Karnataka
26	*C. ecalcarata* Sivar. and Indu	Kerala
27	*C. soloensis* Val.	West Bengal
28	*C. brog* Val.	West Bengal
29	*C. haritha* Mangaly and Sabu	Kerala
30	*C. raktakanta* Mangaly and Sabu	Kerala
31	*C. kudagensis* Velay. *et al.*	Karnataka
32	*C. thalakaveriensis* Velay. *et al.*	Karnataka
33	*C. malabarica* Velay.	Kerala and Karnataka
34	*C. karnatakensis* Velay. *et al.*	Karnataka
35	*C. cannanorensis* Ansari *et al.*	Kerala
36	*C. vamana* Mangaly and Sabu	Kerala
37	*C. lutea* Ansari *et al.*	Kerala and Karnataka
38	*C. coriacea* Mangaly and Sabu	Kerala
39	*C. mutabilis* Skornick. *et al.*	Kerala
40	*C. leucorhiza* Roxb.	West Bengal

2.1.2 *Curcuma* propagation

Curcuma is propagated vegetatively using the underground rhizome. Tissue culture protocols for *in vitro* propagation is also available in some of the *Curcuma* species (Sanghamitra and Nayak, 2000). However, tissue culture propagation of *Curcuma* is not a cost effective method to rhizome propagation as a plantation practice.

2.1.3 Morphology of genus *Curcuma*

2.1.3.1 Underground parts

At the bottom of the aerial shoot, the stem consists of vertical ovoid or globose structure called rhizome, bearing horizontal branches and roots. However branched rhizomes also occur in some species. The roots often produce ellipsoid tubers. Core of rhizomes varies in different colours, i.e., white, cream, yellow, orange, blue, deep blue and bluish-green. Some species have a unique colour of rhizomes which are useful for identification, such as the bluish-black rhizome in *C. caesia* (Syamkumar and Sasikumar, 2007).

2.1.3.2 Leafy shoots

Plants vary from 1-2 m in height. Pseudostem is formed by the leaf sheaths and surrounded by the leafless sheaths at the base. It can be green or with a red or pink tinge low to the ground or rather high up depending on the habit and size of a particular species. Usually leaf blades are large, lanceolate or elliptic, rarely linear. Leaves of some species have purple streak along either side of the midrib while some species do not have it (Sirirugsa *et al.*, 2007).

2.1.3.3 Inflorescence

The inflorescence is either terminal on the leaf-shoot with the peduncle enclosed by the leaf sheaths or lateral on the separate shoot with the peduncle enclosed by the bladeless sheaths. The shape of inflorescence can be cylindrical, conical or oval. Its inflorescence, a compound spike with prominent bracts each subtending a cincinnus of two to ten flowers, which are joined to each other forming pouches at the base, make the recognition of genus *Curcuma* easy (Apavatjrut *et al.*, 1999).

2.1.3.4 Bracts

Generally bracts are large and joined to each other by establishing sacs at the base, the free ends of the bracts are normally wide spread, each subtending a cincinnus of 2-10 flowers. The uppermost bracts called "coma" are larger and longer than the rest and differently coloured in many species. Usually they are sterile (Sirirugsa *et al.*, 2007).

2.1.3.5 Flowers

Flowers are surrounded by bracteoles, consists of the following floral parts: Calyx is tubular, toothed unequally, intensely divided along one side. Corolla-tube is more or less funnel shaped; corolla-lobes are unequal, the dorsal lobes somewhat larger than the lateral lobes and its apex is covered. Staminodes are petaloid, elliptic, oblong or linear. Labellum has a thickened central part and thinner sidewise lobes which overlap the staminodes. Stamen has a short and broad filament and a narrow

apex. Anther is with or without spurs and the anther-peak is usually small. Spurs vary in diverse shapes and sizes. Ovary is pubescent or glabrous and trilocular, ellipsoid seeds with a lacerate aril (Sabu, 1991).

2.1.4 Economic importance

Curcuma is one among the few genus having manifold uses in areas such as religion, folk/traditional/modern medicines, aromatherapy, cosmetics, dye, floriculture and food industry. Several economically important species are reported from the genus *Curcuma. Curcuma longa* aka *C. domestica* is the most exploited species. Table 2 gives the list of some economically important *Curcuma* species and their traditional uses.

Table 2 List of some economically important *Curcuma* species and their traditional uses

Species	Part	Use/Property
C. aeruginosa Roxb.	Rhizome	Used medicinally for asthma, cough and as a paste with coconut oil for dandruff, as purgative during childbirth (Liu *et al.*, 2013)
C. amada Roxb.	Rhizome	Anti-inflammatory, carminative, antipyretic, antifungal, cholesterol lowering agent (Pachauri and Mukherjee, 1970)
C. angustifolia Roxb.	Rhizome	Used for starch extraction (east Indian arrowroot), Wound healing, Anti-inflammatory (Rao and Jamir, 1982)
C. aromatica Salisb.	Entire plant	To treat asthma, tuberculosis and blood impurity (Maikhuri and Gangwar, 1993)
	Green leaf	Used for gastric troubles (Cuellar *et al.*, 1998)
	Rhizome	Anti-inflamatory, against leucoderma and blood diseases (Jangde *et al.*, 1998)
C. caesia Roxb.	Rhizome	Paste made from the rhizome is used to cure blood dysentery and as poultice in rheumatic pain, anti-asthmatic (Satyendra *et al.*, 2013)

C. comosa Roxb.	Rhizome	Choleretic and cholagogue, anti-inflamatory (Piyachaturawat *et al.*, 1996)
C. decipiens Dalz.	Rhizome	Anti-inflammatory, anti-bacterial, anti-diabetic (Jadhao and Bhuktar, 2015)
C. longa Linn.	Dried entire plant leaf	To treat renal calculi (Mukerjee *et al.*, 1984)
	Rhizome	Anthelmintic, counter-irritant on insect stings, cancer remedy, to facilitate the scabbing process in chickenpox and smallpox, to treat urinary diseases, liver diseases and jaundice, to treat constipation, a reddish skin disease and minor skin infections (Bellakhdar *et al.*, 1991)
	Dried rhizome	To protect against snakebite, to treat liver complaints, to treat slow lactation, diabetes and to regulate fat metabolism
	Dried root	Anti-pyretic
C. malabarica Velay.	Rhizome	Anti-bacterial (Wilson, 2005)
C. zedoaria Rosc.	Rhizome	Improves blood circulation, used against abdominal and rheumatic pain, ulcer (Wilson, 2005)

2.2 CHARACTERIZATION IN *CURCUMA*

Genetic markers are sites of variation that can be used to identify individuals or species. Such markers are differentiable from species to species, so the characterization of species can be effectively done. Genetic markers are of three types. They are morphological markers, biochemical markers and DNA markers.

2.2.1 Morphological characterization

Johann Goethe coined the term "morphology" and originated plant morphology as a scientific discipline (Kaplan, 2001). Goethe theorized that knowing the fundamental organizational theme enabled one to predict plant forms

that had not been discovered (Mueller and Engard, 1952). Morphological characterization is done in each accession by using a set of standard descriptors. According to Kaplan (2001) for the comprehension of life cycles, geographical distribution, evolution, conservation status as well as species delimitation and morphological data are very important. However, the phenotypic variation of plants does not always follow the genetic pattern of variation and diversity as of plant populations.

Identification of plant species has long been based on morphological parameters. Plant morphology shows high polymorphism and phenotypic characters may allow plant species classification (Duminil and Di Michele, 2009). Nevertheless, different individuals of the same species may exhibit a variation in their morphology either naturally or in connection with local adaptations. Despite the fact that some species of the same genus represent separate taxonomic entities, they may be grouped into the same species as they share similar morphology (Shaw 2000). Another limitation of phytomorphological study is their accessibility. These drawbacks of the morphological markers led to the development of DNA markers.

Morphological characterization of 19 *Curcuma* species from India was studied by Yusuf *et al.* (2016) using morphological descriptors. Whole plants and their underground parts such as rhizome, roots and root tubers were collected from different parts of South India. 8 morphological characters were taken into consideration by authors in assessing the morphological similarities/dissimilarities in nineteen *Curcuma* species. The nineteen species studied were clustered into three groups in the dendrogram and *C. bhati* was distinct from all other species. *C. montana* and *C. psuedomontana* shared the same node showing that both are synonyms and the need for reassessing their separate species status.

Syamkumar (2008) studied morphological characteristics of 15 *Curcuma* species for 10 aerial morphological characters, 6 floral and 11 rhizome characters. The fifteen species studied were clustered into seven groups in the dendrogram and wide variability was observed among the fifteen *Curcuma* species for the conventional taxonomic and morphological features. Rather than using more

quantitative characters, the reliability of using the qualitative aerial characters (leaf mid rib colour, colour of leaf sheath and leaf texture), floral characters (colour of the fertile bract, colour of the corolla, colour of the labellum and anther spur) and rhizome characters (rhizome colour, aroma of rhizome and rhizome flavour/taste) for grouping of *Curcuma* species were also proved by this study.

2.2.2 DNA markers

A molecular marker is a DNA sequence that is easily detected and whose inheritance can be easily be tracked. The uses of molecular markers rely on DNA polymorphism that forms the foundation for coming up with approaches to utilize it for crop improvement programmes. A marker must be polymorphic i.e. it must exist in distinct forms so that chromosome carrying the mutant genes can be differentiated from the chromosomes with the normal gene by a marker it also carries. DNA markers seem to be the supreme candidates for systematic assessment and selection of plant material. Unlike protein markers, they are independent of the environment. DNA is easily extracted from plant and its analysis can be effective in case of both cost and labour (Kumar, 2009).

2.2.2.1 Restriction Fragment Length Polymorphism (RFLP)

RFLP is a technique in which species are distinguished by analysis of patterns derived from cleavage of their DNA. The similarity of the patterns generated from restriction digestion can be accustomed to differentiate species from one another. This technique is mainly based on the restriction endonucleases (Kumar, 2009).

RFLPs have the advantages of having high reproducibility and showing codominant alleles. They are very reliable markers and can easily determine if a linked trait is present in a homozygous or heterozygous state in species, information highly desirable for recessive traits (Winter and Kahl, 1995).

In a study conducted by Hayakawa *et al.* (2010), PCR-RFLP analysis was accurate and effective in identification of the strain with a high content of curcumin from various strains of *C. longa* Linn.

2.2.2.2 Random Amplified Polymorphic DNA (RAPD)

RAPD is a PCR-based technology which is based on enzymatic amplification of target or random DNA segments with arbitrary primers. In this process, a single primer anneals to the DNA at two different loci on complementary strands of DNA template. If these priming sites are within an amplifiable range of each other, a discrete DNA product is formed through PCR amplification. On an average, each primer directs amplification of several discrete sites in the genome, making the method useful for efficient screening of nucleotide sequence polymorphism between species/individuals (Williams *et al.*, 1993). The major advantage of RAPDs is that they are faster and easy to assay. Only low quantities of template DNA per reaction are required (usually 10-50 ng) as PCR is involved. Since random primers are commercially available no sequence information for primer construction are needed (Kumar, 2009).

RAPD based study of genetic diversity in *C. zedoaria* from Bangladesh was done by Islam *et al.* (2007). The study showed that hilly area populations bear higher genetic diversity which was also found to be distinct from plateau land and plain land populations.

2.2.2.3 AFLP (Amplified Fragment Length Polymorphism)

AFLP is an intermediary between RFLPs and PCR. It is based on selectively amplifying a subgroup of restriction fragments from an intricate mixture of DNA fragments acquired after digestion of genomic DNA with restriction endonucleases. Polymorphisms are identified from dissimilarities in the length of the amplified fragments by polyacrylamide gel electrophoresis (PAGE) (Matthes *et al.,* 1998) or by capillary electrophoresis.

The power of AFLPs lie in their high genomic abundance, significant reproducibility, the formation of several informative bands per reaction, their broad range of applications and the fact that no sequence data for construction of primer are required (Kumar, 2009).

In a study conducted by Das *et al.* (2011) to study the genetic relationships among 9 species of *Curcuma* by using 4 AFLP primers revealed significant correlation between them.

2.2.2.4 Inter Simple Sequence Repeat (ISSR)

ISSRs are DNA fragments of size 100-3000 bp located between neighboring, oppositely aligned microsatellite regions. According to this technique, reported by Zietkiewicz *et al.* (1994) primers based on microsatellites are deployed to amplify inter-SSR DNA sequences. ISSR primers are built to anneal to microsatellites with one extra nucleotide (anchors) in the 3' end which allows amplification only if the primer binds to a 5' end of the microsatellite with an appropriate nucleotide in the flanking sequence. The advantages of ISSR include unnecessity of sequence data for primer construction, low quantities of template DNA requirement etc. This is mostly a dominant marker though sometimes it exhibits codominance.

2.2.2.4.1 ISSR characterization in Curcuma

Molecular marker based genetic diversity of *Curcuma* species from India analyzed by Syamkumar (2008). Molecular genetic fingerprints of 15 *Curcuma* species were developed using Inter Simple Sequence Repeats (ISSR). Out of the 91 bands produced by 8 ISSR markers, 87 were polymorphic.

Das *et al.* (2011) studied the genetic relationship of *Curcuma* species from North-east India using PCR-based markers. The aim of study was to elucidate the intra and inter-specific genetic diversity important for utilization, management and conservation of the species. Twenty ISSR primers were used to characterize nine species of *Curcuma*. High percentage of polymorphism with all the 19 primers (98.55%) was displayed among the nine species with 68 polymorphic bands.

Saha *et al.* (2016) used twenty ISSR primers to ascertain the genetic relationship among four different *Curcuma* species of Tripura. Out of the total 119 amplified products, 103 were polymorphic and 13 bands were monomorphic and these were amplified in the range of 200-5000 bp. The percentage of ISSR polymorphic bands were found to range from 66.67-100.

In order to assess the genetic diversity of indigenous *C. longa* germplasm from India, Rana *et al.* (2015) used 13 ISSR primers. The efficiency and reliability of using ISSR markers and relationships among the indigenous turmeric germplasm were demonstrated by this study. A total of 221 fragments, varying from 10 to 21 were generated of which 175 (79.2 %) were polymorphic.

Wang *et al.* (2015) investigated the genetic diversity and variation of five populations of *C. wenyujin*, using ISSR markers. These markers found to be significant in detecting polymorphism.

ISSR characterization of 18 popular cultivated turmeric genotypes analysis showed the polymorphic index value of 87.27% across all the genotypes. In this study, ISSR markers were found to be ideal for producing genetic maps of individual species (Prasanth *et al.*, 2015).

2.2.2.5 Simple Sequence Repeat (SSR)

Microsatellites or SSRs, which are tandem repeats of 1-6 nucleotide long DNA motifs that are arranged all over the genomes of most eukaryotic species (Powell *et al.*, 1996). If nucleotide sequences in the flanking area of the microsatellite are familiar, specific primers (generally 20-25 bp) can be developed to amplify the microsatellite by PCR. The advantages of microsatellites comprise the codominance of alleles, their high genomic abundance and random distribution throughout the genome with preferential association in low-copy regions (Morgante *et al.*, 2002). Because the marker is PCR-based, only small quantities of template DNA (10-50 ng per reaction) are required. The use of long PCR primers make the reproducibility of microsatellites high and analysis do not require high quality DNA.

2.2.2.5.1 SSR characterization in Curcuma

Molecular identification of 8 elite cultivars and 88 accessions in turmeric were studied by Sahoo *et al.* (2017) using EST-SSR marker based method. Fifty EST-SSR primers were screened against 8 cultivars of turmeric out of which 11 primers showed polymorphic banding pattern.

Taheri *et al.* (2014) used SSR molecular markers to elucidate genetic variation and relationships between five varieties of *C. alismatifolia* cultivated in Malaysia. Of the 17 primers tested 8 SSR were selected for their high rates of polymorphism and reproducibility.

Genetic fingerprints of ten species of Zingiberaceae from Eastern India were developed by Mohanty *et al.* (2011) using PCR-based markers. Eight SSR primers were used to elucidate genetic diversity important for conservation, management and utilization. The primer combinations had amplified 325 loci among which 312 were found to be polymorphic in nature and the rest were monomorphic. Out of the 312 polymorphic bands 73 were found to be unique.

Twenty-one polymorphic microsatellite loci were isolated and characterized from *C. longa* by Senan *et al.*, 2013. These markers were screened across thirty accessions and found to be useful for the genetic variability analysis of *Curcuma* species accessions.

Parthasarathy *et al.* (2010) used expressed sequence tags from turmeric for the screening of type and frequency of Class I (hypervariable) simple sequence repeats. 231 microsatellite repeats were detected that could be used for genetic diversity analysis and resolving the taxonomic confusion prevailing in the genus.

Eleven EST-SSR markers used for the molecular identification of 8 elite cultivars and 88 accessions in turmeric (Sahoo *et al.*, 2017). The SSR markers used were found to be unique and useful in precise cultivar differentiation.

Singh *et al.* (2015) using SSR primers investigated the genetic diversity of 10 turmeric genotypes. Among 10 SSR markers, 7 were with polymorphic loci and a total of 65 alleles were detected among the 10 genotypes of turmeric. The findings can be used in breeding programmes.

Sigrist *et al.* (2010) developed and characterized microsatellite markers for *C. longa*. 17 polymorphic microsatellite loci were developed using a CT/GT/CTT enriched genomic library. All microsatellites resulted in amplified PCR products with 2-11 polymorphic bands per locus enabled the discrimination of genotype.

In the present study the intra and inter-specific variation existing among the 15 selected accessions in 8 species of *Curcuma* germplasm conserved in the field gene bank of ICAR-CTCRI was assessed.

Table 3 lists out some important previous morphological and molecular characterization/ diversity studies in the genus studies in species of *Curcuma.*

Table 3 Morphological and molecular markers used for characterization/ diversity studies in the genus *Curcuma*

Number of species/accessions used	References
Morphological	
19 *Curcuma* species	Yusuf *et al.* (2016)
15 *Curcuma* species	Syamkumar (2008)
Restriction Fragment Length Polymorphism	
12 accessions of *C. longa*	Hayakawa *et al.* (2010)
Randomly Amplified Polymorphic DNA	
Curcuma zedoaria from Bangladesh	Islam *et al.* (2007)
Amplified Fragment Length Polymorphism	
9 species of *Curcuma*	Das *et al.* (2011)
Inter Simple Sequence Repeats	
15 *Curcuma* species	Syamkumar (2008)
7 *Curcuma* species from Northeast India	Das *et al.* (2011)
4 *Curcuma* species of Tripura	Saha *et al.* (2016)
29 genotypes of *C. longa*	Rana *et al.* (2015)
5 accessions of *C. wenyujin*	Wang *et al.* (2015)
18 turmeric genotypes	Prasanth *et al.* (2015)
Simple Sequence Repeats	
8 elite cultivars and 88 accessions in turmeric	Sahoo *et al.* (2017)
5 varieties of *Curcuma alismatifolia*	Taheri *et al.* (2014))

10 species of Zingiberaceae from eastern India	Mohanty *et al.* (2011)
30 accessions of *C. longa*	Senan *et al.* (2013)
20 turmeric accessions	Parthasarathy *et al.* (2010)
88 turmeric accessions	Sahoo *et al.*, 2017
10 turmeric genotypes	Singh *et al.* (2015)
57 turmeric accessions	Sigrist *et al.* (2010)

3. MATERIALS AND METHODS

The present study was carried out at the Division of Crop Improvement, ICAR-Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram during the year 2017-2018. Details regarding the experimental materials used and procedures followed in the study are elaborated in this chapter.

3.1 SOURCE OF GERMPLASM

Fifteen accessions belonging to 8 species of the genus *Curcuma* L. (2 accessions in each of *C. amada* Roxb., *C. angustifolia* Roxb., *C. aromatica* Salisb., *C. decipiens* Dalz., *C. malabarica* Velay., *C. raktakanta* Mangaly and Sabu, *C. zedoaria* Rosc. and one accession of *C. longa* Linn.) were collected from different places of India and conserved in the ICAR-CTCRI germplasm repository were selected for the present study. Accessions were raised in pots using rhizome as the planting material during October 2017. The passport data of accessions selected are listed out in Table 4.

3.2 MORPHOLOGICAL CHARACTERIZATION

Morphological characterization of the 15 accessions was carried out in fully grown plants using selected descriptors from the previous studies of Ravindran *et al.* (2007) and Sasikumar (2005). Certain changes were made according to the handiness for listing out the observations taken and for including additional/modified traits. The observations were made at 8th month of planting in the full growth stage and the tuber characters were recorded at the time of harvest.

Table 4 Passport data of the 15 accessions in 8 species of *Curcuma* selected for the study

Sl. No.	Species	Identity Number	Local name	Place	District	State
1	*Curcuma amada*-1	AKI/BT/2017-1	Inji manga	Cheenikuzhy	Idukki	Kerala
2	*Curcuma amada*-2	AKI/BT/2017-2	Manga inji	Amboori	Thiruvananthapuram	Kerala
3	*Curcuma angustifolia*-1	AKI/BT/2017-3	Tikhur	Moglai	Bastar	Chhattisgarh
4	*Curcuma angustifolia*-2	AKI/BT/2017-4	Tikhur	Shemaljodi	Raipur	Chhattisgarh
5	*Curcuma aromatica*-1	AKI/BT/2017-5	Kasthoori Manjal	Cheenikuzhy	Idukki	Kerala
6	*Curcuma aromatica*-2	AKI/BT/2017-6	Kasthoori Manjal	Vellayani	Thiruvananthapuram	Kerala
7	*Curcuma decipiens*-1	AKI/BT/2017-7	Kuzhi koova	Manchikkal	Idukki	Kerala
8	*Curcuma decipiens*-2	AKI/BT/2017-8	Kuzhi koova	Uppukunnu	Idukki	Kerala
9	*Curcuma malabarica*-1	AKI/BT/2017-9	Neelakkoova	Oliviruppu	Idukki	Kerala
10	*Curcuma malabarica*-2	AKI/BT/2017-10	Kattukoova	Nanminda	Kozhikode	Kerala
11	*Curcuma raktakanta*-1	AKI/BT/2017-11	Koova	Athirappally	Thrissur	Kerala
12	*Curcuma raktakanta*-2	AKI/BT/2017-12	Koova	Neerickode	Ernakulam	Kerala
13	*Curcuma zedoaria*-1	AKI/BT/2017-13	Manjakkoova	Chanthavila	Thiruvananthapuram	Kerala
14	*Curcuma zedoaria*-2	AKI/BT/2017-14	Manjakkoova	Cheenikuzhy	Idukki	Kerala
15	*Curcuma longa*	AKI/BT/2017-15	Manjal	Malayinchi	Idukki	Kerala

3.3 MORPHOLOGICAL OBSERVATIONS

The morphological observations were recorded by measuring and weighing using scale and weighing balance. Qualitative traits (13) were recorded as descriptive data as shown in Table 5 and quantitative traits (15) as numerical data as shown in Table 6. All the morphological data recorded were tabulated and scored in excel worksheet for further statistical analysis.

Table 5 Qualitative characters used for morphological characterization

Sl. No.	Descriptor/Trait	Descriptor states
1	Pseudo stem habit	1. Compact 2. Open
2	Leaf colour on ventral side	1. Light green 2. Green 3. Dark green
3	Leaf venation pattern	1. Close 2. Distant
4	Leaf midrib colour	1. Green 2. Light purple 3. Purple 4. Dark purple
5	Leaf margin	1. Even 2. Wavy
6	Leaf texture	1. Glabrous 2. Pubescent
7	Spike position	1. Absent 2. Lateral 3. Terminal
8	Rhizome shape	1. Oblong 2. Cylindrical
9	Stipitate tubers	1. Absent 2. Present
10	Shape of stipitate tubers	1. Fusiform 2. Long-fusiform
11	Rhizome flesh colour	1. White 2. Creamy white 3. Bluish white 4. Pale Yellow 5. Yellow 6. Reddish yellow

12	Aroma of rhizome	1. Camphoraceous 2. Highly camphoraceous 3. Mango
13	Rhizome flavour/taste	1. Bitter 2. Gingery

Table 6 Quantitative characters used for morphological characterization

Sl. No.	Descriptor/Trait	Descriptor states
1	Pseudostem anthocyanin pigmentation	1. Absent 2. Low 3. High
2	Plant height (cm)	1. Short (< 75) 2. Medium (75-100) 3. Tall (> 100)
3	Number of shoots	1. Few (< 3) 2. Medium (3-5) 3. Many (> 5)
4	Number of leaves on main shoot	1. Few (< 5) 2. Inter mediate (5-10) 3. Many (> 10)
5	Leaf disposition	1. Erect (< 45°) 2. Semi-erect (45-85°) 3. Horizontal (> 85°)
6	Leaf petiole length (cm)	1. Short (< 15) 2. Inter mediate (15-25) 3. Long (> 25)
7	Leaf lamina length (cm)	1. Short (< 30) 2. Medium (30-40) 3. Long (> 60)
8	Leaf lamina width (cm)	1. Narrow (< 10) 2. Medium (10-15) 3. Broad (> 15)
9	Number of days to flowering	1. Absent 2. 100-150 3. > 150
10	Rhizome clump weight (g)	1. Small (< 250 g) 2. Medium (250 g-750 g) 3. Large (> 750 g)

11	Number of mother rhizomes	1. One 2. Two-Three 3. More than Three
12	Weight of mother rhizomes (g)	1. Small (< 100 g) 2. Large (> 100 g)
13	Number of primary rhizomes	1. Two 2. Three-Ten 3. More than ten
14	Rhizome internode length (cm)	1. More than 1 2. Less than 1
15	Status of secondary rhizome	1. Absent 2. Present

3.4 ANALYSIS OF MORPHOLOGICAL DATA

The recorded data was subjected to statistical analysis such as Cluster analysis, Pearson Correlation Coefficient analysis and Principal Component Analysis.

3.4.1 Cluster analysis

With the help of hclust function of R software package 3.5.0 (R Studio) hierarchical clustering was done and a cluster dendrogram drawn. The aim was to group the morphologically similar accessions in same group and distinct accessions to distant groups. The whole analysis was based on Euclidean distance.

3.4.2 Principal Component Analysis (PCA)

Principal Component Analysis was done to obtain the biplot. Biplot was obtained in order to determine which of the traits contribute mostly for the diversity of the accessions.

3.4.3 Pearson Correlation Coefficient (r)

It is a very helpful statistical formula that measures the strength between variables and relationships, where the value r =1 means a perfect positive correlation and the value r =-1 means a perfect negative correlation.

3.5 MOLECULAR MARKER ANALYSIS

3.5.1 Glass ware and other materials

1.5 and 2 ml Eppendorf tubes, PCR tubes, mortar and pestle, micropipette tips for 10 µl, 200 µl and 1000 µl were autoclaved and used. 1.5 ml tube stand, PCR tube holders, micropipettes, ice bags, measuring cylinder, bottles, spatula, polythene covers, labels, wipes and weighing pot are the other materials needed for molecular work.

3.5.2 Instruments

The equipments *viz.*, water bath (Memmert), electronic weighing balance (Afcoset), microwave oven (IFB), ice flaking machine (Icematic), vortexer (Labnet), spinner (Rivotek), cooling centrifuge (Eppendorf), NanoDrop™ spectrophotometer, pH meter, deep freezer (-20 °C (Vestfrost), -80 °C (New Brunswick Scientific)), refrigerator (Whirlpool), electrophoresis apparatus (Cleaver Scientific), gel documentation system (Syngene), PCR machine (Proflex), hot air oven (Beston), autoclave (Hirayama) and distilled water unit (ELGA) were used for the study.

3.5.3 DNA Extraction

DNA was extracted from fresh and tender young leaves of *Curcuma* species accessions using the method of Syamkumar (2008). The procedure is as follows:

Tender leaf samples were collected from the field, 1g samples weighed and homogenized to a fine powder using liquid nitrogen. Pre-warmed CTAB extraction buffer (1.5 ml) was added to the samples and ground once more. The samples were transferred to 2 ml microfuge tubes and vortexed. The samples were incubated at 65 °C for 30 minutes with frequent swirling in water bath. Samples were centrifuged at 12000 rpm for 10 minutes at 4 °C and the supernatant was collected in a fresh microfuge tube. Equal volume of chloroform:isoamyl alcohol (24:1) was added and mixed by gentle inversion. Samples were centrifuged at 12000 rpm for 10 minutes at 4 °C. The supernatant was collected in a fresh microfuge tube and again equal

volume of chloroform:isoamyl alcohol (24:1) was added into the sample. Samples were centrifuged at 12000 rpm for 10 minutes at 4 °C. Supernatant was collected in fresh microfuge tube and added 0.8 volume ice-cold isopropanol and kept in -20 °C freezer for 45 minutes. Microfuge tube centrifuged at 12000 rpm for 10 minutes at 4 °C. Supernatant was discarded and saved the pellet. The DNA pellet was washed with 70% ethanol. Air dried the pellet until the ethanol evaporated and dissolved in 100 µl of 1X TE buffer. 5 µl of RNase (10 mg/ml) were added and incubated at 37 °C for 1 hour. All samples were checked for DNA quality in 1% agarose gel and confirmed.

3.6 ANALYSIS OF THE EXTRACTED DNA

3.6.1 Agarose gel electrophoresis

Agarose gel electrophoresis is a technique used to separate and analyse the nucleic acid molecules based on their size. The phosphate backbone of DNA is negatively charged. So when DNA is placed in an electric field it will migrate towards the positive electrode (anode). Ethidium Bromide (EtBr) is added to agarose solution intercalates between the nitrogenous bases of DNA and it produce visual fluorescence when exposed on UV.

1% agarose gel was used to check the quality and integrity of the extracted DNA. 1% agarose solution was prepared by weighing out 1 g agarose in a conical flask and dissolving it using 100 ml 1X TBE buffer. Agarose was dissolved by heating and after that the flask was allowed to cool and when the temperature of the flask became bearable, 1 µl (10 mg/ml) of EtBr was added directly to the molten gel and gentle mixing was done. Casting tray was prepared with combs and gel was poured into the tray and allowed to solidify. 4 µl of isolated DNA sample mixed with 2 µl of 1X loading dye was loaded into the wells of prepared gel.

Horizontal gel electrophoresis unit was used to run the gel. The gel was run for about 30 minutes at 100V. The run was stopped after the dye front reached beyond $3/4^{th}$ of the gel. Then it was visualized UV light using a gel documentation system.

3.6.2 Quantification of DNA

NanoDrop™ spectrophotometer was used to quantify the isolated DNA. It helped to assess the yield and purity with high accuracy and reproducibility. 1X TE buffer in which DNA was dissolved was used to calibrate the machine to blank i.e. zero absorbance. The advantage of NanoDrop™ spectrophotometer is that it requires only 1 µl sample to measure its quantity and quality. The quantity of DNA was determined by measuring OD at 260 nm and the purity was determined by OD_{260}/OD_{280} ratio. According to the better absorbance value/OD value samples were selected.

3.6.3 Dilution of samples

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

3.7 PRIMER SCREENING

Ten ISSR and 10 SSR primers were selected from previous literature (Saha *et al.*, 2016; Senan *et al.*, 2013) for the initial primer screening. The ISSR primers used were UBC-807, UBC-808, UBC-809, UBC-811, UBC-825, UBC-836, UBC-841, UBC-842, UBC-864, UBC-873 and SSR primers used were CuMiSat-19, CuMiSat-20, CuMiSat-21, CuMiSat-22, CuMiSat-23, CuMiSat-24, CuMiSat-25, CuMiSat-26, CuMiSat-27, CuMiSat-28. The list of primers selected, primer sequence and annealing temperatures of both ISSR and SSR primers are given in Table 7 and Table 8 respectively.

Table 7 List of ISSR primers used with details

Sl. No.	Primer name	Sequence	Annealing Temperature (°C)
1	UBC-807	AGA GAG AGA GAG AGA GT	56.3
2	UBC-808	AGA GAG AGA GAG AGA GC	56.3
3	UBC-809	AGA GAG AGA GAG AGA GG	56.3
4	UBC-811	GAG AGA GAG AGA GAG AC	56.3
5	UBC-825	ACA CAC ACA CAC ACA CT	56.3
6	UBC-836	AGA GAG AGA GAG AGA GYA	56.3
7	UBC-841	GAG AGA GAG AGA GAG AYC	56.3

8	UBC-842	GAG AGA GAG AGA GAG AYG	56.3
9	UBC-864	ATG ATG ATG ATG ATG ATG	56.3
10	UBC-873	GAC AGA CAG ACA GAC A	56.3

Table 8 List of SSR primers used with details

Sl. No.	Primer name	Sequence	Annealing temperature (°C)
1	CuMiSat-19 F: CuMiSat-19 R:	CAT GCA AAT GGA AAT TGA CAC TGA TAA ATT GAC ACA TGG CAG TC	65
2	CuMiSat-20 F: CuMiSat-20 R:	CGA TAC GAG TCC ATC TCT TCG CCT TGC TTT GGT GGC TAG AG	65
3	CuMiSat-21 F: CuMiSat-21 R:	TCA TTC AAA GTC CGA TGG AA TTC GAG TGC AGA AGG AGA ATT A	62
4	CuMiSat-22 F: CuMiSat-22 R:	AAT TTA TTA GCC CGG ACC AC AAG AAA GTG AGT AGA AAC CAA AGC	64
5	CuMiSat-23 F: CuMiSat-23 R:	CGT GGA AGG TGA GTT TGA C CAG AAG GGA ACT GAG ATG G	65
6	CuMiSat-24 F: CuMiSat-24 R:	AGG TAT TCT ACT CGA CCA AG AAA TTC ATA TAG CCC CAT C	58
7	CuMiSat-25 F: CuMiSat-25 R:	TAC ATG AGA AAC AAC AAA GCC C AGT TAG CCA AGT CCC AAT TTA GC	65
8	CuMiSat-26 F: CuMiSat-26 R:	CAT TCC GAT GAA TTG TAT G GCA GTT GTT TTG CTT CAG	58
9	CuMiSat-27 F: CuMiSat-27 R:	TAT AGATAG CCA TGC TGA AG CCA TTT TAG TTC ATT ACG TG	63
10	CuMiSat-28 F: CuMiSat-28 R:	TTC AAC TTC TCC TCG CTC AG GCA AGG TGC TGC ATC TAT TTC TC	65

The genomic DNA of six accessions of *Curcuma* species were taken for initial primer screening. These randomly selected accessions were *C. amada*-1, *C. aromatica*-1, *C. decipiens*-1, *C. malabarica*-1, *C. raktakanta*-1, *C. longa*. The

composition of the reaction mixtures of ISSR and SSR primers were as follows in Table 9 and Table 10 respectively.

Table 9 ISSR reaction mixture

Ingredients	**Stock concentration**	**Required concentration**	**Required volume for one reaction (15 µl)**
EmeraldAmp® GT PCR master mix	2X	1X	7.5 µl
$MgCl_2$	50 mM	1.3 mM	0.4 µl
Primer	100 µM	0.3 µM	0.5 µl
Template DNA	10 ng/µl	40 ng	4.0 µl
SDW	----	----	2.6 µl
Total			15 µl

Table 10 SSR reaction mixture

Ingredients	**Stock concentration**	**Required concentration**	**Required volume for one reaction (20 µl)**
EmeraldAmp® GT PCR master mix	2X	1X	7.5 µl
MgCl2	50 mM	1 mM	0.3 µl
Primer (F)	10 µM	0.3 µM	0.5 µl
Primer (R)	10 µM	0.3 µM	0.5 µl
Template DNA	10 ng/µl	50 ng	5 µl
SDW	----	----	1.7 µl
Total			15 µl

3.7.1 PCR conditions for ISSR marker

PCR was carried out in Proflex™ thermalcycler programmed for an initial denaturation at 94 °C for 5 minutes followed by 38 cycles with denaturation at 94 °C for 30 seconds, primer annealing at 56.3 °C for 1 minute and extension at 72 °C for 1 minute. The final extension was performed at 72 °C for 10 minutes followed by its holding at 4 °C.

3.7.2 PCR conditions for SSR marker

Initial denaturation at 94 °C for 5 minutes followed by 38 cycles with denaturation at 94 °C for 30 seconds, primer annealing at appropriate temperatures as given in Table 8 for 45 seconds and extension at 72 °C for 1 minute. The final extension was performed at 72 °C for 20 minutes followed by its holding at 4 °C.

The amplified products were resolved in a 2% agarose gel using 100 bp and 1 kb ladder for checking amplification, to identify molecular weight of obtained bands and for polymorphism studies.

3.8 ISSR/SSR ANALYSIS

3.8.1 Agarose Gel Electrophoresis

Gel electrophoresis was performed with 2% agarose.

3.8.1.1 Gel preparation

2% of agarose was used to resolve the amplicons obtained after the PCR. For preparing a total of 120 ml of 2% gel, 2.4 g of agarose was weighed out and dissolved in 120 ml of 1X TBE buffer. The flask was undisturbed and using a microwave oven, the gel was melted completely. Added approximately 0.9 µl EtBr into the conical flask containing the melted agarose. Then gently and gradually rotated the conical flask for the uniform distribution of EtBr. After proper mixing it was poured on to the casting tray and allowed to solidify for about 30 minutes.

3.8.1.2 Gel loading and running

The PCR products were arranged in a tray according to the sample number for loading. PCR products were spun down using centrifuge. The combs from the solidified gel were removed slowly without disturbing the wells and the samples were loaded in the same order along with 100 bp and 1 kb DNA ladders. The electrophoresis apparatus was connected to a power pack and the voltage was set at 120V. The movement of the tracking dye was noted and the run was stopped when the dye reached the bottom of the tray.

3.8.1.3 PCR product detection

The gel was taken out and then viewed under the ultraviolet light in gel documentation system. The image was taken under appropriate exposure and saved in sgd (Syngene document) format for scoring.

3.9 REPRODUCIBILITY TESTING

After completing the screening, DNA was again isolated from randomly selected accessions (*C. amada*-1, *C. angustifolia*-1, *C. aromatica*-1) in order to confirm the reproducibility of bands. ISSR primers such as UBC-808, UBC-811 and SSR primers such as CuMiSat-19, CuMiSat-26 were used for screening.

3.10 ANALYSIS OF MOLECULAR MARKER DATA

3.10.1 Band scoring

All the images of resolved PCR products were taken. Clear and reproducible bands were taken for scoring. Binary scoring was carried out by assigning "1" for the presence of a specific sized band and "0" for the absence of a band. The data was entered in excel and was used as an input for statistical analysis.

3.10.2 Cluster Analysis

For clustering of molecular marker data, hclust function of R statistical package 3.5.0 (R studio) was used and hierarchical clustering was done based on Euclidean distance. As a result, a dendrogram of 15 accessions was obtained which is based on molecular traits.

3.11 CORRELATION OF MORPHOLOGICAL AND MOLECULAR DATA

Mantel's test is a versatile statistical tool used to analyse the correlation between two matrices. So this can be employed in evaluating the correlation between morphological and molecular data. The test statistic is the Pearson correlation coefficient r. r falls in the range of -1 to +1, where being close to -1 indicates strong negative correlation and +1 indicates strong positive correlation. An r value of 0 indicates no correlation.

4. RESULTS

The study entitled "Characterization of selected *Curcuma* species germplasm using morphological and molecular markers" was carried out at the Division of Crop Improvement, ICAR-Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram during 2017-2018. The selected 15 accessions of *Curcuma* were characterized based on both morphological and molecular markers. The results are depicted in this chapter.

4.1 MORPHOLOGICAL DATA ANALYSIS

Juvenile stage of the 15 selected accessions of *Curcuma* species are shown in the Plate 1 and that of rhizome cross section are shown in the Plate 2.

C. amada-1

C. amada-2

C. angustifolia-1

C. angustifolia-2

C. aromatica-1

C. aromatica-2

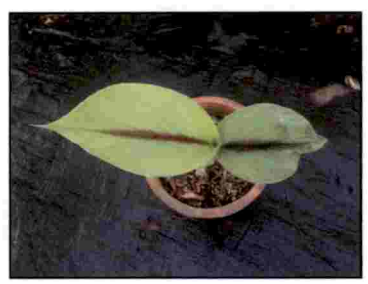
C. decipiens-1

C. decipiens-2

C. malabarica-1

C. malabarica-2

C. raktakanta-1



C. raktakanta-2

C. zedoaria-1

C. zedoaria-2

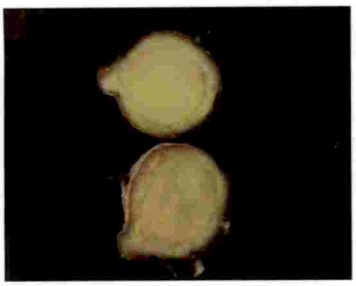
C. longa

Plate 1 - Plant pictures of 15 selected accessions of *Curcuma*

C. amada-1

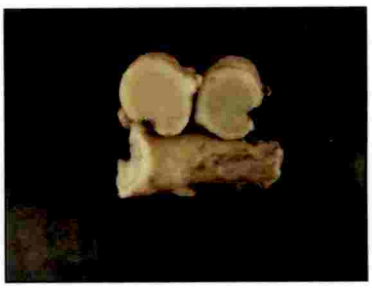
C. amada-2

C. angustifolia-1

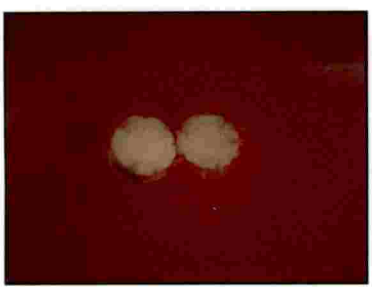


C. angustifolia-2

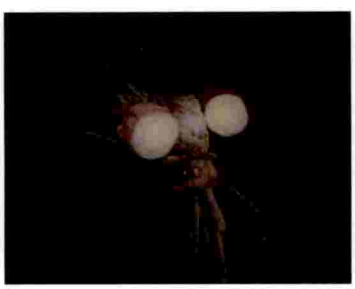
C. aromatica-1



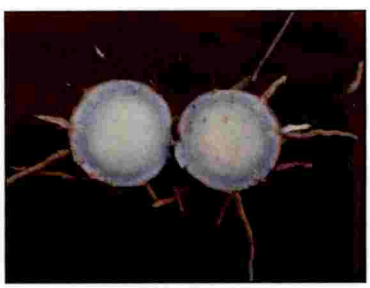
C. aromatica-2



C. decipiens-1



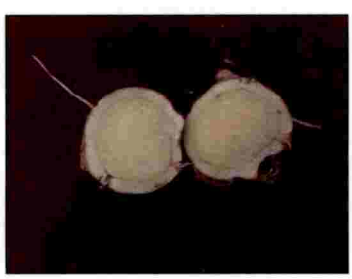
C. decipiens-2



C. malabarica-2

C. malabarica-2

C. raktakanta-1



C. raktakanta-2

C. zedoaria-1

C. zedoaria-2

C. longa

Plate 2 - Rhizome cross section of 15 selected accessions of *Curcuma*

4.1.1 Morphological characters

Important qualitative and quantitative morphological characters of 15 accessions of *Curcuma* species studied are shown in the tables given Table 11 and Table 12 respectively.

Table 11 Qualitative morphological characters

Sl. No.	Accessions	PH	CVS	VP	LMC	LM	LT	SP	RS	ST	SST	RFC	AR	RF
1	*C. amada*-1	Open	Green	Distant	Green	Wavy	Pubescent	Absent	Cylindrical	Present	Fusiform	Pale yellow	Mango like	Gingery
2	*C. amada*-2	Open	Green	Distant	Green	Wavy	Pubescent	Absent	Cylindrical	Present	Fusiform	Pale yellow	Mango like	Gingery
3	*C. angustifolia*-1	Open	Dark green	Distant	Green	Even	Glabrous	Absent	Oblong	Present	Long-fusiform	Pale yellow	Camphoraceous	Bitter
4	*C. angustifolia*-2	Open	Green	Distant	Light purple	Even	Pubescent	Terminal	Oblong	Present	Long-fusiform	Yellow	Camphoraceous	Bitter
5	*C. aromatica*-1	Open	Green	Distant	Green	Wavy	Pubescent	Absent	Cylindrical	Present	Fusiform	Creamy white	Persisting camphoraceous	Bitter
6	*C. aromatica*-2	Open	Light green	Distant	Green	Wavy	Pubescent	Absent	Cylindrical	Present	Fusiform	Creamy white	Persisting camphoraceous	Bitter
7	*C. decipiens*-1	Open	Dark green	Distant	Green	Even	Glabrous	Terminal	Oblong	Present	Long-fusiform	Pale yellow	Camphoraceous	Bitter
8	*C. decipiens*-2	Open	Dark green	Distant	Green	Even	Glabrous	Terminal	Oblong	Present	Long-fusiform	Pale yellow	Camphoraceous	Bitter
9	*C. malabarica*-1	Open	Light green	Distant	Purple	Even	Glabrous	Absent	Oblong	Present	Fusiform	Bluish white	Camphoraceous	Bitter
10	*C. malabarica*-2	Open	Green	Distant	Purple	Even	Glabrous	Absent	Oblong	Present	Fusiform	Bluish white	Camphoraceous	Bitter
11	*C. raktakanta*-1	Open	Green	Distant	Dark purple	Even	Glabrous	Absent	Oblong	Present	Fusiform	Pale yellow	Camphoraceous	Bitter

12	*C. raktakanta*-2	Open	Green	Distant	Dark purple	Even	Glabrous	Absent	Oblong	Present	Fusiform	Pale yellow	Camphoraceous	Bitter
13	*C. zedoaria*-1	Open	Green	Distant	Light purple	Wavy	Glabrous	Absent	Oblong	Present	Fusiform	Reddish yellow	Camphoraceous	Bitter
14	*C. zedoaria*-2	Open	Green	Distant	Purple	Wavy	Glabrous	Absent	Oblong	Present	Fusiform	Reddish yellow	Camphoraceous	Bitter
15	*C. longa*	Open	Light green	Distant	Green	Wavy	Glabrous	Absent	Cylindrical	Absent	Absent	Yellow	Camphoraceous	Bitter

PH-Pseudostem habit, CVS-Leaf colour on ventral side, VP-Venation pattern, LMC-Leaf midrib colour, LM-Leaf margin, LT-Leaf texture, SP-Spike position, RS-Rhizome shape, ST-Stipitate tubers, SST-Shape of stipitate tubers, RFC-Rhizome flesh colour, AR-Aroma of rhizome, RF-Rhizome flavour/taste

Table 12 Quantitative morphological characters

Sl. No.	Accessions	PAC	PH (cm)	NS	LMS	LD	PL (cm)	LL (cm)	LW (cm)	DF	RCW (g)	NMR	WMR (g)	NPR	IL (cm)	SR
1	*C. amada*-1	Absent	129.5	3	9	Erect	20.2	64.3	17.1	Absent	650.7	3	79.4	8	1.4	Present
2	*C. amada*-2	Absent	99.1	3	7	Erect	13.2	46.7	13.7	Absent	881.0	4	87.0	10	1.6	Present
3	*C. angustifolia*-1	Absent	108.2	1	9	Erect	20.1	50.3	15.8	Absent	270.4	2	75.6	5	0.91	Absent
4	*C. angustifolia*-2	Low	86.5	2	9	Erect	12.9	49.2	15.7	185	203.8	2	63.1	5	0.82	Absent
5	*C. aromatica*-1	Absent	74.7	4	8	Erect	9.6	40.5	13.2	Absent	320.0	2	60.3	7	1.1	Present
6	*C. aromatica*-2	Absent	75.7	2	8	Erect	10.5	40.6	12.8	Absent	414.2	3	62.5	6	1.3	Present
7	*C. decipiens*-1	Low	42.5	3	6	Erect	7.1	19.4	9.6	173	181.1	3	29.4	3	1.2	Absent
8	*C. decipiens*-2	Low	37.3	2	8	Erect	8.8	22.5	11.3	178	188.9	4	30.8	2	1.2	Absent
9	*C. malabarica*-1	Absent	119	3	8	Erect	24.3	67.2	15.9	Absent	863.0	5	110.7	6	1.3	Present
10	*C. malabarica*-2	Absent	121.6	4	7	Erect	13.3	60.0	17.5	Absent	934.2	4	150.1	6	1.3	Present
11	*C. raktakanta*-1	High	103.4	3	8	Erect	12.7	55.2	15	Absent	342.5	2	89.6	4	0.7	Present
12	*C. raktakanta*-2	High	109.5	3	6	Erect	11.1	41.6	11.3	Absent	380.7	3	84.2	3	0.7	Present
13	*C. zedoaria*-1	Absent	145	3	9	Erect	20.5	62.8	15.6	Absent	624.1	3	210.1	5	1.9	Absent
14	*C. zedoaria*-2	Absent	109.5	2	6	Erect	11.6	56.1	18.3	Absent	781.4	4	230.8	5	2	Absent
15	*C. longa*	Absent	83.9	5	9	Erect	15.4	64.3	13.4	Absent	317.4	3	86.2	7	1.1	Present

PAC-Pseudostem anthocyanin pigmentation, PH-Plant height, NS-Number of shoots, LMS-Number of leaves on main shoot, LD-Leaf disposition, PL-Petiole length, LL-Leaf length, LW-Leaf width, DF-Number of days to flowering, RCW-Rhizome clump weight, NMR-Number of mother rhizomes, WMR-Weight of mother rhizomes, NPR-Number of primary rhizomes, IL-Internode length, SR-Status of secondary rhizome.

4.1.2 Cluster analysis

On UPGMA cluster analysis, the 15 accessions were grouped into 4 major clusters shown as in the dendrogram (Figure 1). *C. decipiens* accessions formed distant cluster.

Cluster I- *C. amada*-1, *C. amada*-2, *C. aromatica*-1, *C. aromatica*-2, *C. longa*

Cluster II- *C. angustifolia*-1, *C. angustifolia*-2

Cluster III- *C. decipiens*-1, *C. decipiens*-2

Cluster IV- *C. malabarica*-1, *C. malabarica*-2, *C. raktakanta*-1, *C. raktakanta*-2, *C. zedoaria*-1, *C. zedoaria*-2

4.1.3 Pearson correlation coefficient analysis

The Pearson correlation coefficient obtained using the scoring data of morphological characteristics are given in Table 13. The intra and inter-specific variation within the selected accessions were thus estimated.

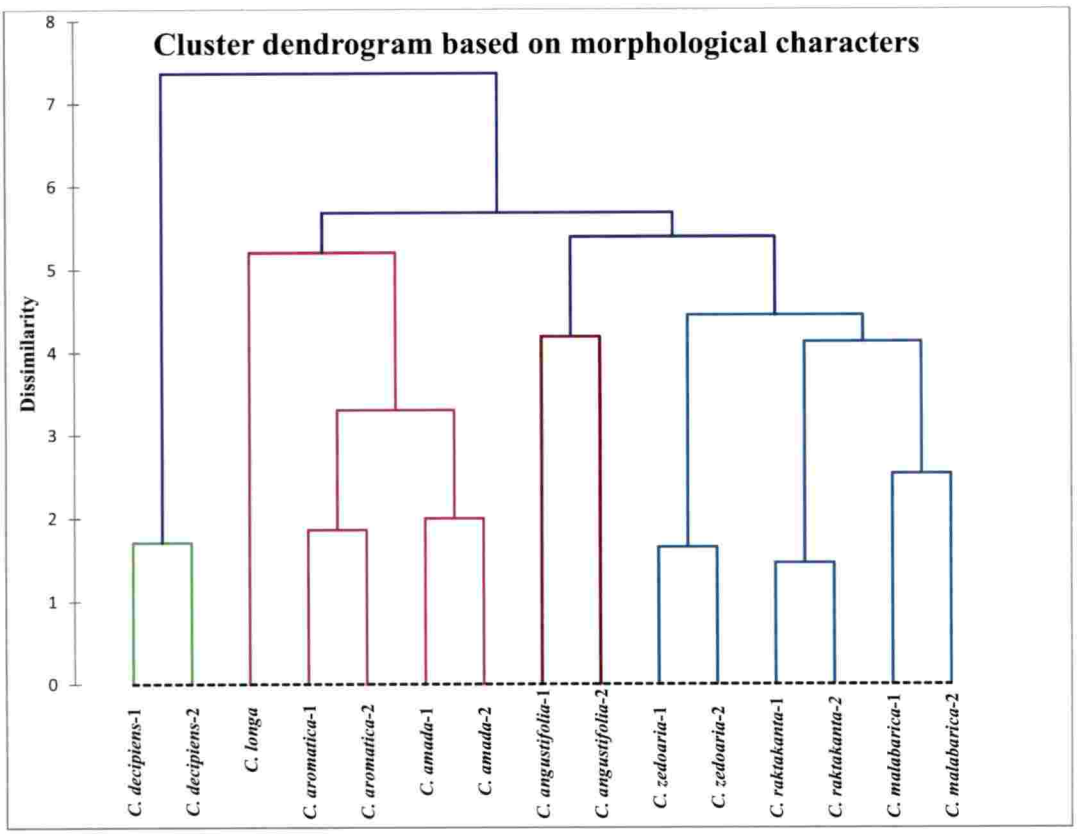


Figure 1 - Cluster dendrogram of 15 accessions in 8 species of *Curcuma* based on morphological descriptors

Table 13 Pearson Correlation Coefficient (PCC) based on morphological characteristics

Accessions	*C. amada*-1	*C. amada*-2	*C. angustifolia*-1	*C. angustifolia*-2	*C. aromatica*-1	*C. aromatica*-2	*C. decipiens*-1	*C. decipiens*-2	*C. malabarica*-1	*C. malabarica*-2	*C. raktakanta-1*	*C. raktakanta*-2	*C. zedoaria*-1	*C. zedoaria*-2	*C. longa*
C. amada-1	**1**	0.8696	0.4185	-0.0734	0.5523	0.6444	-0.0758	-0.0704	0.3484	0.3558	0.1256	0.0000	0.6135	0.4925	0.6309
C. amada-2	0.8696	**1**	0.1875	-0.1625	0.6696	0.6696	0.0479	0.0445	0.0881	0.1350	0.0199	0.0000	0.4850	0.4672	0.5622
C. angustifolia-1	0.4185	0.1875	**1**	0.5199	0.1862	0.3660	0.4495	0.4859	0.5200	0.3102	0.4316	0.2841	0.6322	0.5458	0.2721
C. angustifolia-2	-0.0734	-0.1625	0.5199	**1**	-0.0473	0.0473	0.6624	0.6871	0.1074	0.1280	0.3712	0.2990	0.2723	0.3093	0.0000
C. aromatica-1	0.5523	0.6696	0.1862	-0.0473	**1**	0.8814	0.0419	0.0389	0.2566	0.2555	0.0983	0.1323	0.4056	0.3727	0.6019
C. aromatica-2	0.6444	0.6696	0.3660	0.0473	0.8814	**1**	-0.0558	0.0389	0.3464	0.3472	0.1792	0.1323	0.4775	0.4433	0.5280
C. decipiens-1	-0.0758	0.0479	0.4495	0.6624	0.0419	-0.0558	**1**	0.9281	-0.0845	-0.2427	0.1476	0.1816	0.1564	0.1908	0.0087
C. decipiens-2	-0.0704	0.0445	0.4859	0.6871	0.0389	0.0389	0.9281	**1**	-0.0784	-0.2253	0.1370	0.1685	0.2001	0.2310	0.0081
C. malabarica-1	0.3484	0.0881	0.5200	0.1074	0.2566	0.3464	-0.0845	-0.0784	**1**	0.8379	0.6083	0.5007	0.4779	0.4575	0.2398
C. malabarica-2	0.3558	0.1350	0.3102	0.1280	0.2555	0.3472	-0.2427	-0.2253	0.8379	**1**	0.6503	0.5284	0.4822	0.5101	0.3102
C. raktakanta-1	0.1256	0.0199	0.4316	0.3712	0.0983	0.1792	0.1476	0.1370	0.6083	0.6503	**1**	0.9478	0.5307	0.6564	0.2810
C. raktakanta-2	0.0000	0.0000	0.2841	0.2990	0.1323	0.1323	0.1816	0.1685	0.5007	0.5284	0.9478	**1**	0.4676	0.6029	0.2748
C. zedoaria-1	0.6135	0.4850	0.6322	0.2723	0.4056	0.4775	0.1564	0.2001	0.4779	0.4822	0.5307	0.4676	**1**	0.9369	0.5485
C. zedoaria-2	0.4925	0.4672	0.5458	0.3093	0.3727	0.4433	0.1908	0.2310	0.4575	0.5101	0.6564	0.6029	0.9369	**1**	0.5339
C. longa	0.6309	0.5622	0.2721	0.0000	0.6019	0.5280	0.0087	0.0081	0.2398	0.3102	0.2810	0.2748	0.7485	0.6339	**1**

The intra and inter-specific similarity among the selected accessions based on PCC value are shown in the table below (Table 14).

Table 14 Intra and inter specific similarity

Similarity	Intra-specific	Inter-specific	With *C. longa*
Highest	*C. raktakanta*-1 *C. raktakanta*-2 (0.94)	*C. amada*-2 *C. aromatica*-1 (0.66)	*C. amada*-1 (0.63)
Lowest	*C. angustifolia*-1 *C. angustifolia*-2 (0.52)	*C. decipiens*-1 *C. malabarica*-2 (-0.24)	*C. decipiens*-2 (0.008)

4.1.5 Principal Component Analysis (PCA)

PCA biplot was drawn and morphological characters and selected accessions of *Curcuma* were spatially arranged (Figure 2). The PCA values are shown in Table 15.

Table 15 PCA values for qualitative morphological characters

Morphological traits/Descriptors	PCA Comp.1	PCA Comp.2	PCA Comp.3	PCA Comp.4	PCA Comp.5
Pseudo stem habit	1.158292	0.395255	-0.379896	0.429141	0.651285
Leaf colour on ventral side	-2.116561	-0.965240	-1.811461	-0.247244	-1.101193
Leaf venation pattern	-1.982628	-1.564550	-0.925025	0.542005	0.835568
Leaf mid rib colour	1.441658	2.188356	-1.566246	1.682043	0.513692
Leaf margin	-1.049282	-2.307086	-0.848024	-0.695579	0.082558
Leaf texture	1.158292	0.395255	-0.379896	0.429141	0.651285
Spike position	-3.051094	3.194924	-1.828508	0.171469	0.228178
Rhizome shape	-1.835858	-2.396420	-0.856113	-0.128135	0.667373
Stipitate tubers	0.826306	0.594511	-0.257172	0.860751	0.757283
Shape of stipitate tubers	-3.439761	1.867138	-0.585698	0.882290	-0.499943
Rhizome flesh colour	5.691179	2.175630	-2.911065	-2.988351	-1.216806
Aroma of rhizome	0.910395	2.019701	5.137724	-1.091474	0.791825
Rhizome flavour/taste	-3.138553	-1.322259	-0.021553	-0.317918	-0.951017
Eigen value	**6.0403**	**3.1100**	**2.3666**	**0.9520**	**0.8369**
Percentage variation	**40.2686**	**20.7333**	**15.7776**	**6.3465**	**5.5794**
Cumulative percentage	**40.2686**	**61.0019**	**76.7796**	**83.1260**	**88.7054**

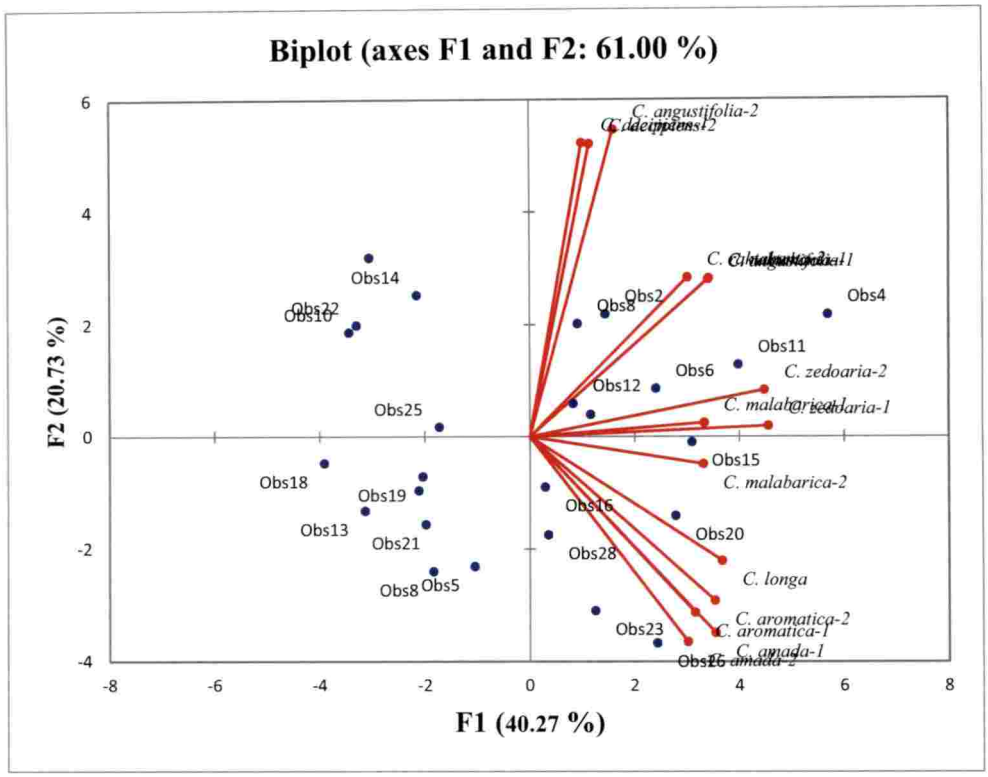


Figure 2 - Biplot showing the spatial distribution of various morphological descriptors and 15 accessions of *Curcuma*

4.2 MOLECULAR DATA ANALYSIS

4.2.1 Standardization of DNA isolation protocol

Isolation of DNA from the 15 accessions of *Curcuma* was tried by using the method of Syamkumar (2008) to extract quality DNA with better concentration. This method of DNA isolation yielded good quality DNA with good spectrophotometric readings (Table 16). The yield of isolated DNA ranged from 312 ng/µl to 2236 ng/µl with high purity. Isolated DNAs were separated on 1% agarose gel to check the quality. It is shown in Figure 3.

Table 16 Spectrophotometric readings of isolated DNA

Sl. No.	Accession name	Concentration (ng/µl)	A_{260}/A_{280}
1	*C. amada*-1	908.60	1.93
2	*C. amada*-2	603.79	1.85
3	*C. angustifolia*-1	1023.10	1.90
4	*C. angustifolia*-2	1789.00	1.86
5	*C. aromatica*-1	312.62	1.91
6	*C. aromatica*-2	369.00	1.84
7	*C. decipiens*-1	770.70	1.81
8	*C. decipiens*-2	696.10	1.87
9	*C. malabarica*-1	974.30	1.90
10	*C. malabarica*-2	1698.00	1.84
11	*C. raktakanta*-1	1307.05	1.89
12	*C. raktakanta*-2	785.67	1.86
13	*C. zedoaria*-1	1077.13	1.95
14	*C. zedoaria*-2	2236.26	1.82
15	*C. longa*	2172.12	1.86

A spectrophotometric value (A_{260}/A_{280}) of 1.81-1.95 and a conspicuous band on 1% agarose gel ensured the high quality of isolated DNA.

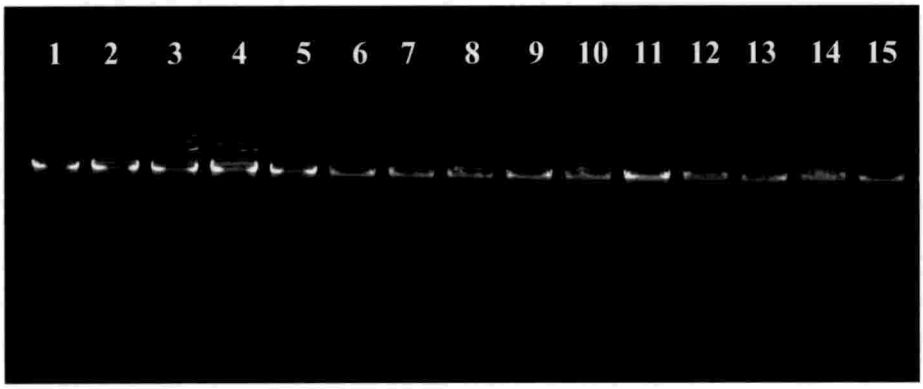


Figure 3 - 1% gel profiles of DNA isolated for 15 accessions of *Curcuma*

Lane-1: *C. amada*-1

Lane-2: *C. amada*-2

Lane-3: *C. angustifolia*-1

Lane-4: *C. angustifolia*-2

Lane-5: *C. aromatica*-1

Lane-6: *C. aromatica*-2

Lane-7: *C. decipiens*-1

Lane-8: *C. decipiens*-2

Lane-9: *C. malabarica*-1

Lane-10: *C. malabarica*-2

Lane-11: *C. raktakanta*-1

Lane-12: *C. raktakanta*-2

Lane-13: *C. zedoaria*-1

Lane-14: *C. zedoaria*-2

Lane-15: *C. longa*

4.2.2 Preliminary screening for ISSR/SSR markers

After initial primer screening, 10 ISSR (UBC-807, UBC-808, UBC-809, UBC-811, UBC-825, UBC-836, UBC-841, UBC-842, UBC-864, UBC-873) and 7 SSR primers (CuMiSat-19, CuMiSat-20, CuMiSat-23, CuMiSat-24, CuMiSat-25, CuMiSat-26, CuMiSat-28) which showed polymorphism were chosen for further analysis. PCR was performed using standardized PCR conditions and the annealing temperature obtained from literature was found to be optimum for each primer.

4.2.3 ISSR analysis

After the completion of PCR using selected ISSR primers, the amplicons were resolved in 2% agarose gel and amplicons obtained are shown in Figures 4-7. A total of 176 scorable bands were produced in the 15 accessions studied, out of which 166 were polymorphic (94.31%). The percentage of polymorphism ranged from a maximum of 100 percentage (shown by 8 primers) and a minimum of 66.67 (shown by UBC-841). The ISSR primers used, the number of bands produced by each primer, number of polymorphic bands and percentage polymorphism are shown in Table 17.

Table 17 PCR analysis using ISSR primers

Sl. No.	Primer	Total number of bands	Number of polymorphic bands	Percentage polymorphism
1	UBC-807	19	14	73.68
2	UBC-808	30	30	100.00
3	UBC-809	15	15	100.00
4	UBC-811	19	19	100.00
5	UBC-825	20	19	95.00
6	UBC-836	15	15	100.00
7	UBC-841	12	8	66.67
8	UBC-842	15	15	100.00
9	UBC-864	15	15	100.00
10	UBC-873	16	16	100.00
Total		**176**	**166**	**94.31**

Molecular characterization using ISSR markers

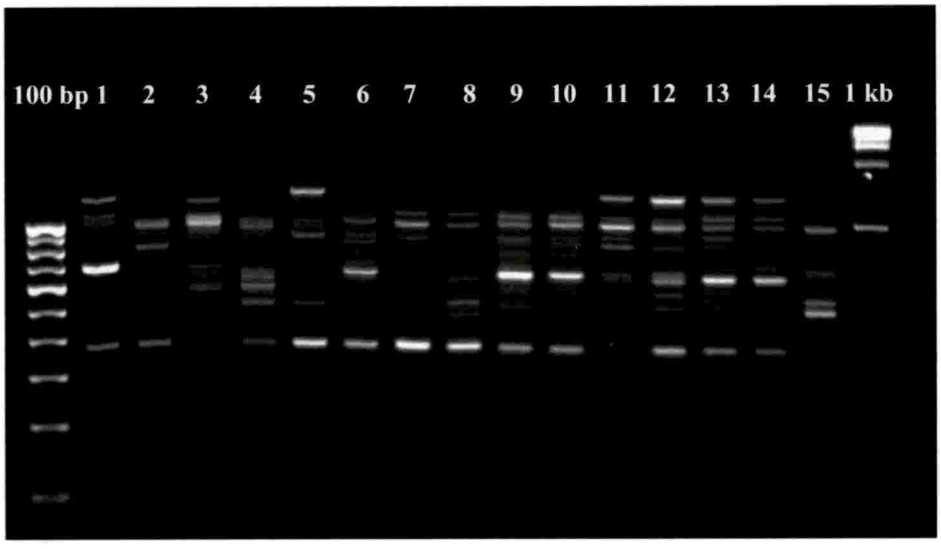


Figure 4 - Agarose gel (2%) profile of the ISSR primer UBC-808 for 15 accessions of *Curcuma*

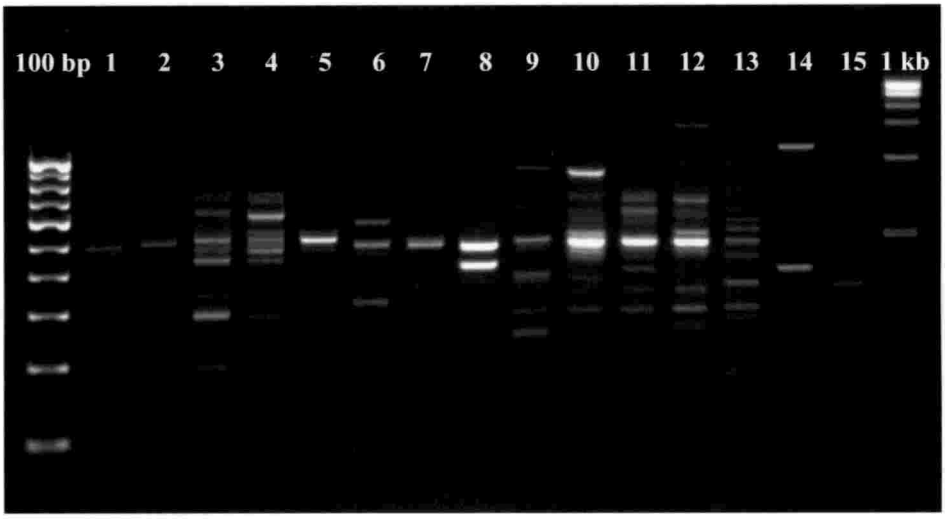


Figure 5 - Agarose gel (2%) profile of the ISSR primer UBC-836 for 15 accessions of *Curcuma*

Lane-1: 100 bp ladder
Lane-2: *C. amada*-1
Lane-3: *C. amada*-2
Lane-4: *C. angustifolia*-1
Lane-5: *C. angustifolia*-2
Lane-6: *C. aromatica*-1
Lane-7: *C. aromatica*-2
Lane-8: *C. decipiens*-1
Lane-9: *C. decipiens*-2
Lane-10: *C. malabarica*-1
Lane-11: *C. malabarica*-2
Lane-12: *C. raktakanta*-1
Lane-13: *C. raktakanta*-2
Lane-14: *C. zedoaria*-1
Lane-15: *C. zedoaria*-2
Lane-16: *C. longa*
Lane-17: 1 kb ladder

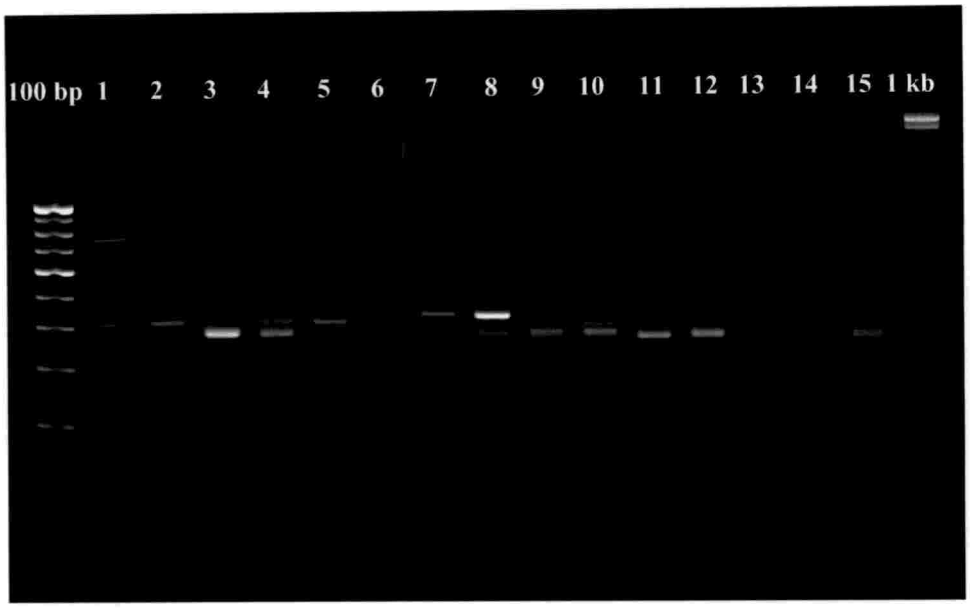


Figure 6 - Agarose gel (2%) profile of the ISSR primer UBC-864 for 15 accessions of *Curcuma*

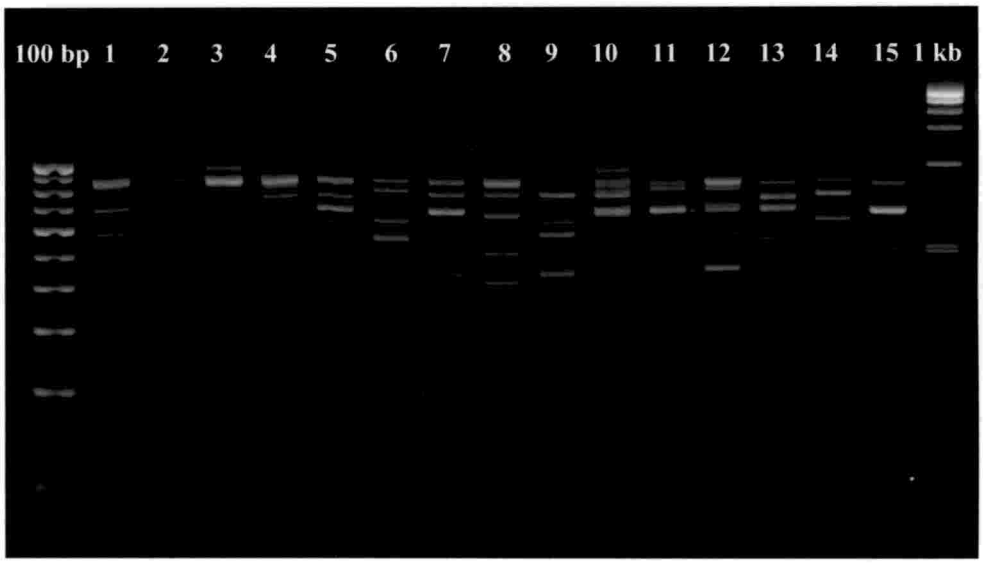


Figure 7 - Agarose gel (2%) profile of the ISSR primer UBC-873 for 15 accessions of *Curcuma*

Lane-1: 100 bp ladder

Lane-2: *C. amada*-1

Lane-3: *C. amada*-2

Lane-4: *C. angustifolia*-1

Lane-5: *C. angustifolia*-2

Lane-6: *C. aromatica*-1

Lane-7: *C. aromatica*-2

Lane-8: *C. decipiens*-1

Lane-9: *C. decipiens*-2

Lane-10: *C. malabarica*-1

Lane-11: *C. malabarica*-2

Lane-12: *C. raktakanta*-1

Lane-13: *C. raktakanta*-2

Lane-14: *C. zedoaria*-1

Lane-15: *C. zedoaria*-2

Lane-16: *C. longa*

Lane-17: 1 kb ladder

4.2.3.1 Cluster analysis

UPGMA cluster analysis grouped the 15 accessions into five major clusters shown as in the dendrogram (Figure 8). They are,

Cluster I- *C. malabarica*-1, *C. malabarica*-2, *C. raktakanta*-1, *C. raktakanta*-2

Cluster II- *C. zedoaria*-1, *C. zedoaria*-2

Cluster III- *C. amada*-1, *C. amada*-2, *C. angustifolia*-1, *C. angustifolia*-2, *C. aromatica*-1, *C. aromatica*-2

Cluster IV- *C. longa*

Cluster V- *C. decipiens*-1, *C. decipiens*-2

4.2.3.2 Pearson correlation coefficient analysis

The Pearson correlation coefficient obtained using the ISSR binary scoring data are given in Table 18 and the intra and inter-specific variation among the selected accessions were thus estimated.

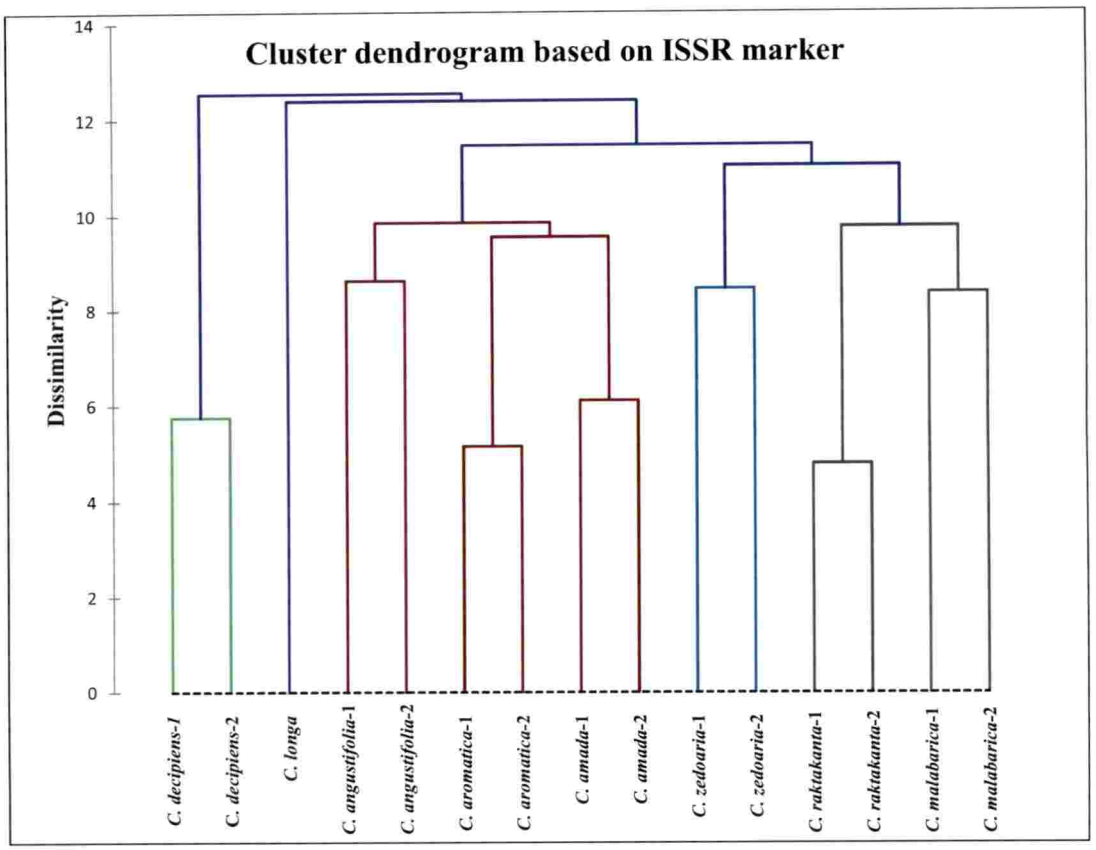


Figure 8 - Cluster dendrogram of 15 accessions in 8 species of *Curcuma* based on ISSR marker

Table 18 Pearson Correlation Coefficient based on ISSR scoring data

Accessions	*C. amada*-1	*C. amada*-2	*C. angustifolia*-1	*C. angustifolia*-2	*C. aromatica*-1	*C. aromatica*-2	*C. decipiens*-1	*C. decipiens*-2	*C. malabarica*-1	*C. malabarica*-2	*C. raktakanta*-1	*C. raktakanta*-2	*C. zedoaria*-1	*C. zedoaria*-2	*C. longa*
C. amada-1	**1**	0.7726	0.3741	0.5103	0.4011	0.4315	0.1006	0.1460	0.2478	0.2109	0.2688	0.2478	0.1944	0.2109	0.1376
C. amada-2	0.7726	**1**	0.3741	0.5103	0.5103	0.4315	0.1006	0.1460	0.2478	0.2109	0.2688	0.2478	0.1944	0.2109	0.1376
C. angustifolia-1	0.3741	0.3741	**1**	0.5466	0.3445	0.3741	0.0596	0.1056	0.1999	0.1631	0.2207	0.1999	0.0596	0.0740	0.1689
C. angustifolia-2	0.5103	0.5103	0.5466	**1**	0.3712	0.4011	0.0792	0.2226	0.2226	0.1858	0.2435	0.2226	0.1693	0.1858	0.1121
C. aromatica-1	0.4011	0.5103	0.3445	0.3712	**1**	0.8378	0.0792	0.1249	0.2226	0.1858	0.2435	0.2226	0.0792	0.0935	0.1952
C. aromatica-2	0.4315	0.4315	0.3741	0.4011	0.8378	**1**	0.1006	0.1460	0.2478	0.2109	0.2688	0.2478	0.1006	0.1147	0.2242
C. decipiens-1	0.1006	0.1006	0.0596	0.0792	0.0792	0.1006	**1**	0.7978	0.0415	0.1674	-0.0272	0.0415	-0.1614	-0.1501	-0.1325
C. decipiens-2	0.1460	0.1460	0.1056	0.2226	0.1249	0.1460	0.7978	**1**	-0.0033	0.0560	0.0114	0.0879	-0.1266	-0.1163	-0.0878
C. malabarica-1	0.2478	0.2478	0.1999	0.2226	0.2226	0.2478	0.0415	-0.0033	**1**	0.5730	0.4827	0.4527	0.2936	0.2284	-0.0103
C. malabarica-2	0.2109	0.2109	0.1631	0.1858	0.1858	0.2109	0.1674	0.0560	0.5730	**1**	0.3411	0.4007	0.1674	0.1863	-0.0450
C. raktakanta-1	0.2688	0.2688	0.2207	0.2435	0.2435	0.2688	-0.0272	0.0114	0.4827	0.3411	**1**	0.8597	0.3202	0.3411	0.0087
C. raktakanta-2	0.2478	0.2478	0.1999	0.2226	0.2226	0.2478	0.0415	0.0879	0.4527	0.4007	0.8597	**1**	0.2096	0.3145	-0.0103
C. zedoaria-1	0.1944	0.1944	0.0596	0.1693	0.0792	0.1006	-0.1614	-0.1266	0.2936	0.1674	0.3202	0.2096	**1**	0.5642	-0.0611
C. zedoaria-2	0.2109	0.2109	0.0740	0.1858	0.0935	0.1147	-0.1501	-0.1163	0.2284	0.1863	0.3411	0.3145	0.5642	**1**	-0.1182
C. longa	0.1376	0.1376	0.1689	0.1121	0.1952	0.2242	-0.1325	-0.0878	-0.0103	-0.0450	0.0087	-0.0103	-0.0611	-0.1182	**1**

4.2.4 SSR analysis of *Curcuma* accessions

After the completion of PCR using selected SSR primers, the amplicons were resolved in 2% agarose gel and obtained amplicons are shown in Figures 9-12. A total of 135 scorable bands were produced out of which 123 were polymorphic (91.11%). The SSR primers used, the number of bands produced by each primer, number of polymorphic bands and percentage polymorphisms are shown in Table 19.

Table 19 PCR analysis using ISSR primers

Sl. No.	Primer	Total number of bands	Number of polymorphic bands	Percentage polymorphism
1	CuMiSat-19	19	19	100.00
2	CuMiSat-20	24	23	95.83
3	CuMiSat-23	8	6	75.00
4	CuMiSat-24	20	17	85.00
5	CuMiSat-25	13	12	92.30
6	CuMiSat-26	20	19	95.00
7	CuMiSat-28	31	27	87.09
	Total	**135**	**123**	**91.11**

4.2.4.1 Cluster analysis

UPGMA cluster analysis grouped the fifteen accessions into six major clusters shown as in the dendrogram (Figure 13). They are,

Cluster I- *C. amada*-1, *C. amada*-2, *C. angustifolia*-1, *C. angustifolia*-2

Cluster II- *C. aromatica*-1, *C. aromatica*-2

Cluster III- *C. malabarica*-1, *C. malabarica*-2, *C. raktakanta*-1, *C. raktakanta*-2

Cluster IV- *C. zedoaria*-1, *C. zedoaria*-2

Cluster V- *C. longa*

Cluster VI- *C. decipiens*-1, *C. decipiens*-2

Molecular characterization using SSR markers

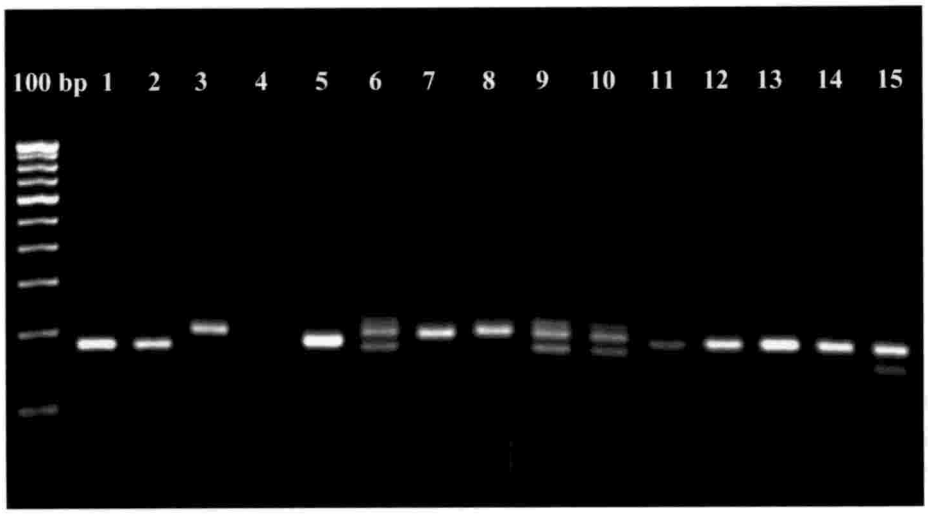


Figure 9 - Agarose gel (2%) profile of the SSR primer CuMiSat-19 for 15 accessions of *Curcuma*

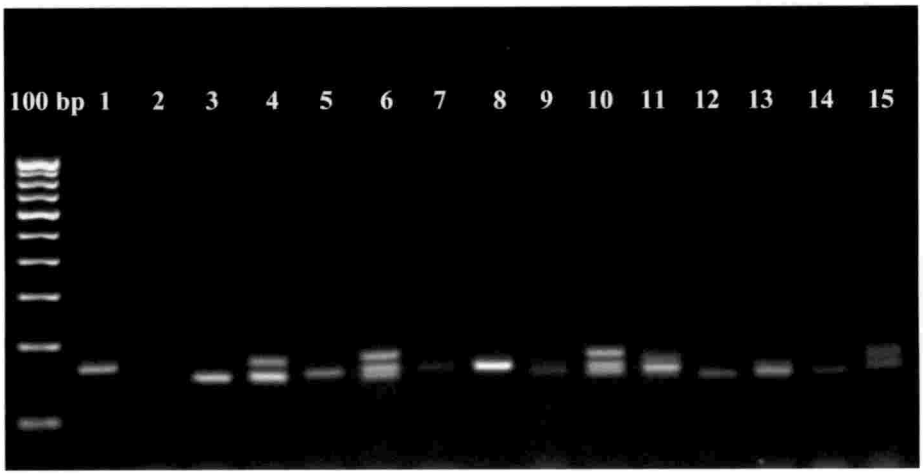


Figure 10 - Agarose gel (2%) profile of the SSR primer CuMiSat-20 for 15 accessions of *Curcuma*

Lane-1: 100 bp ladder
Lane-2: *C. amada*-1
Lane-3: *C. amada*-2
Lane-4: *C. angustifolia*-1
Lane-5: *C. angustifolia*-2
Lane-6: *C. aromatica*-1
Lane-7: *C. aromatica*-2
Lane-8: *C. decipiens*-1
Lane-9: *C. decipiens*-2
Lane-10: *C. malabarica*-1
Lane-11: *C. malabarica*-2
Lane-12: *C. raktakanta*-1
Lane-13: *C. raktakanta*-2
Lane-14: *C. zedoaria*-1
Lane-15: *C. zedoaria*-2
Lane-16: *C. longa*

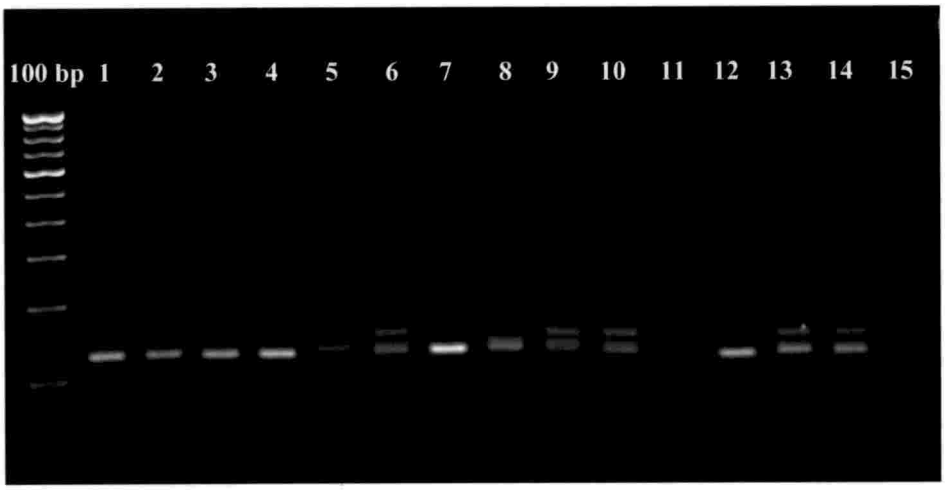


Figure 11 Agarose gel (2%) profile of the SSR primer CuMiSat-24 for 15 accessions of *Curcuma*

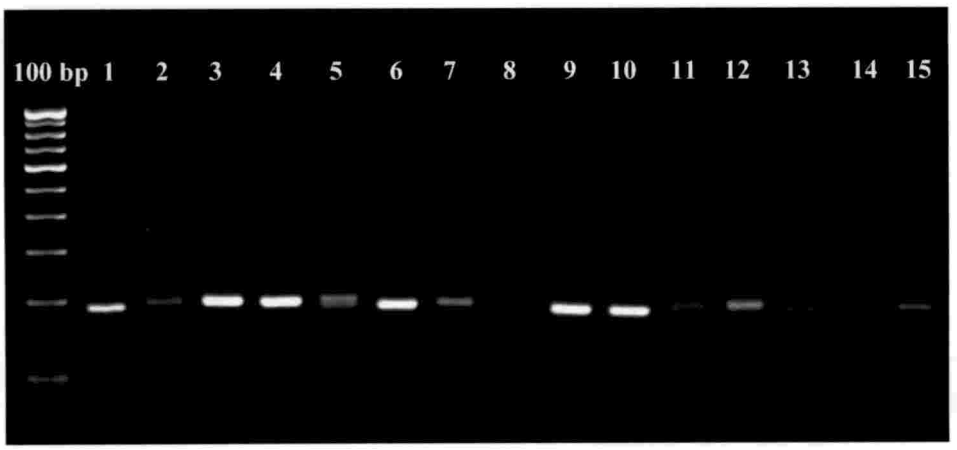


Figure 12 - Agarose gel (2%) profile of the SSR primer CuMiSat-26 for 15 accessions of *Curcuma*

Lane-1: 100 bp ladder
Lane-2: *C. amada*-1
Lane-3: *C. amada*-2
Lane-4: *C. angustifolia*-1
Lane-5: *C. angustifolia*-2
Lane-6: *C. aromatica*-1
Lane-7: *C. aromatica*-2
Lane-8: *C. decipiens*-1
Lane-9: *C. decipiens*-2
Lane-10: *C. malabarica*-1
Lane-11: *C. malabarica*-2
Lane-12: *C. raktakanta*-1
Lane-13: *C. raktakanta*-2
Lane-14: *C. zedoaria*-1
Lane-15: *C. zedoaria*-2
Lane-16: *C. longa*

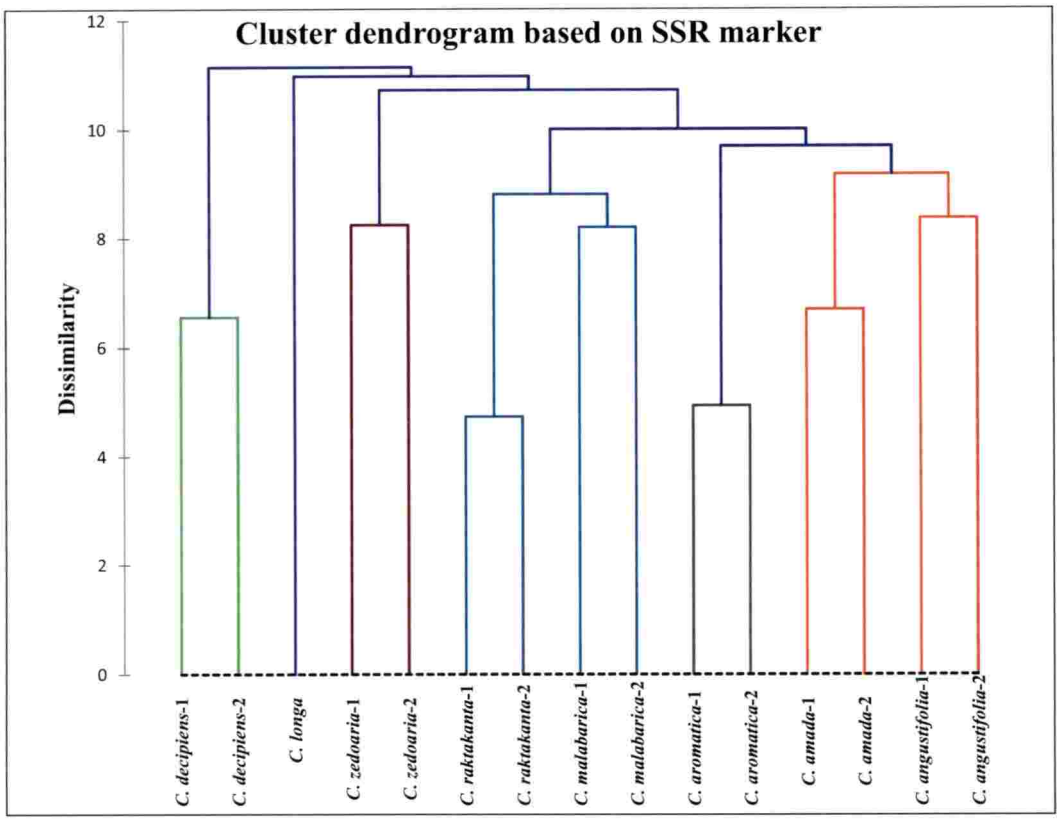


Figure 13 - Cluster dendrogram of 15 accessions in 8 species of *Curcuma* based on SSR marker

4.2.4.2 Pearson correlation coefficient (PCC) analysis

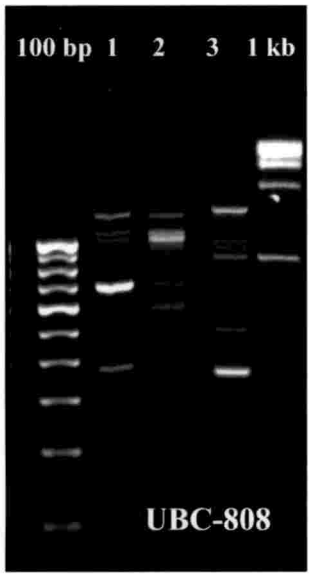
Table 20 Pearson Correlation Coefficient based on SSR scoring data

Accessions	*C. amada*-1	*C. amada*-2	*C. angustifolia*-1	*C. angustifolia*-2	*C. aromatica*-1	*C. aromatica*-2	*C. decipiens*-1	*C. decipiens*-2	*C. malabarica*-1	*C. malabarica*-2	*C. raktakanta*-1	*C. raktakanta*-2	*C. zedoaria*-1	*C. zedoaria*-2	*C. longa*
C. amada-1	1	0.6655	0.2917	0.4771	0.2350	0.2615	-0.0365	0.0083	0.2114	0.1707	0.2350	0.2114	0.1527	0.1707	0.1360
C. amada-2	0.6655	1	0.2615	0.4389	0.4505	0.3597	0.0641	0.1268	0.2998	0.2516	0.3280	0.2998	0.2304	0.2516	0.1062
C. angustifolia-1	0.2917	0.2615	1	0.4771	0.2350	0.2615	-0.0365	0.0083	0.2114	0.1707	0.2350	0.2114	0.0398	0.0546	0.2461
C. angustifolia-2	0.4771	0.4389	0.4771	1	0.2693	0.2961	0.1140	0.1767	0.2454	0.2045	0.2693	0.2454	0.1866	0.2045	0.1699
C. aromatica-1	0.2350	0.4505	0.2350	0.2693	1	0.8181	0.0431	0.1061	0.2686	0.2205	0.2966	0.2686	0.0967	0.1149	0.1797
C. aromatica-2	0.2615	0.3597	0.2615	0.2961	0.8181	1	0.0641	0.1268	0.2998	0.2516	0.3280	0.2998	0.1231	0.1412	0.2109
C. decipiens-1	-0.0365	0.0641	-0.0365	0.1140	0.0431	0.0641	1	0.8312	0.1324	0.2942	0.0431	0.1324	-0.1249	-0.1120	-0.1374
C. decipiens-2	0.0083	0.1268	0.0083	0.1767	0.1061	0.1268	0.8312	1	0.2114	0.2868	0.1061	0.2114	-0.0730	-0.0615	-0.0842
C. malabarica-1	0.2114	0.2998	0.2114	0.2454	0.2686	0.2998	0.1324	0.2114	1	0.4973	0.4942	0.4577	0.1714	0.0911	0.0552
C. malabarica-2	0.1707	0.2516	0.1707	0.2045	0.2205	0.2516	0.2942	0.2868	0.4973	1	0.3261	0.3958	0.0299	0.0490	0.0119
C. raktakanta-1	0.2350	0.3280	0.2350	0.2693	0.2966	0.3280	0.0431	0.1061	0.4942	0.3261	1	0.8324	0.1993	0.2205	0.0795
C. raktakanta-2	0.2114	0.2998	0.2114	0.2454	0.2686	0.2998	0.1324	0.2114	0.4577	0.3958	0.8324	1	0.0726	0.1927	0.0552
C. zedoaria-1	0.1527	0.2304	0.0398	0.1866	0.0967	0.1231	-0.1249	-0.0730	0.1714	0.0299	0.1993	0.0726	1	0.4924	-0.0077
C. zedoaria-2	0.1707	0.2516	0.0546	0.2045	0.1149	0.1412	-0.1120	-0.0615	0.0911	0.0490	0.2205	0.1927	0.4924	1	-0.0783
C. longa	0.1360	0.1062	0.2461	0.1699	0.1797	0.2109	-0.1374	-0.0842	0.0552	0.0119	0.0795	0.0552	-0.0077	-0.0783	1

4.2.5 Reproducibility test for ISSR and SSR markers

This was to confirm whether the reproducibility of banding pattern of DNA. Both ISSR (UBC-808 and 811) and SSR primers (CuMiSat-19 and CuMiSat-26) were found to be reproducible and it is shown in the Plate 12 (Figure 14 and 15).

Reproducibility test for ISSR and SSR markers



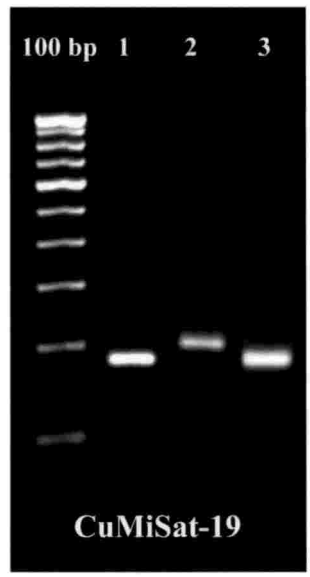


Figure 14 & 15 - Agarose gel (2%) profile of the ISSR primer UBC 808 and SSR primer CuMiSat-19 for 3 selected accessions of *Curcuma*

Lane-1: 100 bp ladder

Lane-2: *C. amada*-1

Lane-3: *C. angustifolia*-1

Lane-4: *C. aromatica*-1

Lane-5: 1 kb ladder

Lane-1: 100 bp ladder

Lane-2: *C. amada*-1

Lane-3: *C. angustifolia*-1

Lane-4: *C. aromatica*-1

4.2.6 Pearson Correlation Coefficient (PCC) for combined ISSR and SSR data

In order to conclude the molecular characterization results, PCC was again calculated by combining the scoring data of both ISSR and SSR marker (Table 21).

Table 21 Combined PCC values

Accessions	*C. amada*-1	*C. amada*-2	*C. angustifolia*-1	*C. angustifolia*-2	*C. aromatica*-1	*C. aromatica*-2	*C. decipiens*-1	*C. decipiens*-2	*C. malabarica*-1	*C. malabarica*-2	*C. raktakanta*-1	*C. raktakanta*-1	*C. zedoaria*-1	*C. zedoaria*-2	*C. longa*
C. amada-1	1	0.7388	0.3487	0.4893	0.3617	0.3901	0.0641	0.0979	0.2719	0.2333	0.2940	0.2719	0.2081	0.2245	0.1071
C. amada-2	0.7388	1	0.3337	0.4715	0.5103	0.4315	0.1006	0.1377	0.3098	0.2683	0.3337	0.3098	0.2413	0.2590	0.0944
C. angustifolia-1	0.3487	0.3337	1	0.5293	0.3061	0.3337	0.0250	0.0595	0.2211	0.1829	0.2429	0.2211	0.0692	0.0835	0.1410
C. angustifolia-2	0.4893	0.4715	0.5293	1	0.3323	0.3602	0.0437	0.1760	0.2451	0.2067	0.2670	0.2451	0.1815	0.1980	0.0822
C. aromatica-1	0.3617	0.5103	0.3061	0.3323	1	0.8378	0.0792	0.1166	0.2825	0.2413	0.3061	0.2825	0.1243	0.1396	0.1537
C. aromatica-2	0.3901	0.4315	0.3337	0.3602	0.8378	1	0.1006	0.1377	0.3098	0.2683	0.3337	0.3098	0.1475	0.1628	0.1809
C. decipiens-1	0.0641	0.1006	0.0250	0.0437	0.0792	0.1006	1	0.8191	0.0931	0.2176	0.0250	0.0931	-0.1227	-0.1105	-0.1682
C. decipiens-2	0.0979	0.1377	0.0595	0.1760	0.1166	0.1377	0.8191	1	0.0883	0.1411	0.0595	0.1339	-0.0912	-0.0797	-0.1338
C. malabarica-1	0.2719	0.3098	0.2211	0.2451	0.2825	0.3098	0.0931	0.0883	1	0.5604	0.5137	0.4820	0.2639	0.1962	-0.0009
C. malabarica-2	0.2333	0.2683	0.1829	0.2067	0.2413	0.2683	0.2176	0.1411	0.5604	1	0.3665	0.4273	0.1371	0.1550	-0.0366
C. raktakanta-1	0.2940	0.3337	0.2429	0.2670	0.3061	0.3337	0.0250	0.0595	0.5137	0.3665	1	0.8549	0.2902	0.3101	0.0187
C. raktakanta-1	0.2719	0.3098	0.2211	0.2451	0.2825	0.3098	0.0931	0.1339	0.4820	0.4273	0.8549	1	0.1785	0.2837	-0.0009
C. zedoaria-1	0.2081	0.2413	0.0692	0.1815	0.1243	0.1475	-0.1227	-0.0912	0.2639	0.1371	0.2902	0.1785	1	0.5642	-0.0611
C. zedoaria-2	0.2245	0.2590	0.0835	0.1980	0.1396	0.1628	-0.1105	-0.0797	0.1962	0.1550	0.3101	0.2837	0.5642	1	-0.1182
C. longa	0.1071	0.0944	0.1410	0.0822	0.1537	0.1809	-0.1682	-0.1338	-0.0009	-0.0366	0.0187	-0.0009	-0.0611	-0.1182	1

4.2.7 Intra and inter-specific similarity

The intra and inter-specific genetic similarity among the selected accessions using the molecular characterization data are shown in the table below (Table 22). Corresponding PCC values are also included.

Table 22 Comparison of results of ISSR and SSR molecular marker characterization

Marker	Similarity	Intra-specific	Inter-specific	With *C. longa*
ISSR	Highest	*C. raktakanta*-1 *C. raktakanta*-2 (0.86)	*C. aromatica*-1 *C. amada*-2 (0.51)	*C. aromatica*-2 (0.22)
	Lowest	*C. angustifolia*-1 *C. angustifolia*-2 (0.55)	*C. decipiens*-1 *C. raktakanta*-1 (-0.027)	*C. decipiens*-1 (-0.13)
SSR	Highest	*C. raktakanta*-1 *C. raktakanta*-2 (0.83)	*C. malabarica*-1 *C. raktakanta*-1 (0.49)	*C. angustifolia*-1 (0.25)
	Lowest	*C. angustifolia*-1 *C. angustifolia*-2 (0.47)	*C. decipiens*-2 *C. raktakanta*-1 (0.043)	*C. decipiens*-1 (-0.13)
ISSR and SSR combined	Highest	*C. raktakanta*-1 *C. raktakanta*-2 (0.85)	*C. malabarica*-1 *C. raktakanta*-1 (0.51)	*C. aromatica*-2 (0.18)
	Lowest	*C. angustifolia*-1 *C. angustifolia*-2 (0.53)	*C. decipiens*-1 *C. raktakanta*-1 (0.025)	*C. decipiens*-1 (-0. 16)

4.3 CORRELATION OF MORPHOLOGICAL AND MOLECULAR DATA

Mantel's test was performed to observe the correlation between morphological and molecular data. The values obtained in Mantel's test was $r = 0.4133$ and $p = <0.0001$. As the computed p-value was lower than the significance level alpha = 0.05, the alternative hypothesis was accepted i.e. a positive correlation existed between the morphological and the molecular characterization data.

5. DISCUSSION

Tropical root and tuber crops form an important staple food source for millions in the tropical and sub-tropical countries. These crops produce high level of calories and carbohydrates besides many vitamins and minerals. They can withstand adverse biotic and abiotic conditions. The major tropical root crops are cassava, sweet potato, yams and aroids. In addition to these major tuber crops there are many types of tuberous and rhizomatous minor root crops such as Chinese potato, Canna, arrowroot, *Curcuma* spp. which are utilized in different parts of the world. Various studies have uncovered the starch attributes of tuber crops and the likelihood of using these natural starches instead of chemically modified starches (Hoover, 2001).

Many of the *Curcuma* species are used for starch extraction. *Curcuma* starches are believed to have medicinal value. As an example, they are used in the treatment of gastric ailments and as an infant food. It has also been reported to have similar digestive properties as arrowroot starch. *C. angustifolia, C. malabarica* and *C. zedoaria* are widely cultivated in Kozhikode and Malappuram districts of Kerala for the extraction of starch and manufacturing value added products. *Curcuma* spp. have high starch value yield potential and they are yet to be appropriately explored. The characterization *Curcuma* sp. is very much needed to assess the extent of genetic variation within and between the species and thus can be utilized in the crop improvement programmes.

In the present study 15 accessions in 8 species of *Curcuma* collected from India maintained in the field gene bank of ICAR-CTCRI, were characterized using morphological and molecular markers (ISSR/SSR).

5.1 Morphological characterization

Morphological characterization is an indispensable step for the fruitful utilization of crop germplasm. The scientific classification of the plant still depends on morphological traits. Furthermore, this technique is easier, cost effective, easy

to score and requires less time and finally it does not need any technical knowledge (Din *et al.*, 2010).

A wide variability was observed among the 15 accessions of *Curcuma* species studied for the morphological characters. Intra and inter-specific morphological characterization and diversity analysis is reported for the first time in these selected species accessions. Selected morphological descriptors of Ravindran *et al.* (2007) and Sasikumar (2005) were efficient in characterizing and differentiating selected accessions into various clusters. Accessions of the same species have shown more than 83% similarity except *C. angustifolia* (52%). *C. angustifolia*-2 have shown intra-specific variability in the key characters such as pseudostem anthocyanin pigmentation, leaf mid rib colour and rhizome flesh colour from *C. angustifolia*-1. In a clump of *C. angustifolia*-2, leaves with both purple and green midrib colour were observed. This notable intra-specific variation may be due to the effect of the environment or due to the species dissimilarity. In the current study, the leaf midrib colour of *C. raktakanta* accessions were observed as dark purple, but this was in non-congruence with the study of Syamkumar (2008) reported as green.

Leaf texture of *C. amada* accessions in the present study was pubescent i.e. in non-congruence with the report of Syamkumar (2008). However, some studies had reported that lower side of the leaves were pubescent (Sabu, 1991 and Sabu, 2006).

Several authors had reported that, a key character in characterizing the *Curcuma* is its inflorescence (Syamkumar, 2008; Velayudhan *et al.*, 1999; Yusuf *et al.*, 2016). *Curcuma* species are perennial plants that show high irregularity in flowering behaviour and are shy flowering (Nayak, 2000; Lebot, 2009). In the present study, only *C. angustifolia*-2, *C. decipiens*-1 and *C. decipiens*-2 were flowered. Most of these accessions were planted in the field gene bank just before the start of this study. In addition, Pathak *et al.* (1960) reported that flowering may vary depending on the species and climatic conditions. These may be the reasons behind the flowering irregularity/absence. So floral characters were omitted in this

study. Otherwise, it may have resulted in the higher variability of these 3 accessions from other 11 accessions.

Curcuma raktakanta accessions have shown highest intra-specific similarity (94%). *C. decipiens* accessions were found to be the highly variable from the most commonly exploited species *C. longa* and *C. aromatica* has shown highest similarity to *C. longa*. This result was in consonance with the report of Syamkumar (2008).

Principal component analysis showed that the characters such as leaf midrib colour, rhizome flesh colour, leaf texture and aroma of rhizome have contributed mostly to the variability among accessions. Component-1 has shown maximum variation (40.27%). This was in congruence with Syamkumar (2008).

5.2 Molecular characterization

Compared to DNA fingerprinting techniques, morphological traits are relatively less reliable and less efficient for accurate discrimination of closely related accessions and analysis of their genetic relationships (Beyene *et al.*, 2005). Yusuf *et al.* (2016) also reported that the evaluation of the morphological characters alone in species characterization has its own limitations for the genus *Curcuma*. Molecular marker may be useful as an adjunct to morphological taxonomic tools. These findings thus deduce the unreliability of solely relying on the conventional taxonomic tools in discriminating *Curcuma* species.

CTAB method of Syamkumar (2008) was used to isolate DNA from 15 accessions of *Curcuma* species. Good quality DNA were obtained using this protocol. ISSR and SSR markers have been applied to estimate the genetic diversity and relationships in a number of crop species (Bart *et al.*, 2002; Gomes *et al.*, 2009; Leal *et al.*, 2010). SSRs are usually codominant, reproducible, and easily detected by PCR. In the present study, ISSR and SSR markers were selected based on the previous studies in *Curcuma* (Saha *et al.*, 2016; Senan *et al.*, 2013). Annealing temperature used for the preliminary screening of both ISSR and SSR primers were same as shown in the literatures referred. Scoring of the PCR product was done

based on presence (1) or absence (0) of bands. Some of primers gave 100% polymorphism. Most of the primers used in the study have shown high polymorphism. This suggested that both ISSR and SSR markers are efficient tools in intra and inter-specific characterization of *Curcuma* species and they could be employed in future genetic variability studies.

Pearson Correlation Coefficients (PCC) were obtained for both ISSR and SSR markers and later they were combined to form a single similarity coefficient. PCC data has shown that a less variation exists within accessions of same species and a high variation exists between accessions of different species. *C. angustifolia*-1 was found to be highly variable from *C. angustifolia*-2. The intra-specific similarity among *C. raktakanta*-1 and *C. raktakanta*-2 were found to be highest than all other accession pair. The same has been observed in the morphological data analysis also. The dendrogram constructed based on the combined scoring data of both ISSR and SSR markers did not vary much from the individual dendrograms. *C. decipiens*-1 and *C. decipiens*-2 clustered as a different group in both ISSR and SSR clusters. This was in congruence with the report of Syamkumar (2008).

DNA was isolated again from 3 accessions and the reproducibility of ISSR and SSR markers were proved by obtaining similar sized banding pattern. This test confirmed that the DNA markers were not affected by environmental factors or the developmental stage of the plant (Winter and Kahl, 1995).

5.3 Comparison between ISSR and SSR data

In both the dendrograms (ISSR and SSR), clustering results were same in all the species except *C. aromatica* accessions which were grouped into separate clusters in SSR dendrogram. Intra-specific similarity was highest among *C. raktakanta* and lowest between *C. angustifolia* in both ISSR and SSR characterization.

5.4 Comparison between morphological and molecular data

A comparison of the dendrograms constructed based on the key morphological characters (Pearson Correlation Coefficients) and the combined

ISSR and SSR marker (PCCs) showed congruence though there was little dissimilarity between the two dendrograms. In the cluster-I of the dendrogram constructed using the key morphological characters and combined molecular markers (ISSR and SSR), *C. decipiens* accessions were clustered as a separate group. In the cluster-IV of both the dendrograms (morphological and molecular), accessions of *C. malabarica* and *C. raktakanta* were clustered together which showed their highest inter-specific similarity among the 8 species.

The congruence or non-congruence between the morphological and molecular clustering of the selected accessions of *Curcuma* species germplasm can be explained as a degree of correlation between the molecular markers and the morphological characters in study. The present study revealed a positive correlation between both the morphological and molecular data.

6. SUMMARY

The genus *Curcuma* comprises of about 120 species of rhizomatous herbs of which 40 species were reported from India and the greatest diversity of the genus occurs in India. *Curcuma* is now attaining universal importance as a mighty cure to counter a variety of sicknesses and as a source of starch besides its known use as a spice. The scientific classification of plants is based on morphological characteristics, but are said to be sometimes inadequate. Molecular markers can be used as an adjunct in the characterization. The present study was thus an attempt to characterize the selected accessions of *Curcuma* species germplasm using morphological and molecular markers.

Fifteen selected accessions of eight species of *Curcuma* were morphologically characterized based on both qualitative and quantitative traits of above ground vegetative characters and below ground tuber characters. Dendrograms constructed based on binary scoring data divided the accessions into 4 major groups. As per Pearson Correlation Coefficient, maximum intra-specific similarity was observed between *C. raktakanta accessions* and maximum inter-specific similarity was observed between *C. amada*-2 and *C. aromatica*-1. PCA analysis revealed that the characters like leaf midrib colour, leaf texture, rhizome flesh colour, aroma of rhizome, rhizome flavour/taste contributed more towards the genetic variability among the accessions *Curcuma*.

Genomic DNA was isolated using the modified CTAB method yielded good quality DNA. The yield of DNA ranged from 312.62 ng/µl to 2236 ng/µl with A_{260}/A_{280} ratio ranged from 1.81 to 1.95.

Two molecular markers viz., ISSR and SSR were used for the molecular characterization. 10 ISSR primers produced a total of 176 bands out of which 166 were polymorphic (94.31%) and 7 SSR primers produced a total of 135 bands out of which 123 were polymorphic (91.11%). The bands produced were found to be reproducible. Dendrograms were constructed separately for both primers using the binary scoring data. In dendrograms and PCC analysis of both the primers, maximum intra-specific similarity was observed between *C. raktakanta* accessions

and maximum inter-specific similarity was observed between *C. malabarica*-1 and *C. raktakanta*-1. *C. decipiens*-1 and 2 were found to be highly variable from *C. longa*.

Mantel's test showed a positive correlation between both the morphological and molecular data.

The present study revealed that there exists a good genetic variability among the 15 accessions of *Curcuma*. The variability among the accessions within species was found to be less as compared to as between the variability among different species. Moreover, it is proved by the study that the morphological as well as the molecular tools were found to be very effective in the characterization of germplasm of *Curcuma* species, which in turn will be useful in the developement of core collections and for further use in the crop improvement programmes.

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APPENDIX I

CTAB extraction buffer

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

APPENDIX II

TE buffer (10 X)

Tris HCl (pH 8.0)	10 mM
EDTA	1 mM

APPENDIX III

TBE buffer (10 X)

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 autoclave before use.

APPENDIX IV

Wash solution

Ammonium acetate	15 mM
Ethanol	70%

APPENDIX V

Chloroform: Isoamyl alcohol

Chloroform	24 ml
Isoamyl alcohol	1 ml

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

APPENDIX VI

70% ethanol

100% ethanol	70 ml
Distilled water	30 ml

CHARACTERIZATION OF SELECTED *CURCUMA* SPECIES GERMPLASM USING MORPHOLOGICAL AND MOLECULAR MARKERS

By

BIMAL THOMAS

(2013-09-101)

Abstract of Thesis

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ABSTRACT

Curcuma L., a perennial rhizomatous herb, is gaining global importance as a source of starch besides its medicinal property and use as a spice. Characterization of germplasm is very essential in crop plants and it is the basis for selection of accessions for use in crop improvement programmes. This research work was an attempt to characterize the fifteen selected accessions in eight species of *Curcuma* collected from different parts of India and maintained in the field gene bank of ICAR-CTCRI using morphological and molecular markers. Two accessions in each of *C. amada, C. angustifolia, C. aromatica, C. decipiens, C. malabarica, C. raktakanta, C. zedoaria* and one of *C. longa* were selected. These 15 accessions were morphologically characterized using 13 qualitative and 15 quantitative traits and a wide variability was observed. Dendrogram based on the morphological characters grouped the genotypes into four clusters. PCC analysis revealed that the accessions of the same species have shown more than 83% similarity except *C. angustifolia*. *C. raktakanta* accessions have shown a highest intra-specific similarity of 94%. *C. decipiens* accessions were found to be the highly variable from the most commonly exploited species *C. longa* while *C. aromatica* has shown highest similarity. PCA showed that the characters such as leaf midrib colour, rhizome flesh colour, leaf texture and aroma of rhizome have contributed mostly to the variability. Molecular characterization was done using 10 ISSR and 7 SSR markers. The total percentage polymorphism obtained by ISSR characterization was 94.31 while it was 91.11 percentage in the SSRs. *C. angustifolia*-1 was found to be highly variable from *C. angustifolia*-2 suggested the occurrence of intra-specific variability. The intra-specific similarity among *C. raktakanta* accessions were found to be highest than all other accession pairs. Clustering based on ISSR markers grouped the genotypes into five clusters while SSRs into six clusters. Mantel's test showed a positive correlation between the morphological and molecular data. The results of the present study indicated that the morphological as well as the molecular tools were found to be very effective in the characterization of germplasm of *Curcuma* species for the developement of core collections and for further use in the crop improvement programmes.