DIVERSITY AND POPULATION STRUCTURE ANALYSIS IN COCONUT (*Cocos nucifera* L.) USING MOLECULAR MARKERS

By

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(2010-11-112)

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COLLEGE OF HORTICULTURE

VELLANIKKARA, THRISSUR - 680 656

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THESIS

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CENTRE FOR PLANT BIOTECHNOLOGY AND MOLECULAR BIOLOGY
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2012

DECLARATION

I hereby declare that the thesis entitled **"Diversity and population structure analysis in coconut (*Cocos nucifera* L.) using molecular markers "** is a bonafide record of research work done by me during the course of research and that it has not been previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other University or Society.

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CERTIFICATE

Certified that the thesis entitled **"Diversity and population structure analysis in coconut (*Cocos nucifera* L.) using molecular markers**" is a record of research work done independently by **Ms. Renju S**. under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, associateship, fellowship to her.

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Renju S

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LIST OF ABREVIATIONS

A	Adenine
AFLP	Amplified Fragment Length Polymorphism
bp	Base pairs
β	Beta
C	Cytosine
COD	Chowghat Orange Dwarf
CGD	Chowghat Green Dwarf
cm	Centimeter
cv	Cultivar
CPBMB	Centre for Plant Biotechnology and Molecular biology
CTAB	Cetyl Trimethyl Ammonium Bromide
^{0}C	Degree Celsius
DNA	Deoxyribo Nucleic Acid
DMSO	Dimethyl sulfoxide
dNTPs	Deoxyribo Nucleoside Triphosphate
EDTA	Ethylene Diamine Tetra Acetic acid
g	Gram
G	Guanine
GB	Gangabondam
ISSR	Inter Simple Sequence Repeats
Kb	Kilo basepairs
KAU	Kerala Agricultural university
KD	Kasaragode
KO	Komadan
KT	Kuttiadi
L	Litre
LO	Laccadive Ordinary
mA	Milli Ampere

Mb	Mega base pairs
Min	Minutes
MP	Malappuram
MYD	Malayan Yellow Dwarf
ml	Millilitre
µg	Microgram
µl	Microlitre
ng	Nanogram
OD	Optical Density
pH	Hydrogen ion concentration
PCR	Polymerase Chain Reaction
PIC	Polymorphism Information Content
PVP	Poly vinyl pyrolidine
%	Percentage
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
rpm	Rotations per minute
SSR	Simple Sequence Repeats
sec	Second (s)
T	Thymine
TAE	Tris Acetate EDTA
TE	Tris EDTA
TI	Tiptur Tall
U	Unit
UPGMA	unweighted pair group method of arithmetic mean
UV	Ultra violet
V	Volts
v/v	Volume by Volume
w/v	Weight by Volume
WCT	West Coast Tall

Introduction

1. INTRODUCTION

The coconut palm (*Cocos nucifera* L.) which is popularly known as "Kalpavriksha" is one of the most remunerative and important palms of the world with recorded history of more than two thousand years. It plays a significant role in the economic, cultural, and social life of over eighty tropical countries. The palm is reckoned as nature's greatest gift to mankind and is lauded by different names as tree of heaven, the tree of abundance, nature's super market, king of palms and the tree of life. Regarding the importance of coconut palm in the world, Asian and Pacific Coconut Community (APCC) is celebrating 2nd September as 'World Coconut Day'.

India is one of the leading coconut producing countries in the world occupying third position in production (15729.75 Million nuts). Kerala ranks first in area and production of coconut in India. Ninety per cent of the area under coconut in India is in the four southern states of Kerala, Tamil Nadu, Karnataka and Andhra Pradesh.

Coconut is a member of the monocotyledonous family Arecaceae (Palmae). It is the only species of the genus cocos belonging to the subfamily Cocoideae which includes 27 genera and 600 species. Coconut is botanically classified into two major groups based on its stature as Talls and Dwarfs. Intermediate form of coconut also exists. The Talls are generally cross fertilized and the Dwarfs are self fertilized. Hybrids between the two forms display pronounced heterosis (Thampan, 1987).

Germplasm collections that contain significant amounts of genetic diversity within and among populations are the mainstay for crop improvement and genetic dissection of complex traits. At the Central Plantation Crops Research Institute (CPCRI), India, a large collection of coconut germplasm is being maintained. India is the site for the International Coconut Gene Bank for South Asia (ICGB-SA) and extensive germplasm collecting is underway to enrich the coconut germplasm centre. World's largest germplasm collection of coconut comprising of four hundred and one

accessions (two hundred and sixty nine indigenous and hundred and thirty two exotic) is being maintained at the institute (CPCRI, 2011).

Assessment of the genetic diversity present within a species is a prerequisite for future sustainable breeding efforts. Understanding genetic diversity, population structure, and the level and distribution of linkage disequilibrium (LD) in target populations is of great importance and a prerequisite for association mapping (Jin *et al*., 2010).

Reliable knowledge of the genetic diversity of breeding material is important in order to select parents for a new breeding cycle. The choice of parent selection in diversity studies is valuable because it is a means of creating useful variations in subsequent progenies. Diversity studies on these crops at their respective primitive levels led to the development of widely distributed cultivars and varieties with proven characteristics based on stability and adaptability of performance with consistent tolerance to adverse weather conditions and resistant to diseases (Aremu, 2011). Understanding genetic relationship within and among cultivars could increase hybrid vigour and reduce or avoid re-selection within existing germplasm.

The characterization and evaluation of coconut populations have relied mostly on morphological and agronomic traits (Sugimora *et al*., 1997). These procedures are time and labour consuming and do not provide an accurate measure of genetic diversity because many characters exhibit complex inheritance and are influenced by both environmental and genetic factors. Germplasm characterization based on morphological and biochemical methods have extensively been used for crop improvement.

Crop improvement programmes in coconut have several limitations such as the perennial nature of the crop, tall stature, long pre bearing period, prolonged development period of nuts, heterozygous nature and requirement of large area for experimentation and field study. Now, the rapid development of biotechnology allows

easy analysis of large number of loci distributed throughout the genome of the plants. Molecular markers have proven to be powerful tools in the assessment of genetic variation and in elucidation of genetic relationships within and among species (Chakravarthi and Naravaneni, 2006).

Molecular markers provide an important technology for evaluating levels and patterns of genetic diversity and population structure studies and have been utilized in a variety of plant species. Molecular markers such as Random Amplified Polymorphic DNA (RAPD), Inter Simple Sequence Repeats (ISSR), Simple Sequence Repeats (SSR), Amplified Fragment Length Polymophism (AFLP), Restriction Fragment Length Polymorphism (RFLP) etc. can be used to estimate genetic diversity and population structure between and among crop plants. These DNA based methods are very informative and attractive because of the immense number of characters they reveal and their capacity to perform with greater speed and accuracy throughout the life cycle independent of the environment.

Use of various marker techniques like RAPD (Upadhyay *et al*., 2004), AFLP (Perera *et al*., 1998) and SSRs (Teulat *et al*., 2000) has been reported in coconut for diversity analysis.

Keeping the above in view, the present study on diversity and population structure analysis in coconut (*Cocos nucifera* L.) using molecular markers was proposed with the following objectives:

1. To estimate the intra and inter population genetic variability of fifty genotypes belonging to ten coconut cultivars commonly used for seed production in Kerala, based on molecular marker analysis
2. To determine the genetic relationship and population structure of the fifty coconut genotypes

Review of Literature

2. REVIEW OF LITERATURE

The research programme entitled "Diversity and population structure analysis in coconut (*Cocos nucifera* L.) using molecular markers" was conducted at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara during the period 2010-2012. The study aimed to determine the diversity and population structure analysis of fifty coconut genotypes commonly used for seed production in Kerala based on molecular marker analysis. The relevant literatures on various aspects of the study are reviewed in this chapter.

2.1. Varietal variability

There exists considerable varietal diversity in coconut. Based on stature, coconut is broadly classified into the Talls and the Dwarfs, but an intermediate type also exists (Peter, 2002). Wide range of variation occur within the same variety in tall palms due to their cross pollinating nature. The variations arise in the height of the palm, colour, shape and size of the nuts as well as yield and quality of copra. In India, both tall and dwarf varieties are grown. Different indigenous types belonging to these varieties are also common.

2.1.1. Tall variety

The tall variety which is largely cross pollinating is being cultivated throughout the coconut tracts of the world. It has a long and stout trunk with a swollen base called 'bole'. This variety is characteristically tall, growing to a height of about 15-18 m. It is comparatively hardy, late bearing, and lives up to a ripe age of 80-90 years (Thampan, 1987).

West Coast Tall, Laccadive Ordinary, Andaman Ordinary, Kappadam etc. are some promising varieties of tall type. Komadan and Kuttiadi are two high yielding types of West Coast Tall (WCT) found in the state of Kerala. Komadan is an ecotype of WCT popular in Pathanamthitta district. In southern districts of Kerala, this ecotype is seen far

superior than WCT. Kuttiadi is seen in northern Kerala, particularly in Kozhikode and Kannur districts. Its performance is as good as Komadan.

2.1.1.1. Laccadive Ordinary

Laccadive Ordinary is a popular stress tolerant variety. This cultivar of Lakshadweep islands is almost similar to WCT in growth habits and characteristics, but gives a higher yield of 120 nuts per palm per year under normal management conditions. The palms of this type are considered very good for tapping sweet toddy as the yield of toddy is nearly twice the one obtained from West Coast Tall. It is commonly known by the name Chandrakalpa and is recommended for commercial cultivation in many states in India (Joseph, 2007).

2.1.1.2. Tiptur Tall

Tiptur Tall is a popular cultivar grown in Karnataka state. It resembles West Coast Tall in most of the morphological characters. This performs well in the low rainfall areas (50-64 cm) of Karnataka. The palm is a good yielder even under rainfed conditions and can be used for commercial planting. This cultivar is suitable for consumption as tender nut. The tender coconut water is tasty and sweet (Joseph, 2007).

2.1.2. Dwarf varieties

Dwarf varieties are seen in all countries where coconut is grown. They are believed to have formed due to mutation of tall varieties or as products of several generations of inbreeding in tall varieties. They are generally self pollinating, but cross pollination can occur to some extent. They are irregular or alternate bearer and are generally planted for ornamental purpose (Joseph, 2007).

2.1.2.1. Chowghat Orange Dwarf (COD)

Chowghat Orange Dwarf is seen in the West coast of India particularly in Kerala. This type is more robust than Chowghat Green Dwarf. Self pollination is the general rule, but cross pollination also takes place to a little extent. About eighty per

cent of progenies breed true to type. This cultivar is extensively used in the production of T x D and D x T hybrids in Kerala. It is sensitive to drought and water logging. This variety is released by Central Plantation Crops Research Institute (CPCRI) for tender coconut production, and is now cultivated in large areas (Joseph, 2007).

2.1.2.2. Chowghat Green Dwarf (CGD)

Chowghat Green Dwarf is also a dwarf variety seen extensively in the West coast, particularly in Kerala. Due to self pollination, this variety maintains cent per cent purity. This is made possible due to complete overlapping of male and female phases. WCT x CGD hybrids had a problem of buckling of bunches and fronds during summer months leading to heavy loss to the farmers (Joseph, 2007).

2.1.2.3. Malayan Yellow Dwarf (MYD)

Malayan Yellow Dwarf is believed to have originated in Java as a mutant and later introduced into Malaysia. Among Malayan dwarfs, red, yellow and green are available in Malaysia. Yellow dwarfs are extensively used in Kerala for hybrid seed nut production (Joseph, 2007).

2.1.3. Semi tall type

Semi tall type forms intermediate between the tall and dwarf types (Joseph, 2007).

2.1.3.1. Gangabondam

Gangabondam is from Andhra Pradesh, where it is grown in certain parts for tender nut purpose. This is a stout and early bearing in nature. It is widely used in the production of hybrid seed nuts in Kerala (Joseph, 2007).

2.2. Isolation of genomic DNA

One prerequisite to reliable molecular biology work is that the isolation of genomic DNA of good quality. The isolation of genomic DNA from coconut is quite

difficult because of the high polyphenol and polysaccharide content of its leaves. The polyphenols are released when tissues are wounded and leads to subsequent browning of leaves (Joslyn and Ponting, 1951). In their oxidized forms, polyphenols covalently bind to proteins and DNA, giving the DNA a brown color and making it useless for most research applications (Katterman and Shattuck, 1983; Guillemaut and Marechal-Drouard, 1992; Aljanabi *et al*., 1999).

Porebski *et al*. (1997) standardized DNA isolation protocol for plant material containing large quantities of polyphenols, tannins and polysaccharides. The method involved a modified CTAB extraction protocol, employing high salt concentrations to remove polysaccharides and use of polyvinyl pyrrolidone (PVP) to remove polyphenols. Average yield of DNA ranged from 20 to 84 µg/g of mature leaf tissue for both wild and cultivated octoploid and diploid *Fragaria* species. The method yielded considerably good quantity of DNA and it is consistently amplifiable in the PCR reaction with as little as 0.5 ng DNA per 25 µl reaction.

Angeles *et al*. (2005) conducted an experiment to determine which protocol to be used and which part of the coconut palm is most appropriate to extract good-quality genomic DNA. They tried the method of Dellaporta *et al*. (1983), some modified protocols of Cheung *et al*. (1993) and Datta *et al*. (1997). Genomic DNA from the solid endosperm was found to be of poor quality because of high levels of lipid and galactomannan contaminants. DNA extracted by modified Cheung *et al*. (1993) method from the young leaves of the first emergent frond provided enzyme digestible, good-quality DNA. The modification involved the use of a higher salt concentration (2 M instead of 0.5 M) in the extraction buffer and the use of polyvinyl polypyrrolidone and this modified protocol did not involve the use of organic solvents.

Sharma *et al*. (2008) modified DNA extraction method in tuber crops. This method followed inactivation of protein contaminants by using CTAB or proteinase K and precipitation of polysaccharides in presence of high concentration of salt.

Maltas *et al.* (2011) reported a method of DNA isolation from *Ginkgo biloba*. DNA extraction of *Ginkgo biloba* is quite difficult to work on because of the high phenolic and polysaccharide content of its leaves. The study aimed to determine which protocol to use and which part of *Ginkgo* tree is most appropriate to extract good-quality genomic DNA. They used cetyl trimethyl ammonium bromide protocol and protocol of commercially available kit by EZ1 Nucleic acid isolation system for extraction of genomic DNA from *G. biloba* leaves. Sufficient yields of high-quality amplifiable DNA was produced with kit by EZ1 Nucleic acid isolation method. The purified DNA has excellent spectral quality and was suitable for long-fragment PCR amplification with kit by EZ1 Nucleic acid isolation method.

2.3. Diversity analysis

Genetic diversity is usually measured as the amount of genetic variability among individuals of a variety, or population of a species (Brown, 1983). Although vast germplasm collections of coconut are accessible, their use in genetic improvement programme is limited because the access of the same is still a challenge. According to Spooner *et al*. (2005) diversity analysis enables plant breeders to select novel plant gene combinations and crop varieties more suited to the needs of diverse agricultural systems. Genetic diversity analysis poses several limitations. There are various methods for determining genetic diversity such as morphological, molecular and biochemical methods.

Genetic diversity using a combination of techniques, such as morphological and molecular markers have been conducted in potato (Fisher *et al*., 2008), banana (Mohamed, 2007), beans (Duran *et al*., 2005), globe artichoke (Crino *et al*., 2008) and wheat (Cox and Murphy, 1990; Vieira *et al*., 2007).

2.3.1. Morphometric diversity in coconut

Morphological markers generally correspond to the qualitative traits that can be scored visually. They have been found in nature or generated as the result of

mutagenesis. Morphological markers are usually dominant or recessive (Chawla, 2010). Studying morphometric variation of coconut can generate two kinds of results. The first is an evaluation of the agricultural potential of the cultivars tested; the second is a better understanding of the genetic diversity, in relation to results obtained with molecular markers.

Knowledge about the genetic diversity serves as a guide to choose the cultivars to be introduced in a breeding programme, to maximize selectable diversity and/or heterosis. Morphological traits are highly influenced by environment and hence, show significant level of genotype x environment interaction. This may be a serious hindrance to study genetic relationships of coconuts, particularly if we intend to compare results obtained from different places. The lack of such G x E interaction explains largely the success of molecular methods. On the other hand, it must be recognized that they would be useless without a good knowledge of the measurable and often agriculturally meaningful variation of phenotypic traits.

Ashburner *et al*. (1997a) made a study of the diversity in the coconut species using fruit component analysis on a representative sample from twenty nine distinct South Pacific populations in order to characterise the germplasm present in that region. A large diversity in fruit morphology in characters such as fruit weight, husk weight, endosperm weight, shell weight, water weight were found that ranged from populations exhibiting wild type characters in Central Pacific to populations displaying domesticated characteristics in Rennell Island, the Sikaiana Islands, the Marquesas Islands, and in Papua New Guinea. They found continuous variation in fruit morphology in these populations, and cluster analysis arbitrarily divided the continuum into discrete groups which were consistent with geographic affinities. The continuum displayed clinal variation from populations with small fruit and low husk content in the west to large fruit and more husk in the east of the region. Most populations consisted of a wide range of fruit morphology, from individuals expressing wild-type characters to those with

domestic type characters. The occurrence of both wild and domesticated populations was observed within the clinal variation.

Relations between morphology, genetic structure and geographical distribution of seventy six individuals of sago palm from different localities of Papua New Guinea were investigated by Kjaer *et al*. (2004). Quantitative morphological variables such as trunk length, diameter of the trunk, length of petiole, length of rachis, number of leaflets, length of longest leaflet, width of longest leaflet, thickness of petiole at first leaflet, width of petiole at first leaflet and number of green leaves in the crown etc. were studied. Most of the morphological variables in the study were mutually correlated, except length of petiole and length of trunk seemed to be less associated with other variables. The length of petiole was highly variable and length of trunk is almost independent of the remaining variables. From ordination diagram, it was revealed that the distances between populations were not reflected in the morphology in a readily interpretable manner. Variation in vegetative morhological characters is not related to underlying genetic variation in the sago palm.

Arunachalam *et al*. (2005) reported phenotypic diversity in two hundred and six individual coconut palms comprising of seven tall groups and four dwarf groups representing seven island territories. Seven traits relevant to wind tolerance, dry matter production and taxonomic discrimination known in palms were used in the study. From Shannon -Weaver index, it was clear that the diversity estimate was the highest in Nicobar tall group whereas it was low in tall genotypes of Fiji and Tonga. Thickness of leaf sheath fibre of weft and warp strands had shown high diversity estimates. Average estimate of total diversity in the study is 0.72 indicating the richness of the coconut diversity in the countries under study. Diversity estimate varied from 0.49 (circumference) to 0.97 (thickness of weft fibre of leaf sheath). They concluded that the diversity in a location is mainly decided by the history of populations.

Baudouin and Santos (2005) reported that morphological traits can be used as an index for determining diversity among coconut population. Studying morphological diversity in coconut involves comparing traits that are measured on different parts of the plant. Both quantitative and qualitative traits are being used for diversity analysis. Quantitative traits include speed of germination, stem morphology, leaf morphology, flower production, fruit set, copra per nut, fruit and bunch return etc. Qualitative traits comprises fruit colour, shape, colour of inflorescence etc. They also suggested that besides its obvious use for characterizing cultivars, the morphometric approach to determine genetic diversity provides valuable information on the role of informal selection on the evolution and the maintenance of genetic diversity in coconut.

Hemavathy and Balaji (2006) used seventeen traits including vegetative, inflorescence and fruit/nut characters for genetic diversity studies of twenty eight coconut genotypes from Coconut Research Station, Veppankulam. Genotypes were grouped into eight clusters. Tall cultivars were grouped into four clusters. Dwarf cultivars were resolved into four clusters. The study also confirmed the distinctiveness of the dwarf and tall varieties. Nut characters were found to be more efficient in assessing genetic divergence. The inter cluster diversity values ranged from 23.69 to 136.6. The genotypes from one location clustered differently supporting the view that geographic distribution and genetic divergence do not follow the same trend.

Selvaraju and Jayalekshmi (2011) reported morphometric diversity of six popular coconut cultivars of Travancore viz., WCT, Komadan, Laccadive Ordinary, Natural Cross Dwarf (NCD), Chowghat Green Dwarf and Chowghat Orange Dwarf. Fourteen biometric traits were taken into account. The six cultivars were grouped into three clusters, cluster I with WCT and NCD, cluster II with COD and CGD and cluster III with Komadan and Laccadive Ordinary. Cluster analysis showed that nut characters are superior in assessing genetic diversity in coconut.

2.3.2. Biochemical markers

Biochemical markers, such as allozymes (Cardena *et al.,* 1998) or polyphenols (Jay *et al.,* 1988), were first used at the beginning of the 1980s to describe the diversity of coconut collections. Unlike morphological traits, biochemical markers do not require measuring different characters from different parts in a full-sized palm. It is enough to take a sample (leaflet, root, etc.) to reveal the biochemical identity of the palm.

2.3.2.1. Allozymes

Allozymes are allelic variants of enzymes encoded by structural genes. Enzymes are proteins consisting of amino acids, some of which are electrically charged. As a result, enzymes have a net electric charge, depending on the stretch of amino acids, comprising the protein. When a mutation in the DNA results in an amino acid being replaced, the net electric charge of the protein may be modified, and the overall shape (conformation) of the molecule can change. Because changes in electric charge and conformation can affect the migration rate of proteins in an electric field, allelic variation can be detected by gel electrophoresis and subsequent enzyme-specific stains that contain substrate for the enzyme, cofactors and an oxidized salt. Usually two, or sometimes even more loci can be distinguished for an enzyme and these are termed isoloci. Therefore, allozyme variation is often also referred to as isozyme variation (Kephart, 1990; May 1992). Allozyme analysis is simple, quick and easy to use. Allozymes are codominant markers that have high reproducibility. Zymograms can be readily interpreted in terms of loci and alleles but they are less abundant, shows low level of polymorphism and they are affected by environmental conditions.

Allozymes found applications in many fields such as diversity analysis (Hamrick and Godt, 1999; Lamboy *et al*., 1994), population genetic studies (Erskine and Muehlenbauer, 1991), population structure and divergence (Freville *et al*., 2001), fingerprinting purposes (Tao and Sugiura, 1987; Maass and Klaas, 1995), interspecific relationships (Garvin and Weeden, 1994).

Parthasarathy *et al*. (2004) analysed forty different coconut cultivars (twenty seven tall and thirteen dwarf) and six different hybrids and their parents based on eleven isozyme systems such as esterase, glutamate, peroxidase etc. The cultivars grouped mainly into six clusters. Most dwarfs clustered together except for Kulasekharam Orange Dwarf (KOD), Gangabondam (GBGD), Gudanjali Dwarf (GDD) and Cameroon Red Dwarf (CRD). Among that KOD, GBGD and GDD were intermediate between tall and dwarf while, CRD was more distinct and it grouped along with the Dwarfs. Among Talls, three ecotypes, San Ramon Tall (SNRT), Straight Settlement Apricot Tall (SSAT) and Zansibar Tall (ZAT) grouped along with the Dwarfs. Philippines Ordinary Tall (PHOT) was intermediate between Talls and Dwarfs. Two morphologically distinct ecotypes, Kappadam Tall (KPDT) with very large fruits and Laccadive Ordinary Tall (LCT) with very small fruits clustered together. In case of hybrids and their parents, the hybrids clustered intermediate between parents. The similarity level was the highest in LCT x GBGD and WCT x SSGT (94.1%), while it was the least in LCT x GBGD and COD (57.5%). Low intra population variation was observed both for Talls and Dwarfs.

Perez *et al*. (2004) electrophoretically analyzed eighteen allozyme loci for estimating the levels and structuring of genetic variation within and among natural populations of the protected endemic palm species (*Phoenix canariensis*) from the Canary Islands to evaluate its genetic relationship with the widespread congener *P. dactylifera*, and to assess comparatively the genetic variation in the populations where the two species coexist with morphologically intermediate plants (mixed populations). It revealed that a close genetic relationship between *P. canariensis* and the mixed populations and showed reduced levels of genetic variation between *P. canariensis* and *P. dactylifera*, the fact that the genetic makeup of the Canarian endemic (with no unique alleles) is a subset of that found in *P. dactylifera,* and the high genetic identity between both species strongly suggested that *P. canariensis* is recently derived from a common ancestor closely related to *P. dactylifera.*

Geethalakshmi *et al*. (2005) studied the genetic diversity among thirty coconut genotypes using ten enzyme systems. Twenty loci and forty alleles were observed of which fourteen loci are polymorphic. Seven loci were heterozygous. Null alleles were observed for acid phosphatase (ACP), alcohol dehydrogenase (ADH) and malate dehydrogenase (MDH). Greater heterozygosity was observed for glucose-6 phosphate hydrogenase and the least for super oxide dismutase. Average heterozygosity was observed to be 26.07 per cent.

2.3.3. Molecular markers

A molecular marker is a DNA sequence which is readily detected and whose inheritance can be easily monitored. They are based on naturally occurring polymorphisms in DNA sequences. The molecular markers are directly linked to the genome. Their number is potentially unlimited in the genome and dispersion across the genome is complete. Molecular tools provide valuable data on diversity through their ability to detect variation at the DNA level. Glaszmann (2010) reported that molecular markers have clarified the structure of genetic diversity in a broad range of crops. Recent developments in molecular biology have made whole-genome surveys and gene-targeted surveys possible, shedding light on population dynamics and on the impact of selection during domestication.

Molecular markers have the advantages of being abundant, phenotypically neutral, show absence of epistasis and are not influenced by the developmental stage or tissue of the plant or environmental conditions (Mohapatra, 2007). Many molecular markers are utilised for numerous purposes such as characterisation of germplasm, varietal identification and clonal fidelity testing, assessment of genetic diversity, validation of genetic relationships and marker-assisted selection (Hoogendijk and Williams, 2001). Molecular markers are highly heritable, obtainable at a high number and frequency and display enough polymorphism in closely related genotypes (Stuber *et al.,* 1999; Archak *et al*., 2003; Weising *et al*., 2005). Morphological markers on the other hand have limited application in breeding as they are few in number as well as

dependent on the season and developmental stage of the plant (Krishna and Singh, 2007) and are influenced by the environment.

A molecular marker should have some desirable properties.

1. Must be polymorphic as it is the polymorphism that is measured for genetic diversity studies
2. Co-dominant inheritance
3. Should be evenly and frequently distributed throughout the genome
4. Easy, fast and cheap to detect
5. Reproducible

Molecular markers found applications in germplasm characterization, genetic diagnostics, characterization of transformants, genome organization and phylogenetic analysis other than diversity and population structure analysis (Sing, 2008).

2.3.3.1. Types of molecular markers

2.3.3.1.1. Random amplified polymorphic DNA (RAPD)

RAPDs are DNA fragments amplified by the Polymerase Chain Reaction (PCR) using short synthetic primers (generally 10 bp) of random sequence. These oligonucleotides serve as both forward and reverse primer, and are usually able to amplify fragments from 1-10 genomic sites simultaneously. Amplified fragments, usually within the 0.5-5 kb size range, are separated by agarose gel electrophoresis, and polymorphisms are detected, after ethidium bromide staining, as the presence or absence of bands of particular sizes. These polymorphisms are considered to be primarily due to variation in the primer annealing sites, but they can also be generated by length differences in the amplified sequence between primer annealing sites. They are quick and easy to assay. Only low quantities of template DNA are required, usually 5-50 ng per reaction. Since random primers are commercially available, no sequence data for primer construction are needed. Moreover, RAPDs have a very high genomic abundance

and are randomly distributed throughout the genome but they are less reproducible (Schierwater and Ender, 1993). RAPD markers are not locus-specific, band profiles cannot be interpreted in terms of loci and alleles (dominance of markers), and similar sized fragments may not be homologous.

RAPDs have been used for many purposes, ranging from studies at the individual level (genetic identity) to studies involving closely related species. RAPDs have also been applied in gene mapping studies to fill gaps not covered by other markers (Williams *et al*., 1990; Hadrys *et al*., 1992) and DNA fingerprinting (Manjunath, 2011) that uses shorter, 5-8 bp primers to generate a larger number of fragments.

Ashburner and Rohde (1994) reported an RAPD data set from twenty coconut populations from the South Pacific region and one from the Indian Ocean Islands. The data showed a moderate and variable level of diversity within population in the Pacific, but very little population divergence. There were relatively large differences between the Pacific and Indian Ocean types. The results indicated the presence of extensive migration of coconut germplasm in the Pacific, which lead to blurring of genetic structure when coupled with a mixed mating system.

Cardena *et al.* (2003) carried out RAPD assay for identifying markers linked with the lethal yellowing resistance of coconut palm. They used three coconut populations which could be used for this purpose, and comprised the susceptible West African Tall (WAT), the resistant Malayan Yellow Dwarf (MYD), and a resistant population of Atlantic Tall (AT) palms. WAT and AT were closely related and both of them were distantly related to MYD. A total of eighty two RAPDs could differentiate the DNA pools from MYD and WAT, and twelve of them appeared at frequencies $\geq$ 0.85 in MYD, and $\leq$ 0.150.15 in WAT.

RAPD assay for the genetic diversity analysis for twenty nine Indian mango cultivars comprising popular landraces and some advanced cultivars was done by

Karihaloo *et al*. (2003). PCR amplification with twenty four primers generated three hundred and fourteen bands, 91.4 per cent of which were polymorphic. Jaccard's similarity between pairs of cultivars ranged between 0.318 and 0.75 with a mean of 0.565. UPGMA dendrogram showed the majority of the cultivars from northern and eastern regions of India clustering together and separate from southern and western cultivars. Analysis of molecular variance revealed that 94.7 per cent of the genetic diversity in mango existed within regions. Northern and Eastern regions formed one zone and western and southern regions formed another zone of mango diversity in India.

Pradeepkumar *et al*. (2003) conducted RAPD analysis in twenty two cultivars of black pepper (*P. nigrum*) from South India and one accession each of *P. longum* and *P. colubrinum*. Twenty four primers generated three hundred and seventy two RAPD markers of which three hundred and sixty seven were polymorphic. Jaccard's similarity between pairs of accessions ranged between 0.11 and 0.66 with a mean of 0.38. Among *P. nigrum* cultivars, the similarity ranged between 0.20 and 0.66 and the mean was 0.42. The study revealed that there is wide genetic diversity and extensive inter and intra population morphological variability in pepper cultivars from South India. Unweighted pair group method with arithmetic mean (UPGMA) dendrogram and PCO plot revealed *P. colubrinum* is the most distant of the three species. Greater divergence was observed among landraces than among advanced cultivars. Landraces grown in southern parts of coastal India and those grown in more northern parts were grouped in separate clusters of the dendrogram. They found that the genetic proximity among *P. nigrum* cultivars could be related to their phenotypic similarities or geographical distribution.

Al-Khalifah and Askari (2003) made RAPD analysis for the genetic diversity analysis among thirteen different cultivars of date palm (*Phoenix dactylifera L*.) of Saudi Arabia. Out of hundred and forty RAPD primers used, only thirty seven gave polymorphism. Cluster analysis by the UPGMA showed two main clusters. Cluster A consisted of five cultivars (Shehel, Om-Kobar, Ajwa, Om-Hammam and Bareem) with 0.59-0.89 Nei and Li's coefficient in the similarity matrix. Cluster B consisted of seven

cultivars (Rabeeha, Shishi, Nabtet Saif, Sugai, Sukkary Asfar, Sukkary Hamra and Nabtet Sultan) with a 0.66-0.85 Nei and Li's similarity range. The average similarity among the thirteen cultivars was more than fifty per cent. Most of the cultivars have a narrow genetic base. Om-Hammam and Bareem were the two most closely related cultivars among the thirteen cultivars with the highest value in the similarity matrix for Nei and Li's coefficient (0.89). The cultivar Barny with thirty four per cent genetic similarity to the rest of the twelve cultivars did not belong to any of the cluster groups.

Upadhyay *et al*. (2004) carried out RAPD analysis of genetic relationship and diversity in fifteen Indian and five exotic coconut accessions revealed that eight primers yielded seventy seven markers. The genetic diversity within accessions and between accessions was 0.58 and 0.42 respectively. Tall accessions had higher proportions of polymorphic bands and genetic diversity. Exotic accessions exhibited more variation than indigenous accessions. Dwarfs from geographically distinct regions did not show much variation.

Verma *et al*. (2004) carried out RAPD assay for detecting genetic diversity in betelvine **(***Piper betle* L.) landraces belong to four groups, namely, '*Kapoori*', '*Bangla*', '*Sanchi*' and '*Others*'. On the basis of the data from eleven RAPD primers, they distinguished genetic variation within and among the four groups of landraces. The results indicated that the '*Kapoori*' group is the most diverse. The neighbour joining (NJ) tree after a bootstrap (5000 replicate) test of robustness clearly shows the four groups to be well separated. All known male or female betelvine landraces have separated in the NJ tree indicating an apparent gender-based distinction among the betelvines.

The RAPD analysis of interrelationships among thirty three coconut germplasm accessions from various coconut growing regions by Manimekalai and Nagarajan (2006a) indicated that forty five random primers could produce a total of three hundred and ninety nine polymorphic markers and fifteen informative and reproducible primers

were identified. The Polymorphism Information Content ranged from 0.031 to 0.392. The Marker Index (MI) ranged from 0.28 to 0.034 among the primers. There was less genetic similarity among South Pacific and South East Asian accessions based on the study.

The first report of the DNA based polymorphism assay to assess the level of variability in three oil palm varieties, *dura*, *pisifera* and *tenera* was made by Sathish *et al*. (2007). The three varieties were evaluated using thirty RAPD primers. Of the thirty primers, twenty six yielded significant polymorphic DNA bands. A total of hundred and eighty five bands were determined (300-1500 bp), of which fifty nine were representing *dura*, sixty five for *pisifera* and sixty one bands for *tenera.* A dendrogram was constructed using UPGMA, grouped all the three varieties into two distinct clusters. In one cluster, the shelled ones (*dura* and *tenera*) were grouped, while the other comprised the shell less one (*pisifera*). The data generated out of the study was sufficient to distinguish each variety.

Paul *et al.* (2008) reported genetic diversity in three yellow dwarf populations of coconut viz, Malayan Yellow Dwarf (MYD), Kulasekharam Yellow Dwarf (KYD) and Andaman Yellow Dwarf (AYD). Mean heterozygosity was higher in KYD (0.157) and MYD (0.153) and less in AYD (0.039). Jaccard's coefficient showed a close association of the two indigenous yellow dwarfs AYD and KYD with the exotic MYD. The cluster analysis produced a dendrogram of two major clusters. The AYD palms formed a distinct sub cluster within the second group, indicating its distinctness and homogeneity. The MYD and KYD palms were scattered in different sub clusters indicating the presence of diversity within accessions. There is greater diversity within the accessions than between the accessions. They made a conclusion that AYD and KYD evolved from a common progenitor, the MYD.

2.3.3.1.2. Inter Simple Sequence Repeats (ISSR)

ISSRs are DNA fragments of about 100-3000 bp located between adjacent, oppositely oriented microsatellite regions. ISSRs are amplified by PCR using microsatellite core sequences as primers with a few selective nucleotides as anchors into the non-repeat adjacent regions (16-18 bp). About 10-60 fragments from multiple loci are generated simultaneously, separated by gel electrophoresis and scored as the presence or absence of fragments of particular size. The main advantage of ISSRs is that no sequence data for primer construction are needed. Because the analytical procedures include PCR, only low quantities of template DNA are required (5-50 ng per reaction). ISSRs are largely distributed throughout the genome.

It is a multi locus molecular technique based on PCR that identifies insertions and deletions in DNA. It is highly sensitive, highly reproducible, provides Mendelian segregation, and has been successfully applied in genetic and evolutionary studies of many species, including coconut (Manimekalai and Nagarajan, 2006b). It can also be applied in studies involving genetic identity, parentage, clone and strain identification, and taxonomic studies of closely related species. In addition, ISSRs are considered useful in gene mapping studies (Godwin *et al*., 1997; Zietkiewicz *et al*., 1994; Gupta *et al*., 1994) and diversity analysis (Manimekalai and Nagarajan , 2006b).

Adawy *et al*. (2002) did genetic variability studies and molecular fingerprinting of forty Egyptian date palm individuals belonging to four cultivars based on RAPD and ISSR markers. All the tested primers exhibited intra varietal polymorphism as well as inter varietal variation. The DNA samples of the ten trees representing each cultivar were bulked to produce a single sample. The ten primers revealed twelve unique polymorphic RAPD markers among the four cultivars. Six ISSR primers revealed twenty four unique markers characterizing the different cultivars.

Balasaravanan *et al*. (2005) determined inter and intra species genetic relationships among one hundred and forty nine individuals belonging to six *Eucalyptus*

species based on inter-simple sequence repeats (ISSR). Seven primers could detect a total of five hundred and eighty three loci. The ISSR fragments indicated significant polymorphism and genetic diversity among the individuals. Cluster analysis and principal component analysis revealed the occurrence of wide genetic diversity among populations of *E. tereticornis* Sm., *E. camaldulensis* Dehnh. and *E. urophylla* S. T. Blake and narrow genetic diversity among populations of *E. citriodora* Hook. and *E. grandis*. Genetic diversity was high in *E. tereticornis* Sm. (47.27%) and low in *E. citriodora* (18.64%). Maximum Nei's genetic identity (0.897) was observed between *E. camaldulensis* and *E. tereticornis* species, whereas maximum genetic diversity (0.286) was found between individuals of *E. citriodora* and *E. grandis*.

Manimekalai and Nagarajan (2006b) carried out a study in which thirty three coconut accessions from worldwide coconut collection were analyzed using ISSR markers with nineteen primers, flanking the Simple Sequence Repeats regions. They were able to locate hundred and fifty four polymorphic ISSR markers. Similarity values ranged between 0.526 and 0.855. The least similarity was found between Nicobar Tall and Chowghat Orange Dwarf. The coconut accessions from South East Asia, South Asia and South Pacific formed separate groups and it was in accordance with the origin and pattern of dispersal of coconut from its centre of origin.

Manimekalai *et al*. (2007) estimated molecular diversity of eight South East Asian accessions comprising thirty two individuals, using ISSR markers. Nineteen ISSR primers detected a total of eighty five polymorphic markers across thirty two individuals. The average genetic distance among the eight populations ranged from 0.7908 to 0.9327 with a mean of 0.8540. Laguna Tall and Kongthienyong Tall showed the highest genetic identity of 0.9327.The lowest genetic identity was between San Romon Tall and Philippines Dalig Tall (0.708). The Shannon's index ranged from 0.1615 to 0.299. The dendrogram constructed based on the genetic distance revealed clustering of Kongthienyong Tall and Laguna Tall while Philippines Dalig Tall was positioned separately.

Thimmappaiah *et al.* (2009) studied genetic diversity among hundred cashew germplasm using RAPD and ISSR markers. They found out that both kinds of markers discriminated the accessions effectively, analysis of combined data of markers (RAPD+ISSR) resulted in better distinction of accessions. By combining markers, a total of hundred and twenty seven bands were detected, of which hundred and nine bands (85.8 per cent) were polymorphic and produced on an average of 5.45 polymorphic bands per primer. Genetic relationship estimated using similarity co-efficient (Jaccard's) values between different pair of accessions varied from 0.43 to 0.94 in RAPD, 0.38 to 0.89 in ISSR and 0.43 to 0.87 with combined markers suggested a diversity (dissimilarity) ranging from 6 to 57%, 11 to 62% and 13 to 57% respectively and the diversity skewed around 50 per cent indicated moderate diversity. Among the accessions studied, NRC-142 and NRC-12 were highly divergent and NRC-231 and NRC-232 were genetically similar.

Karim *et al*. (2010) studied the genetic diversity of ten Tunisian date palm accessions using ISSR markers. Eighty two polymorphic markers were generated out of seven primers used for amplification of date palm accessions. These markers were used to examine the genetic distance and relationships between the ten accessions. The matrix had a genetic distance of 0.3008 to 0.7885 with a mean of 0.505. The phenograms obtained as a result of data analysis were in agreement with those obtained with agronomic traits. The dendrogram revealed that the foreign varieties were unlikely clustered with indigenous accessions. They were unable to identify the tested genotypes as mono varietal groups.

Patra *et al*. (2011) made a comparative study of RAPD and ISSR markers to assess the genetic diversity of fifteen cultivars of betel vine from different parts of Orissa. Five hundred and twenty three amplicons were generated out of twenty RAPD and eighteen ISSR primers. Five hundred and four amplicons were polymorphic and fifty four were found to be unique. Maximum genetic diversity (0.68) was observed

between Balipana and Birkoli and minimum (0.114) was between Banglamandesore chitalpudi and Halisahar Sanchi. All the cultivars were related with each other with a diversity of 0.2913. Dendrogram showed that Godibengala was clustered separately, isolated from all the other species. Correlation between RAPD and ISSR was very low (r=0.17). RAPD showed high correlation with all the primers.

Jiang and Liu (2011) investigated genetic diversity of seventy one accessions representing eleven wild piper species in Hainan island using ISSR markers. Two hundred and forty seven polymorphic bands out of a total of two hundred and forty eight (99.60 per cent) were generated. The overall level of genetic diversity among *Piper spp* in Hainan was high, with the mean Shannon information index of 0.2843 and the mean Nei's genetic diversity of 0.1904. The genetic similarity coefficient ranged from 0.548 to 0.976 among individual plants of *Piper spp* and the within species genetic distance ranged from 0.104 to 0.28. Unweighted pair group method with arithmetic mean (UPGMA) dendrogram showed that *P. kadsura* is the most divergent and the most distant of the eleven species. The diversity analysis unambiguously distinguished all piper species.

Tomar *et al*. (2011) analyzed the phylogenetic relationship among twenty mango cultivars from Gir region of Saurashtra based on ISSR markers. Twenty one primers gave hundred and twenty five reproducible, polymorphic amplicons, and were selected to construct a DNA fingerprinting map to distinguish the genotypes of mango. According to the banding patterns obtained with twenty one selected primers, all cultivars tested in this study except Jamadar and Kesar were distinguished from each other and showed ample genetic diversity. All Gir mango landraces tested were clustered into a three big groups based on UPGMA analysis. This pattern of clustering indicated that some Gir landraces had a close relationship with each other, while some were drastically dissimilar from other landraces.

The variability of *Arthrocnemum macrostachyum* was estimated by Saleh (2011) using ISSR markers. He used three genotypes from Syria. The amplification of the genotypes with seven ISSR primers generated eighty eight bands, and eighty (90.91 per cent) were polymorphic. He concluded that the use of ISSR fingerprints could be a powerful tool to assess the genetic diversity in *A. macrostachyum*.

2.3.3.1.3. Microsatellites or Simple Sequence Repeats (SSR)

Microsatellites are tandem repeat motifs of 1-6 base pairs. Microsatellites and their flanking sequences can be identified by constructing a small-insert genomic library, screening the library with a synthetically labelled oligonucleotide repeat and sequencing the positive clones. Alternatively, microsatellites may be identified by screening sequence databases for microsatellite sequence motifs from which adjacent primers may then be designed. In addition, primers may be used that have already been designed for closely related species. Polymerase slippage during DNA replication, or slipped strand mispairing, is considered to be the main cause of variation in the number of repeat units of a microsatellite, resulting in length polymorphisms that can be detected by gel electrophoresis.

They are codominant, highly abundant in eukaryotic genomes and are randomly distributed throughout the genomes with preferential association in low copy regions. (Morgante *et al*., 2002). Only less quantities of template DNA is required (10-100 ng per reaction). It is highly reproducible and do not require high quality DNA. Although microsatellite analysis is, in principle, a single-locus technique, multiple microsatellites may be multiplexed during PCR or gel electrophoresis if the size ranges of the alleles of different loci do not overlap (Ghislain *et al.,* 2004). High development costs are involved if adequate primer sequences for the species of interest are unavailable, making them difficult to apply to unstudied groups. Although microsatellites are in principle co dominant markers, mutations in the primer annealing sites may result in the occurrence of null alleles (no amplification of the intended PCR product), which may lead to errors in genotype scoring. Null alleles may result in a biased estimate of the allelic and

genotypic frequencies and an underestimation of heterozygosity. They are used in population genetic studies ranging from individual level to closely related species. Microsatellites are also considered ideal markers in gene mapping studies (Hearne *et al*., 1992; Morgante and Olivieri, 1993).

Rivera *et al.* (1999) studied genetic diversity between coconut populations from germplasm collections from different coconut resources. He isolated microsatellites or SSRs from coconut cultivar, Tagnanan Tall. Seventy five per cent of the genomic library contained a microsatellite, of which sixty four per cent are di nucleotide, sixteen per cent were tri nucleotide and thirty per cent were compound repeats. They conducted genetic diversity studies in twenty cultivars from different groups, of which thirty SSRs detected one hundred and ninety eight alleles. Genetic diversity values ranged from 0.14 to 0.809. Heterozygotes were present at high frequencies among some dwarf cultivars. Dwarf showed less genetic diversity. In a wider test on fourty samples, SSRs detected sixty four alleles. These results indicated the high potential of microsatellites to detect genetic diversity in coconut population.

Perera *et al*. (2000) used eight pairs of SSR primers to analyse the genetic diversity in hundred and thirty individuals of coconut (*Cocos nucifera* L.) comprising seventy five tall individuals and fifty five dwarf individuals, representing ninety four different coconut ecotypes throughout the world. A total of fifty one alleles were detected, with an average of 6.4 alleles per locus. Fifty alleles were detected in tall coconuts (mean alleles/locus 6.3) compared with only twenty six (mean/locus 3.3) in dwarfs, and the average diversity value in Talls was 0.589 and was also significantly higher than that in Dwarfs (0.348). The individuals were clustered into five groups, each mainly composed of either Talls or Dwarfs.

Meerow *et al*. (2003) used fifteen simple sequence repeat (SSR) microsatellite DNA loci for analyzing genetic variation within *Cocos nucifera* germplasm collections at two locations in south Florida, representing eight cultivars. A total of sixty seven

alleles were detected. Gene diversity of the fifteen loci ranged from 0.778 to 0.223, with a mean of 0.574. The tall and dwarf palms were clustered differently. The highest gene diversity was found in the tall cultivars (H = 0.583 cumulatively), and the lowest in the Malayan Dwarf (H = 0.202). After the tall coconuts, the Fiji Dwarf was most genetically diverse (Hˆ = 0.436), and had the largest number of unique alleles. The Red Malayan Dwarf is genetically distinct from the Green and Yellow Malayan Dwarf phenotypes, which cannot be distinguished with the SSR loci used.

Noel *et al.* (2007) had done microsatellite analysis for determining the genetic diversity among sixty two palms of eleven coconut accessions from the International gene bank for Africa and Indian Ocean. The mean number of alleles and mean genetic diversity were 3.08+1.1 and 0.536+0.218 respectively. By cluster analysis, the accessions were clustered into two. The South Pacific coconut accessions clustered with Srilankan Dwarf Green accession. The accessions from Africa clustered separately. Among the accessions, the Srilankan Dwarf Green showed the least genetic diversity of 0.198+0.218 and that with the highest was to Tonga Tall of 0.678+0.229.

Elshibli and Korpelainen (2008) investigated genetic diversity in date palm germplasm from Sudan representing thirty seven female and twenty three male accessions using sixteen loci of microsatellite (SSR) primers. A total of three hundred and forty three alleles were detected at the sixteen loci. A high level of expected heterozygosity was observed among Sudan cultivars. The results indicated that the genetic groups of the Sudan cultivars and/or males did not follow a clear geographic pattern. The morocco group showed significant differentiation in relation to the Sudan groups, as measured by FST values and genetic distances. The effect of the methods of pollination and cultivar selection on the genetic structure was clearly detected by the weak clustering association that was observed for the majority of accessions originating from Sudan and Morocco as well.

The extent of genetic diversity in twenty six coconut accessions from the Andaman and Nicobar (A&N) islands was determined using fourteen microsatellite markers by Rajesh *et al*. (2008). A total of hundred and three alleles were detected by the microsatellite markers with an average of 7.35 alleles per locus. The average observed and expected heterozygosity was 0.29 and 0.66 respectively. Heterozygosity was the highest in tall coconut accessions. Majority of rare alleles were observed in tall accessions from the Nicobar islands. The UPGMA dendrogram revealed clustering of majority of tall and dwarf accessions separately.

Dasanayaka *et al*. (2009) analyzed forty three coconut accessions conserved *ex-situ* in field gene banks of the Coconut Research Institute of Sri Lanka (CRISL) using sixteen microsatellite markers. Gene diversity and polymorphism information content (PIC) were relatively higher in the common 'tall' coconut and Pacific Tall coconut than in autogamous dwarf form of coconut. The SSR assessment unveiled the genetic lineages based on evolutionary mechanisms signifying the narrow genetic base of coconut germplasm, with most of the diversity confining to 'tall' coconut. The overall results supported the hypothesis that coconut disseminated from its center/s of origin in Far East to Indo Atlantic regions via America.

Devakumar *et al*. (2010) reported microsatellite variability in a total of nine coconut accessions from Agatti and Kavaratti islands of Lakshadweep as part of a germplasm enrichment programme for island coconut populations of India. This includes three Laccadive Micro types, four types of Laccadive Ordinary Tall differing in nut bearing habit, fruit shape and size, one each of sweet husked Kaithathali tall and dwarf type. The microsatellite analysis of the nine coconut populations with eight primers revealed a total of thirty seven alleles. The estimate of heterozygosity was the highest (>0.5) for the two Laccadive Micro Tall (LMT02 and LMT03) and the Laccadive Small Tall (LCT02) populations and was the lowest (0.24) for the Laccadive Micro Tall (LMT01). The sporadic occurrence of Laccadive Micro Tall is due to high out crossing and inbreeding depression.

Kumar *et al*. (2011) used ten tall and four dwarf coconut accessions from International coconut gene bank for South Asia, Kidu for assessing genetic diversity. A total of eight primers produced twenty eight polymorphic alleles. The primer CNZ 40 recorded the highest PIC value of 0.8791. The highest similarity index was obsered between the accessions Hari papua Dwarf and Kiriwana Tall and the similarity coefficient ranged from 0.1775 to 0.7654 with a mean of 0.4231. The accessions were grouped into three clusters. Cluster one consists of five accessions belonging to New Guinea. Cluster two represents the four accessions from French Polynasia and cluster three includes five accessions from Solomon islands.

2.3.3.1.4. Restriction Fragment Length Polymorphism (RFLP)

RFLPs were developed by Botstein *et al*. (1980). RFLP uses restriction enzymes that cut the DNA molecule at specific sites, called restriction sites, resulting in different fragments of variable lengths. After separation by electrophoresis, fragments are transferred to nitrocellulose or nylon filters through Southern blotting followed by hybridization with radioactively labeled DNA probes and visualisation using photographic film (Varshney *et al*., 2004).

They are codominant markers that are more or less specific depending on the probe used (cDNA, genomic DNA, etc). These markers are difficult and quite expensive to use. They require the extraction of a large quantity of good quality DNA and operations are lengthy. It takes around two weeks after DNA extraction to read the bands. Cytoplasmic genomes are usually much less polymorphic than the nuclear genome. They can be a source of information for establishing phylogenies, or retracing the domestication routes of a plant from its region of origin (Lebrun *et al.,* 1999). The nuclear genome proves to be much more polymorphic than chloroplast genome. Irrespective of the number of probes used, or their origin, the results as regards diversity structuring were comparable. However, this technique is laborious to use (Lebrun *et al.,* 2005).

Lebrun *et al*. (1998) performed genetic diversity study in hundred coconut genotypes (ten Tall and seven Dwarf) from various geographical origins by RFLP analysis. Nine cDNA clones from rice, one mitochondrial DNA clone (*CoxI*) and one genomic clone (rDNA) from wheat were used as probe for southern hybridization. Forty polymorphic bands revealed by rice cDNA clones were studied using a multivariate analysis and allowed to identify two main genetical groups. The first one includes the ecotypes from the Far East and from the South Pacific, whereas the other one comprises the ecotypes from India, Sri Lanka and Western Africa. The Far East and the Pacific regions which were the most likely center of origin also exhibit the widest polymorphism. The Comoro Tall appears to be intermediate between the two main groups. All Dwarf varieties belong to the first group, including those collected from West Africa. Those were probably introduced from Asia and Pacific at the beginning of the century. The cross pollinating Tall ecotypes were generally more polymorphic than the self pollinating Dwarf ecotypes.

Mayes *et al*. (2000) identified RFLP markers for assessing genetic diversity within fifty four palms. A total of hundred and fifty seven RFLP bands were scored. The data was analyzed by calculating genetic distances according to Nei & Li, by correspondence and cluster analysis. The relationships that emerged through this molecular analysis corresponded well with known pedigree and provenance. Four major clusters were identified. The top cluster consists solely, and includes all, pure AVROS palms studied. The bottom cluster consists only, and includes all, pure Deli types. The two less discrete clusters in the centre consist either of DRC origins crossed with Nigerian/Deli material, or of pure DRC or pure Tanzanian or Deli AVROS crosses. The AVROS material showed the least intra group variability, followed by the Deli types.

Barcelos *et al*. (2002) did the genetic diversity study, organization and the genetic relationships within thirty eight oil palm accessions from America and Africa using Restriction Fragment Length Polymorphism (RFLP) and Amplified Fragment

Length Polymorphism (AFLP). They were analyzed using thirty seven cDNA probes. Data was subjected to Factorial Analysis of Correspondence (FAC) and cluster analysis, with parameters of genetic diversity being also studied. Results appeared congruent between RFLP and AFLP. In American palms, AFLP confirmed the strong structure of genetic diversity revealed by RFLP. Four distinct genetic groups were identified viz., Brazil, French Guyana/Surinam, Peru, north of Colombia/Central America. Both markers revealed that genetic divergence between the two species is of the same magnitude as that among provenances American palms.

Maizura *et al*. (2006) assessed genetic diversity in three hundred and fifty seven oil palm accessions from eleven African countries using Restriction Fragment Length Polymorphism. All the accessions exhibited higher levels of diversity than the standard variety, Deli dura. The standard variety, Deli dura, lost thirty six alleles as compared to the natural populations indicating a reduction in genetic variability. Material from Nigeria showed the highest mean number of alleles per locus (1.9) and percentage of polymorphic loci (67.2%). These findings, combined with others, suggested that Nigeria may be the centre of diversity of wild oil palm. It further suggested that oil palm natural populations may be possessing adequate genetic variability that are potentially useful for improvement programmes.

2.3.3.1.5. Amplified Fragment Length Polymorphism (AFLP)

AFLPs are DNA fragments (80-500 bp) obtained from digestion with restriction enzymes, followed by ligation of oligonucleotide adapters to the digestion products and selective amplification by the PCR. The AFLP technique is based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA. AFLPs therefore involve both RFLP and PCR. The technique involves three steps: (i) restriction of the DNA and ligation of oligonucleotide adapters, (ii) selective amplification of sets of restriction fragments, and (iii) gel analysis of the amplified fragments. PCR amplification of restriction fragments is achieved by using the adapter and restriction site sequence as target sites for primer annealing. The selective amplification is achieved

by the use of primers that extend into the restriction fragments, amplifying only those fragments in which the primer extensions match the nucleotides flanking the restriction sites. Using this method, sets of restriction fragments may be visualized by PCR without knowledge of nucleotide sequence. The method allows the specific co-amplification of high numbers of restriction fragments. The number of fragments that can be analyzed simultaneously is dependent on the resolution of the detection system. Typically 50-100 restriction fragments are amplified and detected on denaturing polyacrylamide gels (Vos *et al*., 1995).

The strengths of AFLPs lie in their high genomic abundance, considerable reproducibility, the generation of many informative bands per reaction, their wide range of applications, and the fact that no sequence data for primer construction are required. AFLPs may not be totally randomly distributed around the genome as clustering in certain genomic regions, such as centromers, has been reported for some crops (Alonso-Blanco *et al*., 1998; Young *et al*., 1999; Saal and Wricke, 2002).

AFLP markers have been used to study the genetic diversity of various fruit species including apricot (Hagen *et al*., 2002; Geuna *et al*., 2003;), citrus (Krueger and Roose, 2003), mango (Kashkush *et al*., 2001), olive (Montemurro *et al*., 2005), peach (Aranzana *et al*., 2003), European plum (Goulao *et al*., 2001) and sweet cherry (Zhou *et al*., 2002).

Teulat *et al*. (2000) analyzed the genetic diversity of ten tall and four dwarf populations of coconut from across the geographic range represented by thirty one individual palms using SSR and AFLP markers. An average of 9.14 alleles per locus was observed and diversity ranged from 0.47 to 0.90. Diversity was higher in populations from the South Pacific and South East Asia. The East African populations had higher heterozygosities than those from West Africa, and the populations from Tonga and Fiji generally had distinct alleles from those of the South Pacific. AFLP analysis with twelve primer combinations gave a total of 1106 bands, of which 303 were

polymorphic (27%). Dendrograms and plots revealed similar relationships among the populations for both approaches. There was generally a good separation of populations.

El-Assar *et al*. (2005) studied the genetic analysis of forty seven Egyptian date palm accessions, collected from six locations in Egypt using four sets of AFLP markers with near infrared fluorescence labeled primers. A total of three hundred and fifty bands were scored and two hundred and thirty three (66.6 per cent) were polymorphic. Twenty seven Egyptian accessions and 'Medjool' and 'Deglet Noor' accessions from California could be classified into the major cluster. This major cluster may represent a major group of date palm germplasm in North Africa. There were four other clusters, each containing one or two accessions. They suggested that the variety 'Halawy' and one accession of unknown provenance were most likely from hybridization between two clusters. Six groups of accessions of which had the same names, revealed similar but not identical AFLP profiles suggesting that these accessions might be derived from seedlings rather than through clonal offshoot propagation.

Nazeem *et al*. (2007) detected genetic variability and relatedness in forty nine black pepper varieties through RAPD and AFLP analyses. Through RAPD analysis, a total of 139 markers were detected and 629 markers for AFLP. The dendrogram revealed an average similarity of 63 per cent among accessions. Two selections from the variety Karimunda, named Sreekara and Subhakara grouped in a single cluster with almost 92 per cent similarity. The dissimilarity observed between the varieties Panniyur 1 and Panniyur 3, the progenies of the same parentage Uthirenkotta and Cheriyakanyakodan was only eighteen per cent. Based on RAPD analysis, thirty four varieties were grouped into five clusters. Distinct clustering was not observed in AFLP analysis.

Galvez-Lopez *et al*. (2010) studied the molecular diversity and genetic relationships of forty one local mango accessions from Mexico through AFLP analysis. AFLP analysis indicated high levels of polymorphisms among accessions (> 84 per

cent). Analysis of Molecular Variance (AMOVA) indicated significant genetic differentiation (F**ST** = 0.23743) among and within mango accessions. Cluster analysis showed two groups. *Mangifera odorata* was different than all other *M. indica* accessions. The highest heterozygosity was found in mangoes from Tuxtla Chico (0.260) and Escuintla (0.254). Genetic differences among accessions and cultivars were associated with their geographical origin and indicated new genetic diversity of mangoes from Chiapas due to free-pollination and the use of recombinant plants.

2.4. Population structure analysis

Zizumbo-Villarreal *et al*. (2006) studied the genetic diversity, structure, genetic relationships and their percentage of mortality due to lethal yellowing between four Mexican tall ecotypes, four imported tall ecotypes and Malayan Yellow Dwarf using SSR markers. They concluded that the Mexican pool had high genetic diversity (HT 50.34 ±0.02) similar to that of the populations which have been imported from the world's main gene pools (HT 50.36 ± 0.01). High positive correlations were found between genetic distance and lethal yellowing (LY) mortality percentages under severe incidence conditions suggesting that genetic distance may be useful for the estimation of the potential LY mortality in regions as yet unaffected and identification of potential parents for LY resistance breeding.

Mauro-Herrera *et al*. (2007) evaluated the population structure of 110 genotypes including nine Atlantic Tall genotypes, 36 Fiji Dwarfs , 9 Green Malayan Dwarfs (plus two off-types), 10 Green Ninos, 6 Panama Talls, 19 Red Malayan Dwarfs (plus one off-type), 11 Red Spicatas and 5 Yellow Malayan Dwarfs. One Maypan, a hybrid between a Panama Tall and a Malayan Dwarf variety, and an undetermined tall were also included. They identified a total of 37 alleles with all 13 WRKY-derived markers, ranging between two and four alleles per marker. Gene diversity ranged from 0.075 to 0.542. The STRUCTURE simulation analyses with the WRKY data identified six populations in all genotypes evaluated; five of them could unambiguously be associated with a particular cultivar. Most of the genotypes of Atlantic Tall, Fiji Dwarf, Red

Malayan Dwarf, Green Nino and Red Spicata resolved consistent membership in their corresponding cultivar groups.

Ribeiro *et al*. (2010) studied about the population structures of Brazilian tall coconut by microsatellite markers. They used 195 palms from ten populations. Thirteen simple sequence repeats loci produced 68 alleles. The mean values of gene diversity and observed heterozygosity were 0.459 and 0.443 respectively. Estimates of genetic distances between the populations varied from 0.034 to 0.390. They observed a spacial structuring of the genetic variability among populations and geographically closer populations exhibited greater similarities.

Inghelandt *et al*. (2010) made a study to determine population structure and genetic diversity in a set of 1567 maize inbred lines representing founder (6 per cent) as well as elite (94 per cent) inbred lines of Europe and North-America. The study aimed to examine the population structure and the genetic diversity in elite maize germplasm based on simple sequence repeat (SSR) markers. The average number of alleles per locus, group specific alleles, and the gene diversity was high. Modified Roger's distance (MRD) estimates and membership probabilities of the STRUCTURE matrices was also high. They proposed that SSR is a reliable marker system for analyzing population structure and genetic diversity.

In order to determine the genetic diversity and population structure of one hundred and twenty eight foxtail millet accessions collected from eight different provinces in China, Liu *et al*. (2011) used SSR analysis. 1109 alleles were detected with an average of 14 alleles per SSR and an allele frequency of 0.38. The mean genetic diversity was 0.75 and the mean PIC was 0.72. Population structure analysis using STRUCTURE identified six groups, which matches with their pedigree information, in general, but not with their geographic origins.

Materials and Methods

3. MATERIALS AND METHODS

The research programme entitled "Diversity and population structure analysis in coconut (*Cocos nucifera* L.) using molecular markers" aimed to determine the variability and genetic relatedness of nine coconut cultivars planted in the coconut farm at Vellanikkara by the Coconut Development Board and being maintained by the Central Nursery, Kerala Agricultural University and one at the Regional Agricultural Research Station, Pilicode. The materials used and methodologies adopted are described in this chapter.

3.1. MATERIALS

3.1.1. Plant materials

The experimental material was collected from the coconut farm at Vellanikkara, established by the Coconut Development Board, which is being maintained by the Central Nursery, Kerala Agricultural University and the Regional Agricultural Research Station, Pilicode. Nine cultivars comprising of forty five palms were collected from coconut farm at Vellanikkara and one cultivar including five palms, from Regional Agricultural Research Station, Pilicode.

The tall cultivars *viz*., Laccadive Ordinary, Tiptur Tall, Kasaragode, Komadan, Kuttiadi and Malappuram belong to the blocks I, II, III, IV, V and VII of the coconut farm respectively. The intermediate type Gangabondam and the dwarf cultivar Chowghat Green Dwarf were planted in the blocks VIII and IX respectively. Chowghat Orange Dwarf was planted in IIIrd and IVth blocks. Malayan Yellow Dwarf was collected from blocks J and L of the coconut farm at Pilicode. The block diagram representing the study area is provided in Plate1. The coconut palms selected are depicted in Plate 2.

Spindle leaves were collected from these genotypes for isolation of DNA.

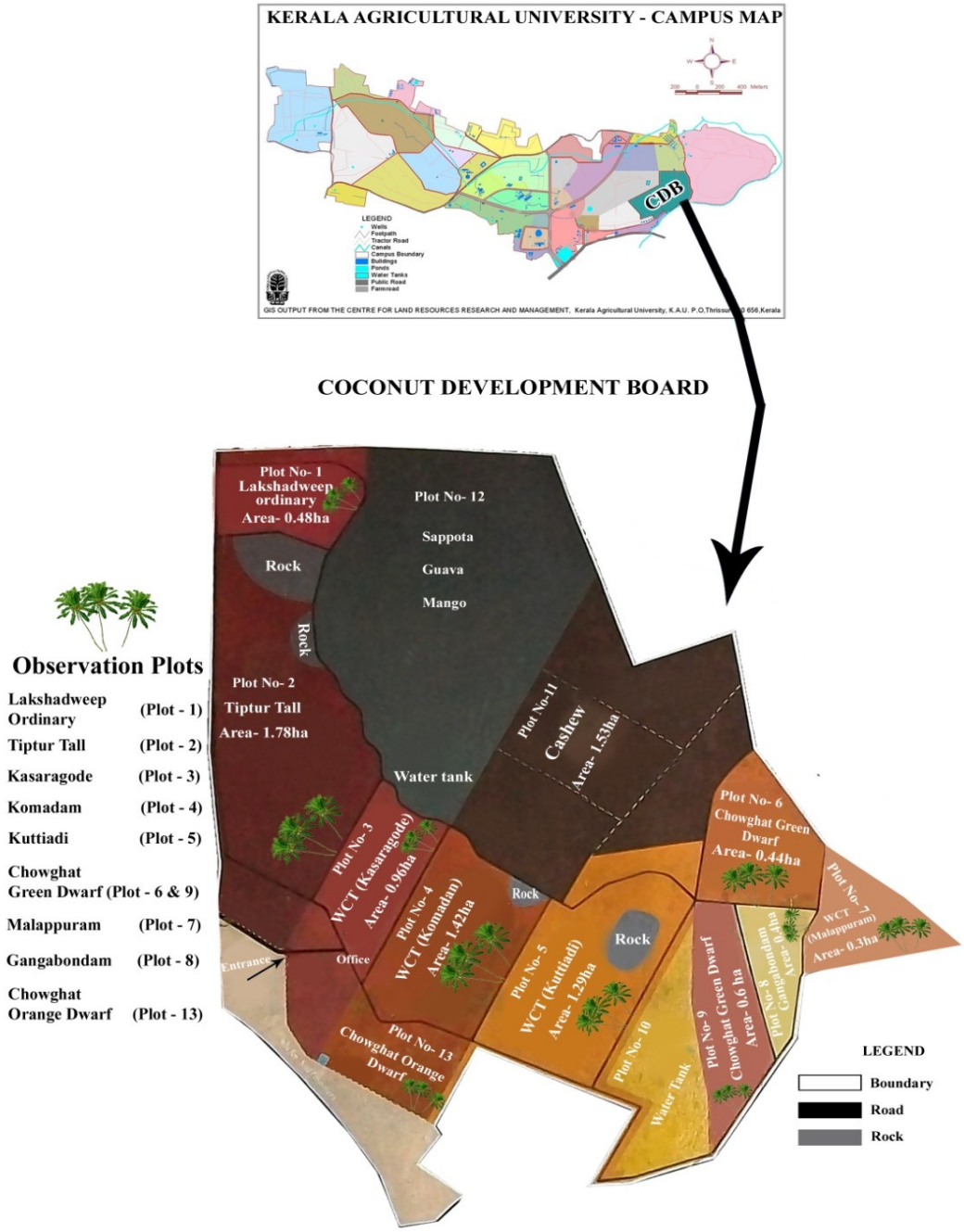


Plate 1. Location map of the study area at KAU main campus, Vellanikkara

Tall cultivars

Laccadive Ordinary Tall

Tiptur Tall

Komadan

Kuttiadi

Malappuram

Kasaragode

Plate 2a. Palms identified for the study

Dwarf cultivars

Chowghat Orange Dwarf

Chowghat Green Dwarf

Malayan Yellow Dwarf

Intermediate type

Gangabondam

Plate 2b. Palms identified for the study

3.1.2. Laboratory chemicals and glassware

The chemicals used in the study were of good quality (AR grade) procured from Merck India Ltd., SRL, HIMEDIA and SISCO Research Laboratories. The Taq DNA polymerase, dNTP's, Taq buffer and molecular weight marker (Lamda DNA *Eco* RI/ *Hind* III double digest, 100bp ladder, 50 bp ladder) were obtained from Bangalore, Genei. The random decamer primers used for the study were supplied by Operon Technologies, USA and were synthesised from Sigma Aldrich Chemicals, Bangalore. ISSR and SSR primers were obtained from Sigma Aldrich Chemicals Pvt. Ltd. The plasticwares were purchased from Tarsons India Ltd. and Axygen, USA.

3.1.3. Equipments and machinery

The equipments available at the Centre for Plant Biotechnology and Molecular Biology and the Bioinformatics Centre, College of Horticulture, Vellanikkara were used for the study. Incubation was done in water bath. Centrifugation was done in High speed refrigerated centrifuge, (KUBOTA 6500). The DNA quantification was done using Nanodrop(R) ND 1000 spectrophotometer. The Polymerase Chain Reaction was done in Eppendorf Master Cycler (Eppendorf, USA) as well as Varity (Applied Biosystems) and agarose gel electrophoresis was done in horizontal gel electrophoresis system (BIO-RAD). BIO-RAD gel imaging system was used for imaging and documenting the agarose gel. The details of laboratory equipments used for the study are provided in Appendix I.

3.2. METHODS

Molecular analysis of the fifty coconut genotypes (ten cultivars) was carried out with three different marker systems- RAPD, ISSR and SSR.

3.2.1. Standardization of genomic DNA extraction

Isolation of good quality genomic DNA is an important parameter for molecular marker analysis. Several procedures were tested for obtaining good quality genomic DNA from coconut leaves. The procedures reported by Rogers and Bendich (1994) with modifications, Upadhyay *et al*. (1999) with certain

Plate 3. Tender spindle leaflets used for DNA isolation

modifications were tried for DNA isolation and the quality of DNA obtained by each of these methods were compared. In all the above methods tried, protein and polysaccharide contamination was a major problem. A good quantity of DNA with desirable quality was obtained by the protocol by Porebski *et al*. (1997) with certain modifications as detailed below. The quality of DNA isolated was compared by gel electrophoresis and Nanodrop method. DNA was extracted from newly emerged spindle leaf (Plate 3). DNA was isolated from the tip and middle portion of the leaves since it was tender and easy to grind.

3.2.1.1. Protocol reported by Upadhyay *et al*. (1999)

Reagents

1. Extraction buffer (2x)

    100 mM Tris (pH 8)

    20 mM EDTA (pH 8)

    1% SDS

    5% PVP

2. 0.2% β -mercaptoethanol

3. Chloroform:Isoamyl alcohol (24:1)

4. Isopropanol

5. 100% ethanol

6. 76% ethanol

7. Sterile distilled water

Procedure

1. Ground one gram of cleaned leaf tissues in a pre-chilled mortar and pestle in the presence of liquid nitrogen.
2. Added 50µl of β-Mercaptoethanol and powdered well.
3. The powdered material was transferred to a 50 ml oakridge tube containing 5 ml hot 2x extraction buffer.
4. The contents were mixed well and incubated at 65ºC for one hour with intermittent mixing by gentle inversion.
5. Added 3 ml of chloroform: isoamyl alcohol (24:1) and mixed by inversion to emulsify. Spun at 12,000 rpm for 20 minutes at 4ºC
6. Aqueous phase containing the nucleic acids were removed by a wide-bore pipette and transferred to a fresh autoclaved oakridge tube
7. Again added 3 ml of chloroform: isoamyl alcohol (24:1) and mixed by inversion to emulsify. Spun at 12,000 rpm for 20 minutes at 4ºC
8. Aqueous phase containing DNA was removed by a wide-bore pipette and transferred to a fresh autoclaved oakridge tube
9. Added 0.6 volumes of chilled isopropanol and mixed by quick gentle inversion till the DNA got precipitated
10. Centrifuged at 10,000 rpm for 10 minutes at 4ºC. Gently poured off the supernatant
11. Washed the DNA pellet with 76% ethanol followed by 100% ethanol
12. Centrifuged at 12,000 rpm for 3 minutes, decanted the ethanol and drained well
13. Air dried the pellet, dissolved in 50ml of autoclaved distilled water and stored at-20ºC

3.2.1.2. Protocol of Porebski *et al*. (1997) (modified)

Reagents

1. Extraction buffer (2x)

   2% CTAB

   100 mM Tris

20 mM EDTA

1.4M NaCl

2% PVP

2. Chloroform: isoamyl alcochol
3. 5M NaCl
4. Ethanol 95% and 70%

Procedure

1. Ground one gram of cleaned leaf tissues in a pre-chilled mortar and pestle in the presence of liquid nitrogen
2. Added 50µl of β-Mercaptoethanol and powdered well
3. The powdered material was transferred to a 50 ml oakridge tube containing 7 ml hot 2x extraction buffer
4. The contents were mixed well and incubated at 65ºC for 30 minutes with intermittent mixing by gentle inversion
5. Added equal volume of chloroform: isoamyl alcohol (24:1) and mixed by inversion to emulsify. Spun at 10,000 rpm for 15 minutes at 4ºC
6. Aqueous phase containing the nucleic acids were removed by a wide-bore pipette and transferred to a fresh autoclaved oakridge tube
7. Again added equal volume of chloroform: isoamyl alcohol (24:1) and mixed by inversion to emulsify. Spun at 10,000 rpm for 15 minutes at 4ºC
8. Aqueous phase containing DNA was removed by a wide-bore pipette and transferred to a fresh autoclaved oakridge tube
9. Added 0.5 volumes of chilled 5M NaCl and 1.5 volumes of 95% ethanol and mixed by quick gentle inversion till the DNA get precipitated and kept in freezer for 10 minutes for the complete precipitation of DNA
10. Centrifuged at 10,000rpm for 10 minutes at 4ºC. Gently poured off the supernatant
11. Washed the DNA pellet with 70% ethanol
12. Centrifuged at 12,000 rpm for 3 minutes, decanted the ethanol and drained well

13. Air dried the pellet, dissolved in 50ml of autoclaved distilled water and stored at -20ºC

Major modification includes use of chloroform: isoamyl alcochol instead of phenol: chloroform.

3.2.3. Electrophoresis of DNA

The quality of isolated DNA was assessed by agarose gel electrophoresis.

Reagents and equipments

1. Agarose – 0.8 per cent (for genomic DNA)
   – 1.3 per cent (for RAPD samples)
   – 2 per cent (for ISSR and SSR samples)
2. 50X TAE buffer (P^H 8.0)
   - Tris buffer
   - 0.5mM EDTA
3. Tracking/loading dye (6X)
   -Bromophenol blue
   -Glycerol
4. Ethidium bromide (stock 10 mg/ml; working concentration 0.5 µg/ml)
5. Electrophoresis unit, power pack, gel casting tray, comb
6. UV transilluminator (Herolab ^{R})
7. BIO-RAD Gel documentation and analysis system

Composition of reagents is provided in Appendix II.

Procedure

1. Cleaned the work area, swabbed gel tray and comb with 100% alcohol
2. Prepared gel tray by sealing the ends with tape. Comb was placed in the gel tray about 1 inch from one end of the tray and positioned the comb vertically such that the teeth are about 1 to 2 mm above the surface of the tray

3. 0.8% agarose was prepared by dissolving 0.8 g agarose in 100 ml 1X TAE buffer and boiled in a micro wave oven until the agarose melted completely and the solution became clear
4. Agarose solution was allowed to cool to about 42 to 45^0C and ethidium bromide was added at a concentration of 0.5µg/ml (4µl) and mixed well
5. Poured the warm gel solution into the tray. Allowed the gel to solidify for about 30 to 45 minutes at room temperature
6. The comb and tape (used for sealing the tray) were removed carefully without disturbing the gel and the gel was placed in the electrophoresis tank containing 1X TAE buffer
7. The tray was kept in such a way that the well side directed towards the cathode
8. The samples were prepared by adding 5µl of DNA sample with 2 µl of tracking dye, mixed well and was loaded into the wells using a micro pipette
9. Loaded suitable molecular weight marker (λDNA *Eco*RI/ *Hind*III double digest) in one lane
10. Electrophoresed at 100 volts until dye has migrated two third the length of the gel
11. Intact DNA appeared as orange fluorescent bands

3.2.4. Gel documentation

The gel documentation was carried out using the BIO-RAD imaging system using PD Quest software. PD Quest is a software package for imaging, analyzing, and data basing 2-D electrophoresis gels. PD Quest can acquire images of gels using any of several BIO RAD imaging systems. An image of a gel is captured using the controls in the imaging device window and displayed on the computer screen. The image was documented in gel documentation system. The gel picture was examined for intactness, clarity of band, presence of contamination such as proteins and RNA.

3.2.5. Determination of the quality and quantity of DNA by Nanodrop method

Nanodrop ND-1000 spectrophotometer was used for detecting the quantity and quality of DNA. It is full spectrum and measures absorbance from 1 µl sample accurately. It measures the concentration of nucleic acids in the samples based on Beer-Lambert Law. Nucleic acid shows absorption maxima at 260 nm whereas proteins show peak absorbance at 280 nm. Absorbance recorded at both wavelengths indicated by the ratio OD_{260}/OD_{280}. A pure solution of double stranded DNA at 50 µg/ml has an optical density of 1.0 at 260 nm and an OD_{260}/OD_{280} ratio of 1.8. Contamination with protein or polyphenol will give OD_{260}/OD_{280} values significantly less than 1.8 and contamination with RNA gives a ratio greater than 1.8 (for pure RNA, OD_{260}/OD_{280} is 2.0). The values between 1.8 and 2.0 indicate that the DNA is pure and free from proteins and other contaminants. The quantity of DNA in the pure sample was calculated using the relation 1 OD_{260} equivalent to 50µg double stranded DNA/ml sample.

1OD at 260 nm = 50 µg DNA(ds)/ml sample

Therefore $OD_{260} \times 50$ gives the quantity of DNA in µg/ml.

3.3. Molecular marker analysis

Three different types of marker system were used for the study which includes RAPD (Random Amplified Polymorphic DNA), ISSR (Inter Simple Sequences Repeats) and SSR (Simple Sequence Repeats).

3.3.1. RAPD assay of coconut genotypes

3.3.1.1. Standardization of PCR conditions

PCR reaction mixtures containing different concentrations and quantities of components such as template DNA (1 µl, 1.5 µl, 2.5 µl), assay buffer B, $MgCl_2$ (1 µl, 1.5 µl, 2.5 µl), Taq DNA polymerase, dNTPs (1 µl and 1.5 µl) and primers (1 µl and 1.5 µl) were tried for the effective amplification of coconut samples. Based on preliminary observations, PCR amplification was performed in a 20 µl reaction mixture in an Eppendorf Master Cycler (Eppendorf, USA) as detailed below.

DNA amplification conditions for PCR

a) Genomic DNA (29.0 ng) - 2.0 µl

b) 10X Taq assay buffer B - 2.0 µl

c) 2.5 mM $MgCl_2$ - 2.0 µl

d) 10mM dNTP mix - 1.5 µl

e) Taq DNA polymerase (1U) - 0.3 µl

f) Primer (100 pM) - 1.5 µl

g) Autoclaved distilled water - 10.7µl

Total volume = 20.0µl

The amplification was done using the following temperature settings

Thermal profile for RAPD assay (modified from Paul *et al*. (2008) in which primer annealing temperature of 42^0C was used)

Initial denaturation - 94^0C for 2 minutes

Final denaturation - 94^0C for 1 minute
Primer annealing - 37^0C for 1 minute
Primer extension - 72^0C for 2 seconds
} 40 cycles

Final extension - 72^0C for 7 minutes

4^0C for infinity to hold the sample

3.3.1.2. Screening of RAPD primers

Thirty five decamer primers in the series of OPA, OPC, OPD, OPF, OPL (Operon Technologies, USA), RN (Reverse Neo) and PNK (49-R) were screened for the DNA amplification using the DNA from Laccadive Ordinary and Chowghat Green Dwarf (25 to 30 ng). Details of the primers used for screening is given in Table1.

The amplified products were loaded to 1.3 per cent agarose gel using 1X TAE buffer stained with ethidium bromide along with 100 bp ladder for comparison.

3.3.1.3. RAPD assay of the selected genotypes

For detecting the variability in the fifty coconut accessions (ten cultivars) selected, RAPD was carried out for the amplification of DNA using the protocol

Table 1. List of Operon decamer primers used for screening coconut genotypes

Sl. No	Primer	Nucleotide Sequence
1	OPA 08	5'GTGACGTAGG3'
2	OPA 10	5'GTGATCGCAG3'
3	OPA17	5'GACCGCTTGT 3'
4	OPA 24	5'AATCGGGCTG3'
5	OPA 26	5'GGTCCCTGAC3'
6	OPA 28	5'GTGACGTAGG3'
7	OPA 29	5'GGGTAACGCC3'
8	OPA 30	5'AGGTGACCGT3'
9	OPA 38	5'CAGCGGTGAC3'
10	OPA 39	5'CAAACGTCGG3'
11	OPC 08	5'TGGACCGGTG3'
12	OPD 10	5'GGTCTACAC3'
13	OPD 20	5'ACCCGGTAAC3'
14	OPE 7	5'AGATGCAGCC3'
15	OPF 9	5'CCAAGCTTCC3'
16	OPL 04	5'GACTGCACAC3'
17	OPS 1	5'GTTTCGCTCC3'
18	OPS 2	5'TGATCCCTGG3'
19	OPS 3	5'CATCCCCCTG3'
20	RN 1	5'CTCACGTTGG3'
21	RN 2	5'ACCAGGGGCA3'
22	RN 3	5'GGTACTCCCC3'
23	RN 4	5'GACCGACCCA3'
24	RN 5	5'ACTGAACGCC3'
25	RN 6	5'GAGACGCACA3'
26	RN 7	5'CAGCCCAGAG3'
27	RN 8	5'ACCTCAGCTC3'
28	RN 9	5'TGCCGGCTTG3'
29	RN 10	5'ACAACTGGGG'3'
30	RN 11	5'TCGCCGCAAA3'
31	RN 12	5'AAGCCTGCGA3'
32	RN 13	5'AGCGTCACTC3'
33	RN 14	5'TCGTGCGGGT3'
34	RN 15	5'CAGCGACTGT3'
35	PNK 49-R1	5'TGCTCTTCCC3'

standardized. Ten decamer primers which gave good amplification of the sample DNA were selected after an initial screening of 35 primers. A negative control (without DNA) was also used. The profile was visualized under UV transilluminator and documented using gel documentation system. The documented RAPD profiles were carefully examined and the bands were scored manually based on intensity and clarity.

3.3.2. Inter Simple Sequence Repeats (ISSR) assay of coconut genotypes

The good quality genomic DNA of (27 to 30ng/µl) isolated from coconut leaf samples were subjected to ISSR analysis as per the procedure reported by Manimekalai and Nagarajan (2006) with slight modification of reaction conditions (2 µl DNA and 1.5 µl dNTP mix)as detailed below. ISSR primers with good resolving power were used for amplification of DNA. The ISSR primers for analysis were selected after an initial screening study of primers.

The amplification was carried out in an Eppendorf Master Cycler (Eppendorf, USA). PCR amplification was performed in a 20 µl reaction mixture and the composition of the reaction mixture consists of

a) Genomic DNA (25 ng)	- 2.0 µl
b) 10X Taq assay buffer B	- 2.0 µl
c) $MgCl_2$	- 2.0µl
c) dNTP mix (10mM each)	- 1.5 µl
d) Taq DNA polymerase (1U)	- 0.3 µl
e) Primer (10 pM)	- 1.5 µl
f) Autoclaved distilled water	- 10.7 µl
Total volume	- 20.0 µl

The amplification was carried out with the following programme

94^0C for 4 minutes	-	Initial denaturation	
94^0C for 45 seconds	-	Denaturation	40 cycles
51^0C for 1 minute	-	Primer annealing	
72^0C for 2 minutes	-	Primer extension	
72^0C for 8 minutes	-	Final extension	

4^0C for infinity to hold the sample

3.3.3.1. Screening of primers for ISSR assay

Forty one primers (ISSR Technologies, USA) were screened for ISSR analysis and are listed in Table 2. Primers were selected from literature based on previous studies in ISSR analysis in coconut.

The amplified products were run on 2 % agarose gel using 1X TAE buffer stained with ethidium bromide along with 100 bp ladder. The profile was visualized under UV transilluminator and documented using gel documentation system Gel DOC-ItTS^{TM} Imaging system (UVP Inc. CA.). The documented ISSR profiles were carefully examined for amplification of bands. The type of bands, pattern of amplification, total number of bands, number of monomorphic bands and polymorphic bands etc. were noted.

3.3.4. SSR (Simple Sequence Repeat) assay

The good quality genomic DNA (30 to 35ng/µl) isolated from coconut genotypes were subjected to SSR analysis as per the procedure reported by Rajesh *et al.* (2008). SSR primers supplied by Sigma, USA with good resolving power were used for amplification of DNA. Highly polymorphic SSR primers were screened for detecting the genetic diversity and population structure of ten coconut cultivars selected for the study. The primers were selected from literatures based on earlier study on coconut for SSR assay.

The amplification was carried out in an Eppendorf Master Cycler (Eppendorf, USA). PCR amplification was performed in a 20 µl reaction mixture consists of

a) Genomic DNA (20 ng)	-	2.0µl
b) 10X Taq assay buffer A	-	2.0µl
c) dNTP mix (10mm each)	-	1.5µl
d) Taq DNA Polymerase (1U)	-	0.3µl
e) Forward Primer (10pM)	-	1.0µl
f) Reverse Primer (10pM)	-	1.0µl

Table 2. List of ISSR primers used for screening of coconut genotypes

Sl. No	Primer	Nucleotide Sequence
1	UBC 354	5'CTAGAGGCCGGCCGC3'
2	UBC 807	5'AGAGAGAGAGAGAGAGT3'
3	UBC 808	5'AGAGAGAGAGAGAGAGC3'
4	UBC 811	5'GAGAGAGAGAGAGAGAC3'
5	UBC 812	5'GAGAGAGAGAGAGAGAA3'
6	UBC 813	5'CTCTCTCTCTCTCTCTT3'
7	UBC 814	5'CTCTCTCTCTCTCTCTA3'
8	UBC 815	5'CTCTCTCTCTCTCTCTG3'
9	UBC 817	5'CACACACACACACACAA3'
10	UBC 818	5'CACACACACACACACAG3'
11	UBC 820	5'GTGTGTGTGTGTGTGTC3'
12	UBC 825	5'ACACACACACACACACT3'
13	UBC 826	5'ACACACACACACACACC3'
14	UBC 834	5'AGAGAGAGAGAGAGAGYT3'
15	UBC 835	5'AGAGAGAGAGAGAGAGYC3'
16	UBC 836	5'AGAGAGAGAGAGAGAGYA3'
17	UBC 840	5'GAGAGAGAGAGAGAGAYT3'
18	UBC 841	5'GAGAGAGAGAGAGAGAYC3'
19	UBC 843	5'CTCTCTCTCTCTCTCTRA3'
20	UBC 844	5'CTCTCTCTCTCTCTCTRC3'
21	UBC 845	5'CTCTCTCTCTCTCTCTRG3'
22	UBC 846	5'CACACACACACACACART3'
23	UBC 847	5'CACACACACACACACARC3'
24	UBC 848	5'CACACACACACACACARG3'
25	UBC 854	5'TCTCTCTCTCTCTCTCRG3'
26	UBC 855	5'ACACACACACACACACYT3'
27	UBC 857	5'ACACACACACACACACYG3'
28	UBC 863	5'AGTAGTAGTAGTAGTAGT3'
29	UBC 865	5'CCGCCGCCGCCGCCGCCG3'
30	UBC 866	5'CTCCTCCTCCTCCTCCTC3'
31	UBC 890	5'VHVGTGTGTGTGTGTGT3'
32	UBC S2	5'CTCTCTCTCGTGTGTGTG3'
33	ISSR 1	5'TTATTATTATTACTAGTGT3'
34	ISSR 2	5'ATTATTATTATTATTCAT3'
35	ISSR 3	5'TTATTATTATTACTT3'
36	ISSR 4	5'ATTATTATTATTATTGTT3'
37	ISSR 5	5'ATTATTGTTGTTGTTTTC3'
38	ISSR 6	5'TTATTATTATTATAA3'
39	ISSR 7	5'ATTATTGTTGTTGTTGTA3'
40	ISSR 8	5' ATTATTATTATTATTGTA3'
41	ISSR 9	5'TTATTATTATTATTATTACT3'

g) Autoclaved Distilled Water - 12.2µl

Total volume - 20.0µl

The thermocycler was carried out with the following programme

94^0C for 5 minutes - Initial denaturation

94^0C for 1 minute - Denaturation

Specific annealing temperature for one min is given to SSR primers } 35 cycles

72^0C for 2 minutes - Primer extension

72^0C for 5 minutes - Final extension

4^0C for infinity to hold the sample

3.3.4.1. Screening of SSR Primers for SSR Analysis

Forty three primers were screened for amplification of coconut DNA for SSR analysis and are listed in Table 3. The PCR conditions for standardization of the reaction and the thermal profile of amplification were standardized by altering quanties of reaction components and conditions provided for amplification. The standardized conditions for amplification of DNA were used for primer screening and final amplification of ten coconut genotypes.

3.3.4.2. **SSR primers selected for diversity and population structure analysis of coconut genotypes**

Out of forty three primer sets screened for SSR analysis, ten primers gave best amplification profile. They were selected for further amplification after standardizing annealing temperatures of each primer by subjecting the DNA to different annealing temperatures in a gradient thermocycler (Eppendorf, USA). The primers were selected based on their banding pattern.

The amplified products were run on 2 per cent agarose gel using 1X TAE buffer stained with ethidium bromide along with 100 bp ladder. The profile was visualized under UV transilluminator and documented using gel documentation system Gel DOC-ItTS^{TM} Imaging system (UVP Inc. CA.). The documented SSR profiles were carefully examined for amplification of DNA. The total number of

Table 3. List of SSR primers used for screening of coconut genotypes

Sl No	Name of Primers	Sequence
1	SSR 34	F 5'TTCGGATAAAGCAATCCAC3' R5'TCGATTGTGTACCAACGTCC3'
2	SSR 43	F 5'CTCCAAATTGGGCAATAACA3' R5'TTAGGAAGTTGCATTAGGCCA3'
3	SSR 47	F5'TCCTCAAGAAATGAAGCTCTGA3' R 5'CCTTGGAGATAACAACCACAA3'
4	SSR 72	F 5'GGTTCCCTTCTCTCTTTGTCC3' R 5'GCGTGTTCTTCGATTTGACA3'
5	SSR 74	F 5'ACTCACCATGGCTGCTTCTT3' R 5'TTTCTTGAAGGGTCTTTCCC3'
6	SSR 85	F 5'ATCCGTTAGCTATTGTGCCG3' R 5'TTGCCATGCACTTATCTTCG3'
7	SSR 94	F 5'AATCAGATCCTTGCCCTTGA3' R 5'AGCTGAGAAAGAGCAGCCAT3'
8	SSR 108	F 5'TGTGTTGGATGTTTGGCACT3' R 5'GCCATTGAAACTTGCAGAGA3'
9	SSR 122	F 5'ACGGGATTGTACCCAATCAA3' R 5'AGGGTTTGAAGAGGAGGAGG3'
10	SSR 188	F 5'TGCAGTGAGTCTCGATTTGC3' R 5'GGTCTCATTGCAGATAGGGC3'
11	SSR 214	F 5'AAATTCCCAACACTTGCCAC3' R 5'CCCACCCACTATCCAAACCC3'
12	SSR 218	F 5'GTGGTTATCCCAAGACCCAA3' R 5'CGCCAGTCTTCCTCTGACTT3'
13	SSR 223	F 5'TGGCTGCCCTCTTCTCTGTTT3' R 5'TTTCTTGAAGGGTCTTTCCC3'
14	SSR 248	F 5'GCATTCGCTGTAGCTCGTTT3' R 5'GGGAGCTTCATCATAGTAACG3'
15	SSR 276	F 5'CTCCGGCAAGAGTGAACATT3' R 5'CGACGGAGTACTTCGCATTT3'
16	SSR 286	F 5'AGCTATGGAGTTTCAGGACCA3' R 5'ATTCAGGTAGCATGGAACGC3'
17	SSR 296	F 5'CCGGAACAAGTCCCTTCATA3' R5'TCAGCCAAGTTCATGGTACATC3'
18	SSR 301	F 5'TTTCCACCTCAAACCACTCC3' R 5'CCCTTTGACCTGTGCCA3'
19	SSR 304	F 5'TCCTCCGGTTGTTACTCCAC3' R 5'TTAGCACTTCCACCGATTCC3'
20	SSR 318	F 5'GCAGAGGATATTGCATTCGC3' R 5'CAAACCGAACTCATCAAGGG3'
21	SSR 326	F 5'TTAGGGCAGCTTACGACTGGA3' R 5'GAGCCAGAGGTCCTTCAGTG3'
22	SSR 350	F 5'GGAATAACCTCTAACTGCGGG3' R 5'CGATGCCTTCATTTGGACTT3'
23	SSR 360	F 5'ATCAGCCCTTCCACTGATTG 3' R 5'TAAGCAACCACCAATGTTCA3'
24	SSR 378	F 5'-TGTGTCTTTTATATTTTTGATG-3' R 5'-TATTAGTAGTTCTCCCTTTTGA-3'

Table 3 continued. List of SSR primers used for screening coconut genotypes

Sl No	Name of Primers	Sequence
25	SSR 479	F 5'TGTAAGAGTGTCTGCCTGCAC 3' R 5'ATGGGTTCGGGTTAGCTCTT3'
26	SSR 526	F 5'AGGGTCCTTCGTTTGGAACT3' R 5'GCATTCCACTTGTGAAGCAT3'
27	SSR 555	F5'TTGATATTAACCATGGCAGCAG3' R 5'TTGATGGGATTGCACAGAAA3'
28	SSR 565	F 5'GAGGTGATGAGAACTCGCC3' R 5'TCAGAGGCTTCTGGGTCAGT3'
29	SSR 578	F 5'ATTCCCAGCACAACCAGACT3' R 5'GTTGGTGGATGAAATTTGTG3'
30	SSR 593	F 5'TGGCATGAACAACAACCAAT3' R 5'AGGSSGTTGCATTAGGCCAT3'
31	SSR 603	F5'GAAGGGACAATTCACAGAGTTTG3' R 5'CCTTCAACTTCACCACCACC3'
32	SSRB 60800	F5'AGATCAAGATTAAGACCACCGGAGC3' R5'TCAGGTGCGAGTGTTACAATCTCTG3'
33	SSR PT 30452	F5'TTCGGTGGGTCTTCCAAATA3' R5'GACTTCAATTTCTGGGAGCG3'
34	CnCir B6	F 5'GAGTGTGTGAGCCAGCAT3' R 5'ATTGTTCACAGTCCTTCCA3'
35	CnCir B12	F 5'GCTCTTCAGTCTTTCTCAA3' R 5'CTGTATGCCAATTTTTCTA3'
36	CnCir C3	F 5'AGAAAGCTGAGAGGGAGATT3' R 5'GTGGGGCATGAAAAGTAAC3'
37	CnCir C7	F 5'ATAGCATATTGGTTTTCCT3' R 5'TGCTCCAGCGTTCATTCA3'
38	CnCir C12	F 5'ATACCACAGGCTAACAT3' R 5'AACCAGAGACATTTGAA3'
39	CnCir E2	F 5'TCGCTGATGAATGCTTGCT3' R 5'GGGGCTGAGGGATAAACC3'
40	CnCir E10	F 5'TGGGTTCCATTTCTTCTCTCATC3' R 5'GCTCTTTAGGGTTCGCTTTCTTAG3'
41	CnCir F2	F 5'GGTCTCCTCTCCCTCCTTATCTA3' R 5'CGACGACCCAAAACTGAACAC3'
42	CnCir G11	F 5'AATATCTCCAAAAATCATCGAAAG3' R 5'TCATCCCACACCCTCCTCT3'
43	CnCir H4	F 5'TTAGATCTCCTCCCAAAG3' R 5'ATCGAAAGAACAGTCACG3'

amplicons, number of polymorphic, monomorphic amplicons and the banding pattern were analyzed.

3.3.4.3. Data analysis of molecular markers

Reproducible, well resolved gel profiles were scored manually. Clear and distinct bands were considered scorable and each band was scored as presence (1) or absence (0) with respect to the 10 genotypes.

PIC was calculated according to Anderson (1993) as $PIC = 1 - \sum_{1}^{n} Pi^2$

where Pi is the frequency of the i^{th} band and n is the number of bands observed.

Diversity and population genetic structure analysis was carried out using the software NTSYS and DARwin.

Results

4. RESULTS

Genetic diversity studies provides the understanding of genetic relationships among populations and hence directs assigning lines to specific heterogenous groups usable in identification of parents for hybridization. So the research programme on diversity and population structure analysis in coconut was conducted to determine the diversity and population structure analysis in ten coconut cultivars commonly used for seed production in Kerala. The results of various aspects of the experiment are presented in this chapter.

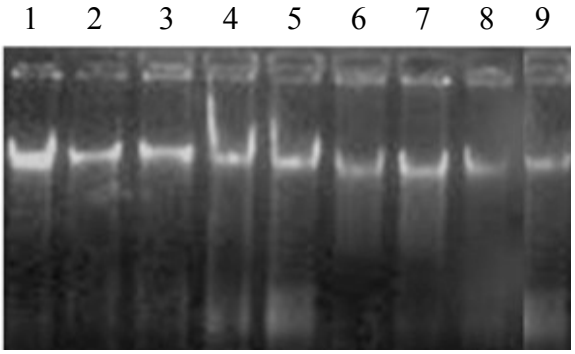
4.1. Standardization of genomic DNA extraction

Tender leaves collected from the first emerged frond of coconut palm were used for DNA isolation. The leaf sample used was from the tip and middle portion of the coconut leaf as it was very soft and tender for easy grinding. The DNA was isolated from fifty coconut genotypes belonging to ten coconut cultivars.

The protocols reported by Rogers and Bendich (1994), Upadhyay *et al*. (1999) and Porebsk*i et al*. (1997) with certain modifications (the use of phenol and sodium acetate (2M) during extraction is avoided and chloroform:isoamyl alcohol (24:1) was used instead of phenol:chloroform reported by Porebsk*i et al*. (1997)). were tried for the isolation of good quality genomic DNA from coconut samples. The quality of the DNA isolated by these methods was tested by agarose gel electrophoresis and spectrophotometer. Among them, the protocol reported by Porebski *et al*. (1997) with certain modifications yielded good quality DNA with sufficient quantity (Plate 4). The DNA isolated by this method was pure and intact. The DNA obtained by other methods was contaminated with protein and polysaccharides (Plate 4). So modified protocol by Porebsk*i et al*. (1997) was selected for genomic DNA extraction from fifty coconut genotypes.

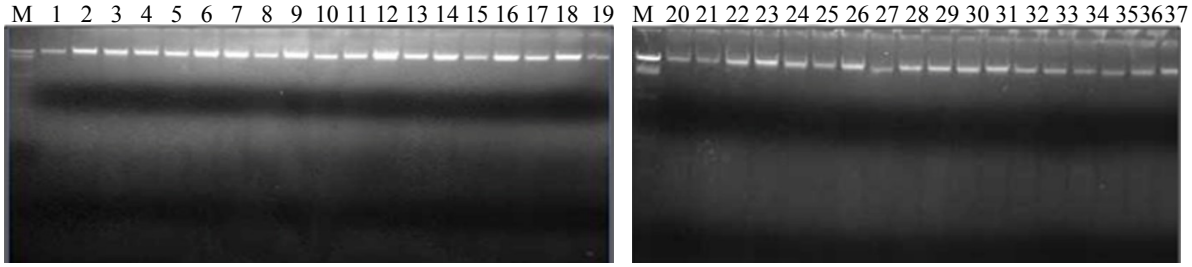
4.2 Determination of the quality and quantity of DNA

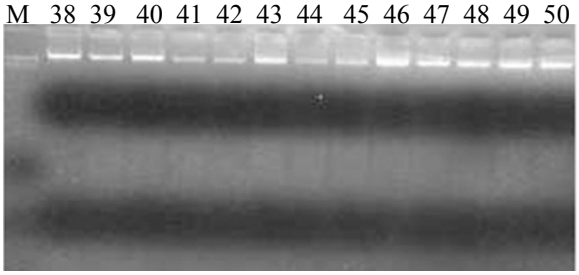
The quality and quantity of DNA isolated was determined by agarose gel electrophoresis (Plate 5) and Nanodrop ND-1000 spectrophotometer. In all fifty



1 & 8 : modified Porebski *et al*. (1997) method ; 2 & 3 : modified SDS method;
4 & 5 : modified CTAB method; 6 & 7: SDS method; 9 :CTAB method

Plate 4. Gel profile of coconut DNA samples by different methods





M : Marker Lambda DNA (*Eco* RI/ *Hind* III digest)

1-5 : Laccadive Ordinary Tall 1 to 5
6-10 : Tiptur Tall1 to 5
11-15 : Komadan1 to 5
16-20 : Kuttiadi1 to 5
21-25 : Malappuram1 to 5
26-30 : Kasaragode 1 to 5
31-35 : Chowghat Orange Dwarf 1 to 5
36-40 : Chowghat Green Dwarf 1 to 5
41-45 : Malayan Yellow Dwarf 1 to 5
46-50 : Gangabondam

Plate 5. Gel profile of fifty coconut DNA samples

coconut samples isolated, intact, clear band was observed on agarose gel which indicated good quality DNA without degradation. Spectrophotometer method showed that the ratio of UV absorbance ranged between 1.8 to 2.0 which indicates the purity of DNA. The quantity of DNA was also good and is ranged from 800 to 3500 ng/µl per gram of leaf sample (Table 4).

4.3. Molecular marker analysis

The protocols (reaction mixture and thermal profile) for different marker systems such as RAPD, ISSR and SSR were standardized with the DNA from the cultivar, Chowghat Green Dwarf. A total of 119 primers suitable to the three markers were screened with the genomic DNA of one selected cultivar (Chowghat Green Dwarf) with the standardized protocols. The best 10 primers in each marker (RAPD and SSR) and 11 primers for ISSR were finally used for amplifying the DNA from the different genotypes.

4.3.1. RAPD assay of coconut genotypes

4.3.1.1. Standardization of PCR conditions

Both the proportion of components in the reaction mixture and the thermal profile were standardized for good amplification of coconut samples.

4.3.1.2. Screening of RAPD primers

A total of 35 decamer primers - 19 from Operon (USA) series, 15 from RN (Reverse Neo) series and one from PNK (49-R) were screened for amplification of DNA from the genotypes, Laccadive Ordinary and Chowghat Green Dwarf using the standardized protocol for RAPD assay. The amplification patterns of these primers are presented in Plate 6.

Out of the 19 Operon primers, only two were selected for the final amplification of the fifty coconut genotypes *viz*., OPA 28 which generated four distinct and clear amplicons with high intensity and OPA 39, which produced seven clear and intact bands among them four were with high intensity and the three were

Table 4. Quality and quantity of DNA isolated from fifty coconut genotypes

Sl. No	Varieties	UV absorbance at 260 nm (A_{260})	UV absorbance at 280 nm (A_{280})	$A_{260}/_{280}$	Quantity (ng/µl)
1	Laccadive Ordinary Tall 1	49.714	26.034	1.91	2485.70
2	Laccadive Ordinary Tall 2	52.884	27.717	1.91	2644.45
3	Laccadive Ordinary Tall 3	49.770	25.551	1.95	2488.48
4	Laccadive Ordinary Tall 4	49.618	25.528	1.94	2480.92
5	Laccadive Ordinary Tall 5	50.224	25.366	1.93	2811.20
6	Tiptur Tall 1	55.509	28.399	1.95	2775.45
7	Tiptur Tall 2	34.075	17.040	2.00	1703.75
8	Tiptur Tall 3	38.227	19.543	1.96	1911.36
9	Tiptur Tall 4	57.444	29.054	1.98	2872.19
10	Tiptur Tall 5	61.118	32.089	1.90	3055.91
11	Komadan 1	55.509	28.399	1.95	2623.70
12	Komadan 2	34.075	17.040	2.00	2177.03
13	Komadan 3	38.227	19.543	1.96	2306.90
14	Komadan 4	57.446	29.054	1.98	2593.03
15	Komadan 5	61.118	32.089	1.90	2948.60
16	Kuttiadi 1	69.341	36.256	1.91	3467.06
17	Kuttiadi 2	51.675	26.479	1.95	2584.25
18	Kuttiadi 3	27.641	14.247	1.94	1382.03
19	Kuttiadi 4	45.671	23.276	1.96	2283.55
20	Kuttiadi 5	53.692	27.808	1.93	2684.62
21	Malappuram 1	43.535	22.433	1.94	2176.75
22	Malappuram 2	36.231	18.529	1.96	1811.56
23	Malappuram 3	16.373	8.617	1.90	2818.66
24	Malappuram 4	37.879	19.04	1.94	1893.93
25	Malappuram 5	22.314	12.061	1.85	1615.68

Table 4 continued. Quality and quantity of DNA isolated from fifty coconut genotypes

Sl. No	Varieties	UV absorbance at 260 nm (A_{260})	UV absorbance at 280 nm (A_{280})	$A_{260}/_{280}$	Quantity (ng/µl)
26	Kasaragode 1	69.341	36.256	1.91	3467.06
27	Kasaragode 2	51.675	26.474	1.95	2584.25
28	Kasaragode 3	27.641	14.247	1.94	1382.03
29	Kasaragode 4	45.671	23.276	1.96	2283.55
30	Kasaragode 5	33.692	27.808	1.93	2684.62
31	Chowghat Orange Dwarf 1	76.907	40.303	1.85	3845.72
32	Chowghat Orange Dwarf 2	71.749	36.596	1.88	3587.44
33	Chowghat Orange Dwarf 3	38.146	18.448	1.93	1907.30
34	Chowghat Orange Dwarf 4	51.181	25.466	1.92	2559.07
35	Chowghat Orange Dwarf 5	44.460	21.579	1.98	2223.28
36	Chowghat Green Dwarf 1	34.570	17.283	2.00	1728.49
37	Chowghat Green Dwarf 2	55.215	27.428	2.00	2760.74
38	Chowghat Green Dwarf 3	46.059	22.780	1.98	2302.93
39	Chowghat Green Dwarf 4	60.524	30.347	1.91	3026.19
40	Chowghat Green Dwarf 5	39.008	19.224	2.03	2520.76
41	Malayan Yellow Dwarf 1	71.166	36.770	1.96	3598.25
42	Malayan Yellow Dwarf 2	59.218	29.369	2.00	2960.68
43	Malayan Yellow Dwarf 3	57.610	28.676	1.98	2880.51
44	Malayan Yellow Dwarf 4	41.541	21.273	1.95	2077.03
45	Malayan Yellow Dwarf 5	27.112	13.517	2.00	1355.58
46	Gangabondam 1	38.702	18.752	2.03	1935.10
47	Gangabondam 2	24.478	11.774	1.98	1225.92
48	Gangabondam 3	43.778	21.853	1.95	2188.92
49	Gangabondam 4	25.801	12.553	1.80	1290.07
50	Gangabondam 5	40.476	20.243	1.87	2023.78

faint. In the remaining ones, clear and intense amplicons were limited to one, two or three (OPA08, OPA10, OPA17, OPA24, OPA 29, OPA 38, OPD10, OPD20, OPF9, OPA26, OPA30, OPE7 and OPL04) or completely failed to amplify coconut DNA (OPC08, OPS 01, OPS 02 and OPS 03) (Plate 6; Table 5). Hence these primers were not selected for the final amplification of the fifty coconut genotypes.

Out of 15 RN primers screened, good amplification of the coconut DNA was obtained only in eight primers *viz*., RN03, RN 04, RN 05, RN 07, RN 08, RN 09, RN 11and RN 12 as detailed in Table 5. These primers produced 5-9 bands which could be scored without any ambiguity (Plate 6). The remaining seven primers were not able to generate sufficient number of clear and distinct amplicons. So these were not selected for final amplification of the fifty coconut genotypes.

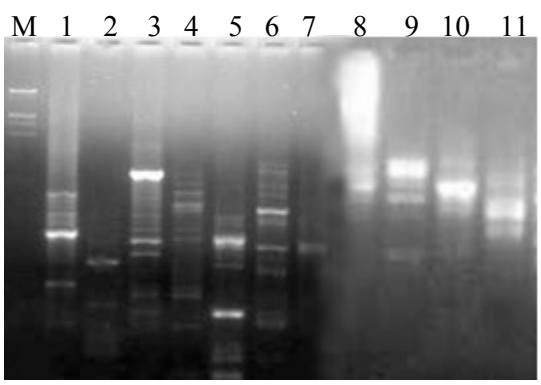
One primer was tested under PNK series *viz.*, PNK 49-R1. It could generate only one amplicon. Hence it was also not selected for final amplification of coconut genotypes.

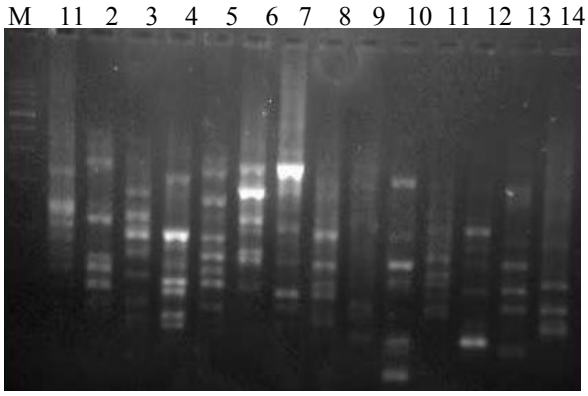
4.3.1.3. Diversity analysis within each cultivar using selected RAPD primers

Out of thirty five RAPD primers screened for DNA amplification of coconut genotype, the best ten primers producing good banding pattern were selected (Table 6) for the final amplification of fifty coconut palms belonging to ten cultivars.

Amplification of DNA from individual palms within each cultivar was carried out using selected primers from each marker system. However RAPD assay with primer RN3 and RN5 showed that the variability within each cultivar is very less (Plates7 and 8).

Amplification of fifty genotypes with RN3 (Plate 7) produced five amplicons in Laccadive Ordinary and two polymorphic bands were absent in palm no 2. The individual palms (five) of Tiptur Tall, Kuttiadi, Kasaragode, Malappuram, Chowghat Green Dwarf, Malayan Yellow Dwarf and Gangabondam generated identical amplification pattern with respect to RN3. In Komadan, only palm no1 differed from

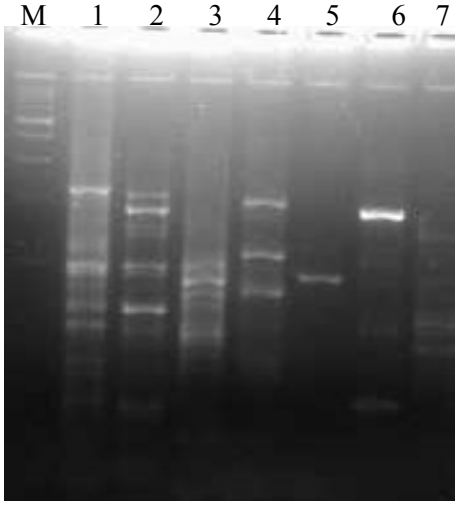


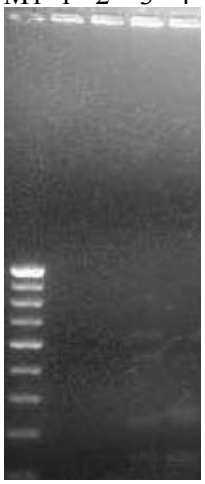


M : Marker Lambda DNA (*Eco* RI/ *Hind* III double digest)

1 : OPL 04
2 : OPD 10
3 : OPA 17
4 : OPA 26
5 : OPA 29
6 : OPA 39
7 : PNK 49-R1
8 : RN 06
9 : OPF 09
10 : OPA 08
11 : OPA 10

1 : RN1
2 : RN2
3 : RN3
4 : RN4
5 : RN5
6 : RN7
7 : RN8
8 : RN9
9 : RN10
10 : RN11
11 : RN12
12 : RN13
13 : RN14
14 : RN15





M : Marker Lambda DNA (*Eco* RI/ *Hind* III digest)
1 : OPA 24
2 : OPA 28
3 : OPA 30
4 : OPA 38
5 : OPD 10
6 : OPD 20
7 : OPE 07

M1: 100 bp ladder
1 : OPC 08
2 : OPS 01
3 : OPS 02
4 : OPS 03

Plate 6. Screening of RAPD primers for amplification of coconut DNA

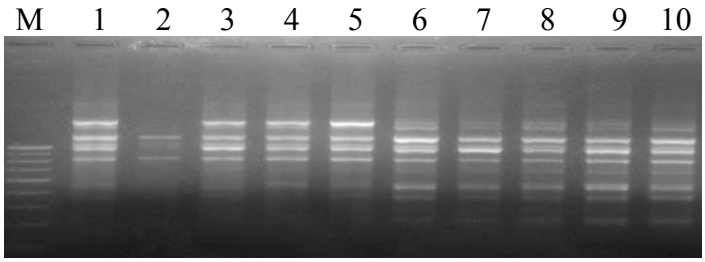


Plate 7 (a)

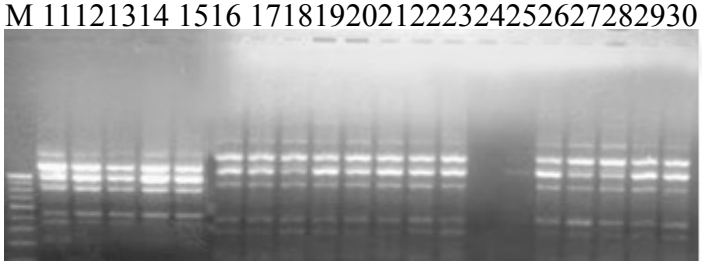


Plate 7 (b)

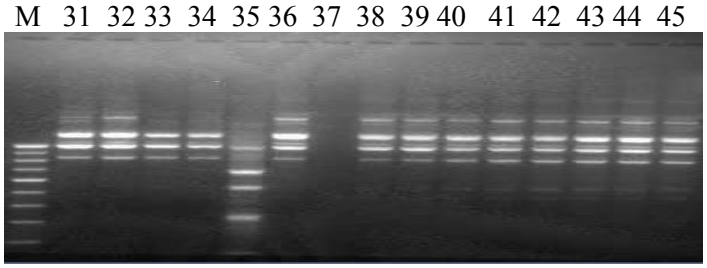


Plate 7 (c)

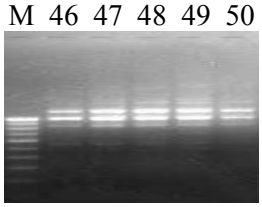


Plate 7 (d)

M : 100 bp ladder

1-5 : Laccadive Ordinary Tall 1-5 6-10 : Tiptur Tall 1-5 11-15 : Komadan1-5
16-20 : Kuttiadi 1-5 21-25 : Malappuram1-5 26-30 : Kasaragode1-5

Plate 7. Amplification (RAPD) of fifty (a)1-15 (b) 16-30(c)31-45(d) 46-50 coconut genotypes with selected decamer primer RN3

Table 5. Screening of 35 random primers for RAPD assay in coconut

Sl No	Primer	Amplification pattern			
		No of amplicons	Type of amplicons		Remarks
			with high intensity	faint	
1	OPA 08	3	1	2	----
2	OPA 10	3	1	2	----
3	OPA17	6	2	4	----
4	OPA 24	5	2	3	----
5	OPA 26	6	2	4	----
6	OPA 28	6	4	2	Selected
7	OPA 29	3	2	1	----
8	OPA 30	4	0	4	
9	OPA38	3	3	0	-----
10	OPA 39	7	4	3	Selected
11	OPC 08	2	1	1	----
12	OPD 10	1	1	0	----
13	OPD 20	2	2	0	----
14	OPE 7	4	1	3	----
15	OPF 9	3	1	2	----
16	OPL 04	6	1	5	----
17	OPS 1	0	0	0	----
18	OPS 2	0	0	0	----
19	OPS 3	0	0	0	----
20	RN 1	4	1	3	----
21	RN 2	6	2	4	----
22	RN 3	7	5	2	Selected
23	RN 4	8	5	3	Selected
24	RN 5	9	6	3	Selected
25	RN 6	1	0	1	----
26	RN 7	7	5	2	Selected
27	RN 8	8	5	3	Selected
28	RN 9	8	6	2	Selected
29	RN 10	1	0	1	----
30	RN 11	7	4	3	Selected
31	RN 12	5	3	2	Selected
32	RN 13	5	2	3	----
33	RN 14	6	2	4	----
34	RN 15	3	2	1	----
35	PNK 49-R1	1	1	0	----

Table 6. Sequence data of the random decamer primers selected for coconut genotypes

Sl. No	Primer	Nucleotide Sequence
1	OPA 28	5’GTGACGTAGG3’
2	OPA 39	5’CAAACGTCGG3’
3	RN 3	5’GGTACTCCCC3’
4	RN 4	5’GACCGACCCA3’
5	RN 5	5’ACTGAACGCC3’
6	RN 7	5’CAGCCCAGAG3’
7	RN 8	5’ACCTCAGCTC3’
8	RN 9	5’TGCCGGCTTG3’
9	RN 11	5’TCGCCGCAAA3’
10	RN 12	5’AAGCCTGCGA3’

the rest of the four palms by the presence of single polymorphic amplicon, all others gave identical banding pattern. Palm no 1 and 2 gave identical amplification pattern in Chowghat Orange Dwarf and differed from palm no 3 and four by the presence of single polymorphic band. Palm no 5 in this group showed different amplification pattern to that of others by the presence of three polymorphic bands.

In Laccadive Ordinary, the primer RN5 produced a total of seven amplicons in which 6 were monomorphic. The single polymorphic band was absent in only one palm (Laccadive Ordinary4), in the rest of the four palms all the seven bands being identical. Similarly in Tiptur Tall, only palm no 3 differed from the rest of the four palms with respect to the absence of a single amplicon. Two palms in Komadan (palm no 2 and 5) differed from the rest by the absence of one or two amplicons. All the five palms in Kuttiadi gave identical banding pattern with respect to the primer RN5 (Plate 8). In case of Malappuram, this primer produced eight amplicons. Among this, two palms (palm no 4 and 5) differed from the rest by the absence of two amplicons. Palm no 2 was not amplified by this primer. Palm no 4 among Kasaragode group amplified only two bands against eight bands being amplified by other palms in this group. RN5 produced eight monomorphic bands in Chowghat Orange Dwarf and Chowghat Green Dwarf. Malayan Yellow Dwarf also produced eight amplicons and one single polymorphic band was absent in palm no2. The rest four palms produced similar banding pattern. All the five palms in the Gangabondam group gave identical amplification pattern.

With the two selected RAPD primers (RN3 and RN5), each cultivar (represented by five palms) produced amplicons ranging from 10-15 (Laccadive Ordinary-12, Tiptur Tall-15, Komadan-13, Kuttiadi-14, Malappuram-14, Kasaragode-13, Chowghat Orange Dwarf-12, Chowghat Green Dwarf-13, Malayan Yellow Dwarf-14 and Gangabondam-10). The polymorphism percentage calculated was less than 10 among the cultivars, Tiptur Tall, Kuttiadi, Kasaragode and Chowghat Orange Dwarf and less than 20 per cent in case of Laccadive Ordinary,

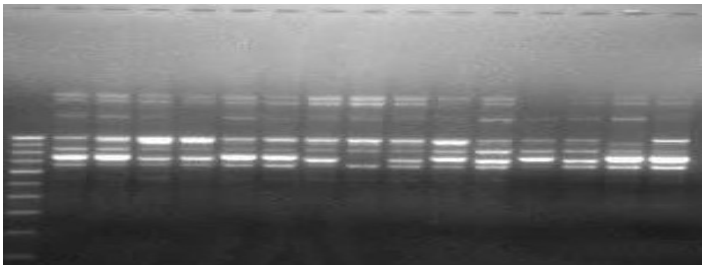


Plate 8 (a)

M 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30

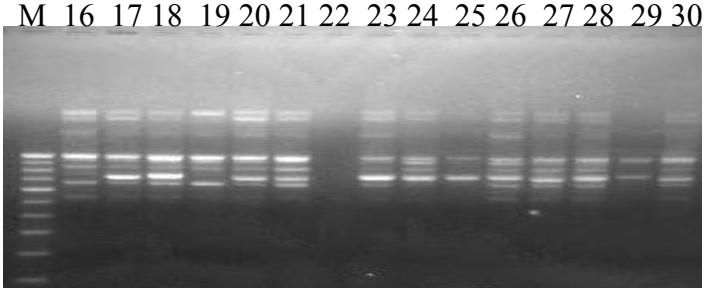


Plate 8 (b)

M 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45

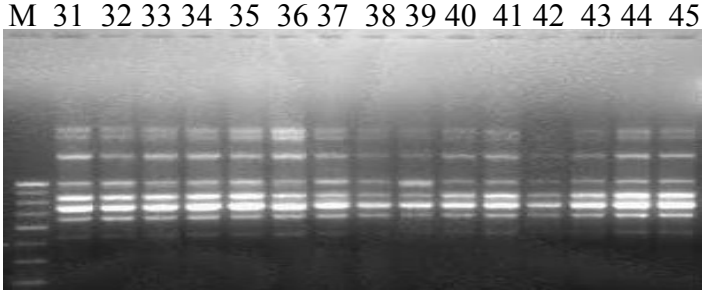


Plate 8 (c)

M 46 47 48 49 50

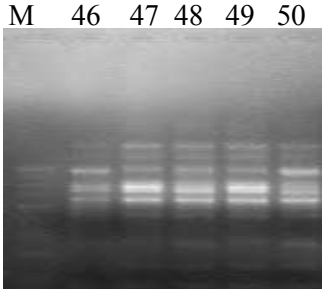


Plate 8 (d)

M : 100 bp ladder

1-5 : Laccadive Ordinary Tall 1-5 6-10 : Tiptur Tall 1-5 11-15 : Komadan1-5
16-20 : Kuttiadi 1-5 21-25 : Malappuram1-5 26-30 : Kasaragode1-5

Plate 8. Amplification (RAPD) of fifty (a) 1-15 (b) 16-30 (c) 31-45 (d) 46-50 coconut genotypes with selected decamer primer RN5

Komadan and Malappuram. The three cultivars Chowghat Green Dwarf, Malayan Yellow Dwarf and Gangabondam could not produce any polymorphic bands.

Since RAPD marker system failed to bring out the variability between palms within a cultivar, the DNA from the five palms in each cultivar was pooled to study the diversity between the ten cultivars.

4.3.1.4. Diversity and population structure analysis of ten coconut cultivars using selected RAPD primers

The gel profile for amplification of 10 coconut cultivars with selected RAPD primers is provided in Plate 9a and 9b. Per cent of polymorphism was worked out for each primer. The details of amplification are presented in Table 7. Observations were as follows.

OPA 28

The primer OPA 28 could generate a total of seven clear, distinct and reproducible bands out of which five were polymorphic. The per cent of polymorphism was 71.42. The amplification profile is given in Plate 9a. 1.

OPA 39

A total of four amplicons were produced by the primer OPA 39. They were clear, distinct and reproducible. It could generate three polymorphic bands out of four amplicons (Plate 9a. 2) and the per cent of polymorphism was 75.

RN 3

RN 3 generated four clear, distinct and reproducible amplicons and only one of them was polymorphic (Plate 9a. 3). The per cent of polymorphism was very less (25%) compared to other primers.

RN 4

The primer RN 4 was able to generate three amplicons. Two bands were very clear, distinct and reproducible and one was faint. It could not generate any polymorphic band and the per cent of polymorphism was zero (Plate 9a. 4).

RN 5

Amplification of ten coconut cultivars with the selected primer RN 5 produced eleven clear, distinct and reproducible amplicons (Plate 9a. 5). Five amplicons were polymorphic giving 45.45 per cent polymorphism.

RN 7

The decamer primer RN 7 could generate five clear, distinct and reproducible amplicons, only one was polymorphic (Plate 9b. 1) giving a polymorphism percentage of 20.

RN 8

Ten amplicons were produced by this primer and three of them were polymorphic. The amplicons were clear, distinct and reproducible (Plate 9b. 2). The polymorphism percentage recorded was 30.

RN 9

RN 9 was able to amplify a total of six amplicons and out of which only two were polymorphic. The bands were clear, distinct and reproducible (Plate 9b. 3). The polymorphism percentage calculated was 33.33.

RN 11

Seven amplicons were produced by this primer and four of them were polymorphic. The bands were clear, distinct and reproducible (Plate 9b. 4). The polymorphism percentage recorded was 57.14.

RN 12

RN 12 could generate a total of eight amplicons which were clear, distinct and reproducible. The polymorphism percentage was less (25) as it could detect only two polymorphic amplicons out of eight amplicons.

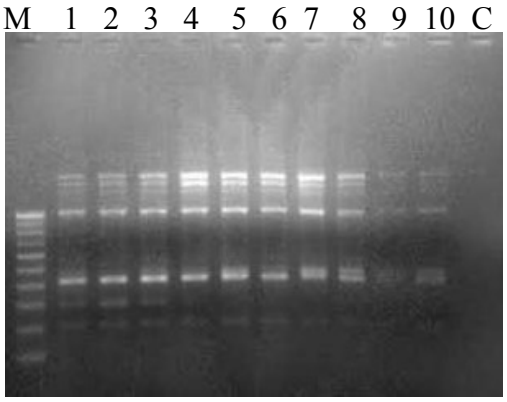
The gel profile showing the amplification pattern of RN12 with the ten coconut cultivars was presented in Plate 9b. 5.

RAPD analysis using ten selected primers produced a total of sixty five markers between the ten coconut genotypes (Table 7). The number of scorable markers produced by each primer ranged from three (RN 3) to eleven (RN 5) with an average of 6.5 markers per primer. The molecular weight of these markers ranged from 200 bp to 1856 bp, with an average molecular weight of 900 bp. The polymorphic bands were 40 per cent of the total, each primer detecting on an average 2.6 polymorphic bands.

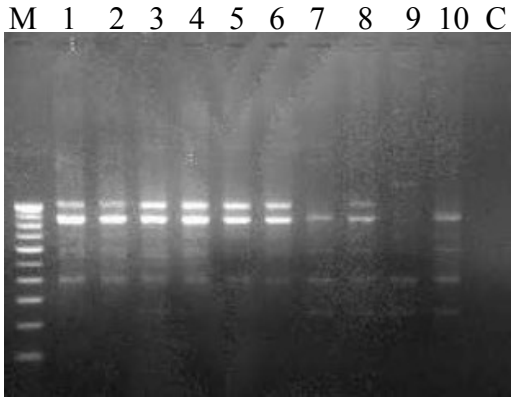
One unique amplicon of 400 bp with RN8 primer was found in the cultivar 'Laccadive Ordinary'. Another amplicon of 262 bp with the OPA39 primer was specific to the Dwarfs and intermediate type. An amplicon (300 bp) with the primer OPA28 was found specific to the three tall cultivars namely Laccadive Ordinary, Tiptur Tall and Komadan. Another unique amplicon of 400 bp with RN5 primer was found only in the cultivar Chowghat Green Dwarf. An amplicon of 400 bp was found only in Laccadive Ordinary and Komadan.

The presence or absence of data was entered into a binary data matrix and was used for calculating the similarity coefficient using Dice coefficient (Nei and Li, 1979) using software DARwin (Version5.0.158) (bootstrapping 5000). Cluster analysis was done using the UPGMA method and dendrogram was constructed by neighbor joining (Saitou and Nei, 1987). The dendrogram generated using RAPD data is given in Fig 1a. The details of clusters are given in Table 8a.

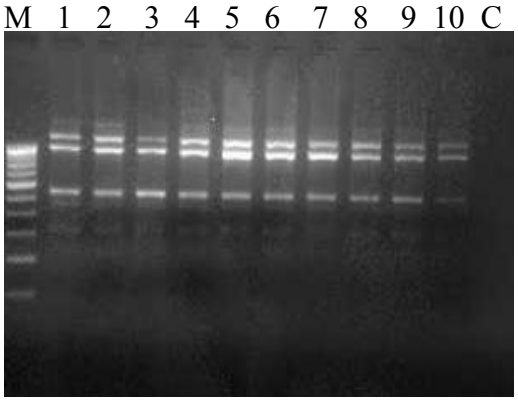
1. Primer OPA 28



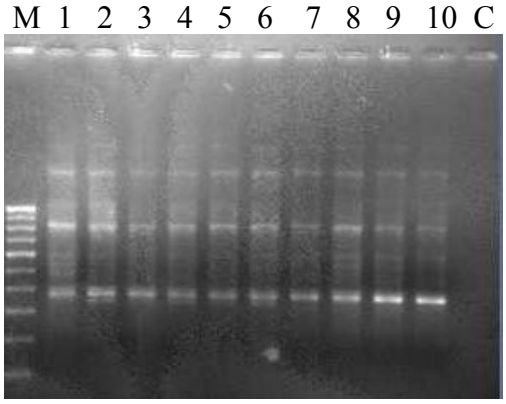
2. Primer OPA 39



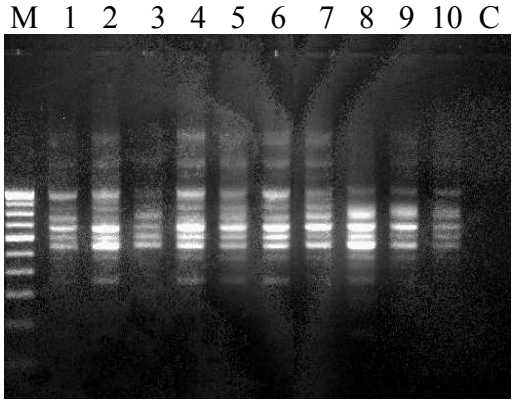
3. Primer RN3



4. Primer RN4



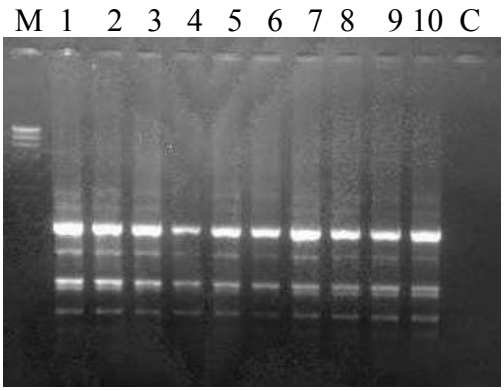
5. Primer RN5



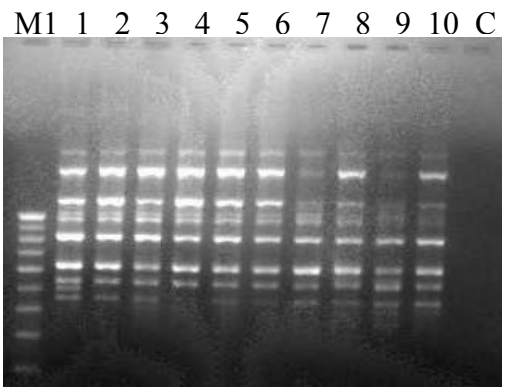
M : 100 bp ladder
1 : Laccadive Ordinary Tall
2 : Tiptur Tall
3 : Komadan
4 : Kuttiadi
5 : Kasaragode
C : Control
6 : Chowghat Orange Dwarf
7 : Chowghat Green Dwarf
8 : Malayan Yellow Dwarf
9 : Gangabondam
10 : Malappuram

Plate 9a. Amplification of coconut genotypes with selected decamer primers for RAPD assay

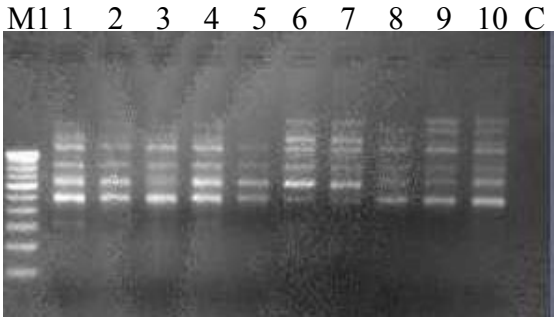
1. Primer RN7



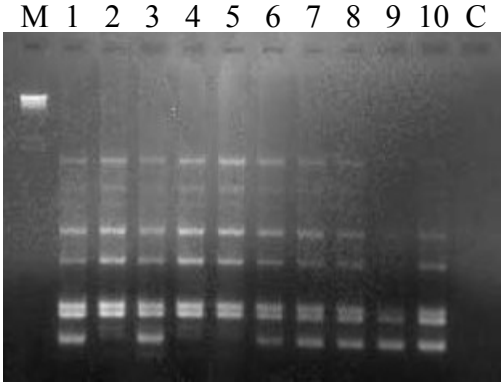
2. Primer RN8



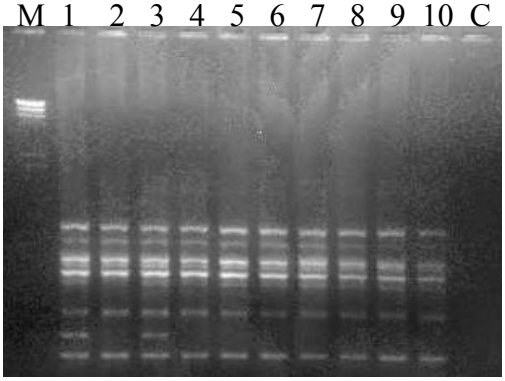
3. Primer RN9



4. Primer RN11



5. Primer RN12



M : Marker Lambda DNA(EcoR1/HindIII double digest)
M1 : 100 bp ladder C : Control

1 : Laccadive Ordinary Tall
2 : Tiptur Tall
3 : Komadan
4 : Kuttiadi
5 : Kasaragode
6 : Chowghat Orange Dwarf
7 : Chowghat Green Dwarf
8 : Malayan Yellow Dwarf
9 : Gangabondam
10 : Malappuram

Plate 9 b. Amplification of coconut genotypes with selected decamer primers for RAPD assay

Table 7. Details of amplification with selected primers for RAPD assay in coconut

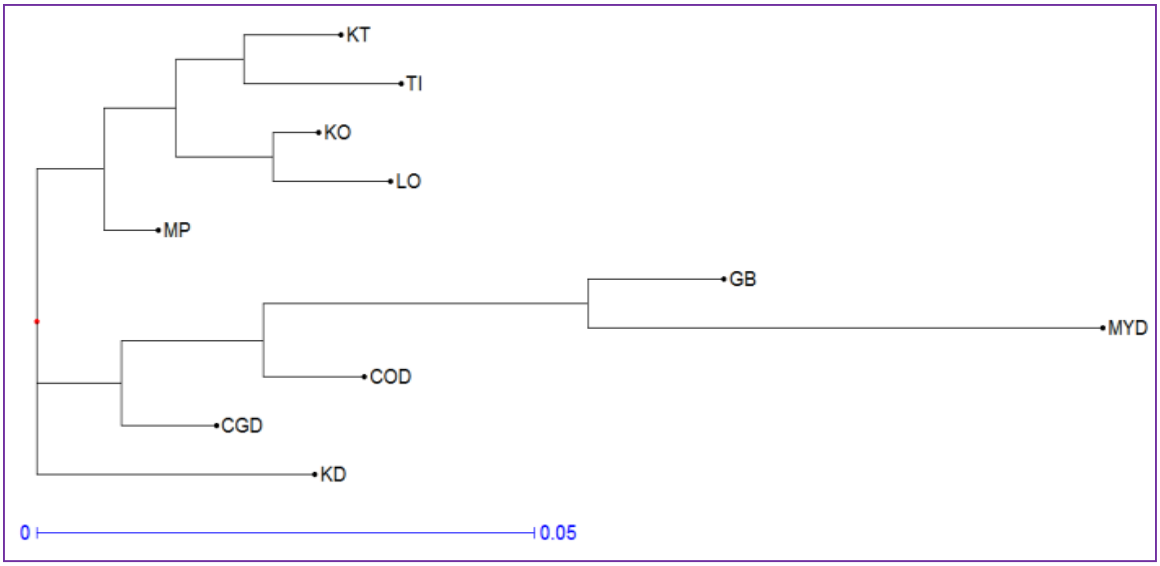
Sl No	Primer	Total no of amplicons	No of polymorphic amplicons	No of monomorphic amplicons	Polymorphism (%)
1	OPA 28	7	5	2	71.42
2	OPA 39	4	3	1	75.00
3	RN 3	4	1	3	25.00
4	RN 4	3	0	3	0.00
5	RN 5	11	5	6	45.45
6	RN 7	5	1	4	20.00
7	RN 8	10	3	7	30.00
8	RN 9	6	2	4	33.33
9	RN 11	7	4	3	57.14
10	RN 12	8	2	6	25.00
Total		65	26	39	40.00
Average		6.5	2.6	3.9	

The dendrogram showed grouping the cultivars into three major clusters. Cluster I consisted of four tall cultivars namely Kuttiadi, Tiptur Tall, Malappuram and Kasaragode. Cluster II included three Dwarf cultivars viz., Chowghat Green Dwarf, Chowghat Orange Dwarf, Malayan Yellow Dwarf and the intermediate type, Gangabondam. Cluster III included the two tall cultivars, Laccadive Ordinary and Komadan. In the cluster for Talls, Kasaragode formed a separate cluster. Laccadive Ordinary and Komadan clustered together in one group. Kuttiadi was found genetically more similar to Tiptur Tall. Malappuram was distantly clustered in the first cluster for Talls. In clusterII, Chowghat Orange Dwarf and Chowghat Green Dwarf clustered separately and the cultivar Malayan Yellow Dwarf got clustered along with Gangabondam.

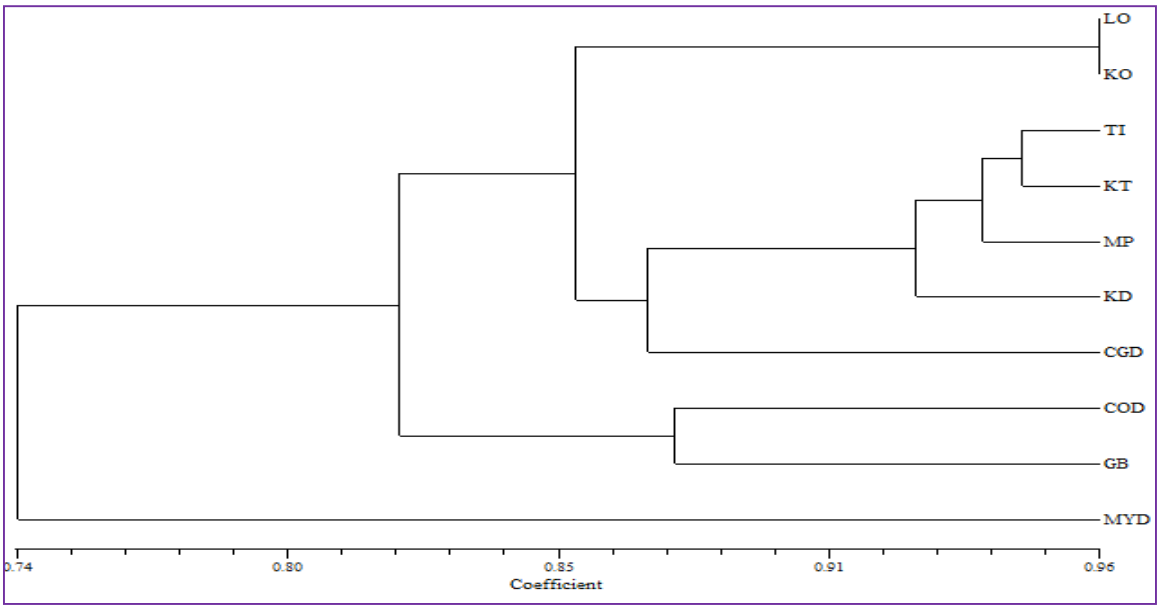
The same binary data matrix was used for calculating the similarity coefficient using Jaccard's coefficient (Jaccard, 1908). The mean similarity coefficient for each accession pair was calculated and used for cluster analysis using the UPGMA method and a dendrogram was constructed using the software package NTSYS-PC (Rohlf, 1993). The dendrogram generated using NTSYS is given in Fig1b and details of clusters are given in Table 8.b.

The dendrogram generated using NTSYS grouped the cultivars into two main clusters. Cluster I includes all the other cultivars (Laccadive Orinary, Tiptur Tall, Komadan, Kuttiadi, Malappuram, Kasaragode, Chowghat Orange Dwarf, Chowghat Green Dwarf and Gangabondam) except Malayan Yellow Dwarf. Malayan Yellow Dwarf formed the second cluster. In clusterI, the tall cultivars Laccadive Ordinary and Komadan were found to be more similar. The clusterI was divided into two major sub clusters. Tiptur Tall and Kuttiadi clustered together. Kasaragode formed a distant clade. The Dwarf cultivar Chowghat Green Dwarf was distantly clustered with the tall group. Chowghat Orange Dwarf and Gangabondam clustered in one group.

The dissimilarity matrix of different coconut genotypes based on the proportion of shared RAPD fragments was also generated. Dissimilarity values



a. DARwin ver.5.0



b. NTSYS

LO: Laccadive Ordinary, TI: Tiptur Tall, KO: Komadan, KT: Kuttiadi, MP: Malappuram, KD: Kasaragode, COD: Chowghat Orange Dwarf, CGD: Chowghat Green Dwarf, MYD: Malayan Yellow Dwarf, GB: Gangabondam

Fig. 1. Dendrogram generated for coconut cultivars out of RAPD data using the softwares, DARwin and NTSYS

Table 8. Grouping of accessions based on RAPD data using the softwares DARwin ver.5.0 and NTSYS

a. Grouping of accessions based on RAPD data using the software DARwin ver.5.0

Cluster No	No of cultivars in each cluster	Name of the cultivars
Cluster I	4	Kuttiadi, Tiptur Tall, Malappuram, Kasaragode
Cluster II	4	Chowghat Orange Dwarf, Chowghat Green Dwarf, Malayan Yellow Dwarf, Gangabondam
Cluster III	2	Laccadive Ordinary, Komadan,

b. Grouping of accessions based on RAPD data using the software NTSYS

Cluster No	No of cultivars in each cluster	Name of the cultivars
Cluster I	9	Laccadive Ordinary, Komadan, Tiptur Tall, Kuttiadi, Malappuram, Kasaragode, Chowghat Green Dwarf, Chowghat Orange Dwarf, Gangabondam
Cluster II	1	Malayan Yellow Dwarf

ranged from 0.0182 (Laccadive Ordinary and Komadan) to 0.1845 (Tiptur Tall and Malayan Yellow Dwarf) (Table 9). The average GD value was the highest for cultivar Malayan Yellow Dwarf (0.13).The number of markers varied in different cultivars. In general, more markers were present in Tall accessions than in Dwarfs. Based on the dissimilarity matrix it was found that within the Dwarf (involving three cultivars) the genetic diversity (GD) was the highest (average dissimilarity 0.11) compared to that within the tall group involving six cultivars (average dissimilarity 0.06). Between Talls and Dwarfs the average dissimilarity was 0.10. Between Talls and intermediate type the genetic diversity observed was 0.13, between Dwarfs and intermediate type the genetic diversity was 0.08. The genetic diversity calculated for all cultivars studied was 0.11.

4.3.2. Inter Simple Sequence Repeats (ISSR) assay of coconut genotypes

4.3.2.1. Standardization of PCR conditions

Both the proportion of components in the reaction mixture and the thermal profile were standardized for good amplification of coconut samples. The good quality genomic DNA isolated from selected coconut genotype, Chowghat Green Dwarf was subjected to ISSR analysis as per the procedure reported by Manimekalai and Nagarajan (2006b) with slight modifications of reaction conditions and thermal profile

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4.3.2.2. Screening of ISSR primers

Forty one primers-32 belonging to UBC (University of British Columbia) series and 9 belonging to ISSR (ISSR technologies, USA) series were screened for the amplification of DNA from the genotypes, Laccadive Ordinary and Chowghat Green Dwarf using the standardized protocol for ISSR assay. Primers were selected based on earlier studies of diversity analysis in coconut using ISSR markers. The amplification patterns of these primers are presented (Plate 10 a and b ; Table 10).

Table 9. Dissimilarity matrix of different coconut cultivars based on the proportion of shared RAPD fragments

	LO	TI	KO	KT	MP	KD	COD	CGD	MYD
TI	0.0796								
KO	0.0182	0.0631							
KT	0.0893	0.0265	0.0727						
MP	0.0796	0.0351	0.0631	0.0265					
KD	0.0796	0.0526	0.0811	0.0265	0.0351				
COD	0.0796	0.0877	0.0811	0.0796	0.0526	0.0526			
CGD	0.0893	0.0796	0.0727	0.0714	0.0442	0.0796	0.0796		
MYD	0.1569	0.1845	0.1600	0.1765	0.1650	0.1456	0.1262	0.1373	
GB	0.1132	0.1589	0.1154	0.1509	0.1215	0.1215	0.0654	0.0943	0.0833

LO: Laccadive Ordinary, TI: Tiptur Tall, KO: Komadan, KT: Kuttiadi, MP: Malappuram, KD: Kasaragode,

COD: Chowghat Orange Dwarf, CGD: Chowghat Green Dwarf, MYD: Malayan Yellow Dwarf, GB: Gangabondam

Out of 32 primers in UBC series, eleven primers showed good amplification pattern with clear and distinct bands and were selected for the experiment (Table 11). These primers produced 6-11 bands which could be scored without any ambiguity (Plate 10 a and b). In the remaining primers some were not able to generate sufficient number of clear and distinct amplicons and the primers UBC808, UBC812, UBC813, UBC815, UBC817, UBC818, UBC820, UBC826, UBC840, UBC843, UBC847, UBC848, UBC863 and UBC866 failed to amplify the coconut DNA (Plate 10 a and b). Hence these primers were not selected for the final amplification of the fifty coconut genotypes.

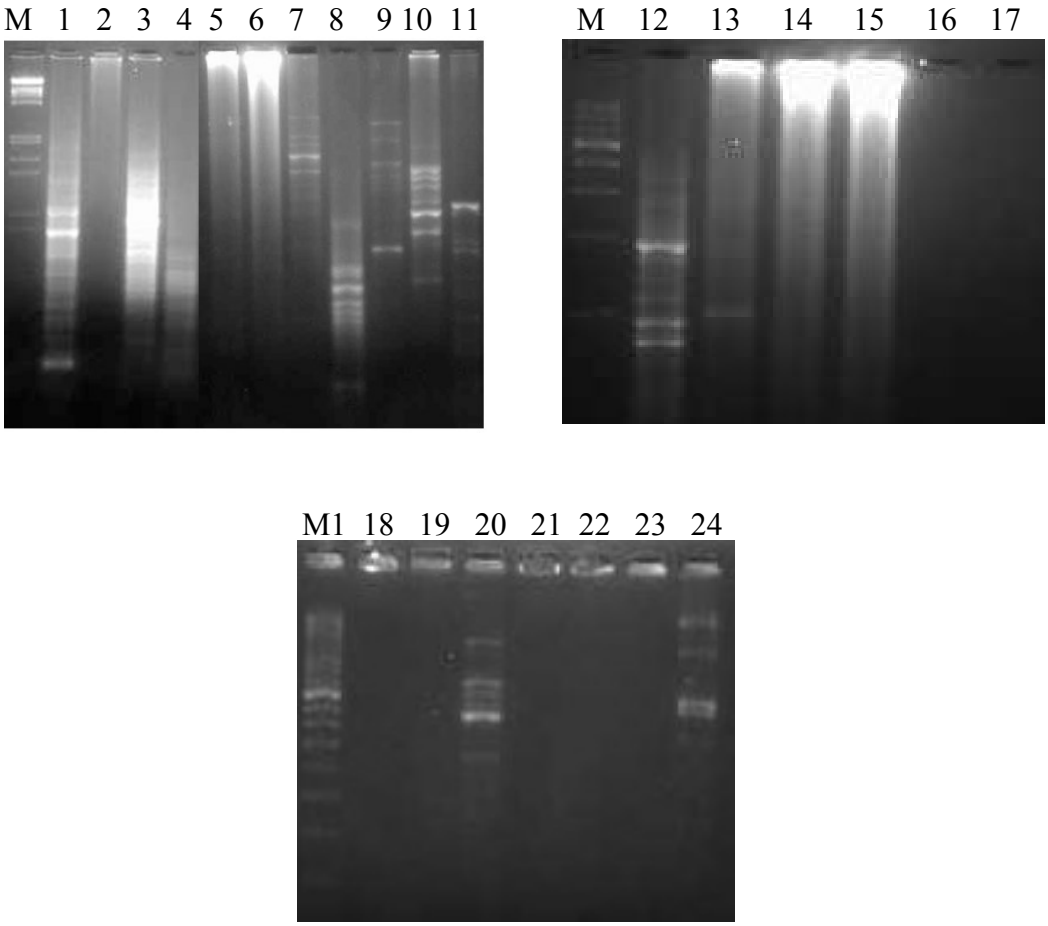
Nine primers from ISSR series (ISSR technologies, USA) were tested viz., ISSR1, ISSR2, ISSR3, ISSR 4, ISSR5, ISSR6, ISSR7, ISSR8 and ISSR9. These could not amplify the coconut genotype except ISSR5. The number of distinct bands produced by ISSR5 was less. Hence they were not selected for final amplification of fifty coconut genotypes. The pattern of amplification is presented in Plate 10 a and b.

4.3.2.3. Diversity analysis within each cultivar using selected ISSR primers

Out of forty one primers screened for ISSR analysis, eleven primers (listed in Table11) gave best amplification profile. They were selected for final amplification of coconut genotypes. The primers were selected based on the banding pattern and number of amplicons produced with clarity, distinctness and reproducibility.

Initially, the fifty genotypes are amplified with the selected primers, UBC 846 and 857.

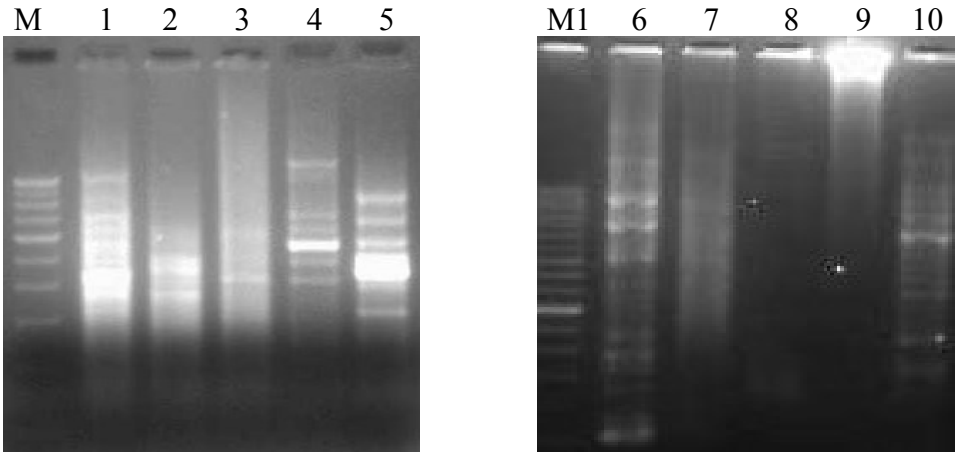
Five palms representing the cultivars Laccadive Ordinary, Tiptur Tall, Komadan, Malayan Yellow Dwarf and Gangabondam showed similar banding pattern within each cultivar by the amplification with the primer UBC 846 (Plate 11). In Kuttiadi, palm no 5 differed from others by the absence of two amplicons. Similarly, in Malappuram, palm no 1 and 2 differed from the rest three palms by the

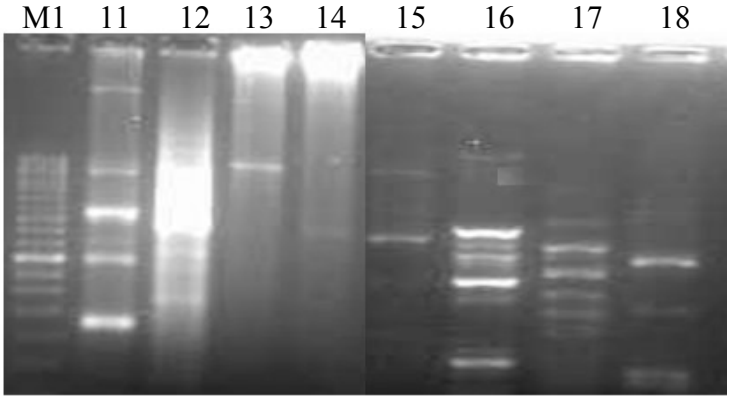


M : Marker Lambda DNA (*Eco* RI/ *Hind* III digest)
M1: 50 bp ladder

1 : UBC 354	7 : UBC 825	13 : UBC 812	19 : ISSR 4
2 : UBC 815	8 : UBC 834	14 : UBC 813	20 : ISSR 5
3 : UBC 826	9 : UBC 835	15 : UBC 820	21 : ISSR 6
4 : UBC 840	10 : UBC 836	16 : ISSR 1	22 : ISSR 8
5 : UBC 817	11 : ISSR 7	17 : ISSR 2	23 : ISSR 9
6 : UBC 818	12 : UBC 811	18 : ISSR3	24 : UBC 844

Plate 10 a. Screening of ISSR primers for amplification of coconut DNA





M : 100 bp ladder M1: 50 bp ladder

1: UBC 841	7 : UBC 815	13: UBC 807
2: UBC 847	8 : UBC 843	14: UBC 808
3: UBC 863	9 : UBC 848	15: UBC 845
4: UBC 865	10: UBC S2	16: UBC 857
5: UBC 855	11: UBC 846	17: UBC 890
6: UBC 854	12: UBC 866	18: UBC 814

Plate 10 b. Screening of ISSR primers for amplification of coconut DNA

Table 10. Screening of 41 primers for ISSR assay in coconut

Sl No	Primer	Amplification pattern			
		No of amplicons	Type of amplicons		Remarks
			Distinct	faint	
1	UBC 354	11	5	6	selected
2	UBC 807	1	0	1	---
3	UBC 808	0	0	0	---
4	UBC 811	8	4	4	selected
5	UBC 812	0	0	0	---
6	UBC 813	0	0	0	---
7	UBC 814	3	2	1	---
8	UBC 815	0	0	0	---
9	UBC 817	0	0	0	---
10	UBC 818	0	0	0	---
11	UBC 820	0	0	0	---
12	UBC 825	7	3	4	selected
13	UBC 826	0	0	0	---
14	UBC 834	6	3	3	selected
15	UBC 835	5	1	4	---
16	UBC 836	7	3	4	selected
17	UBC 840	0	0	0	---
18	UBC 841	0	0	2	
19	UBC 843	0	0	0	---
20	UBC 844	4	2	2	---
21	UBC 845	2	2	0	---
22	UBC 846	6	5	1	Selected
23	UBC 847	0	0	0	---
24	UBC 848	0	0	0	---
25	UBC 854	10	5	5	selected
26	UBC 855	6	5	1	selected
27	UBC 857	8	5	3	Selected
28	UBC 863	0	0	0	---
29	UBC 865	6	1	5	---
30	UBC 866	0	0	0	---
31	UBC 890	7	4	3	Selected
32	UBC S2	10	3	7	Selected
33	ISSR 1	0	0	0	---
34	ISSR 2	0	0	0	---
35	ISSR 3	0	0	0	---
36	ISSR 4	0	0	0	---
37	ISSR 5	6	2	4	---
38	ISSR 6	0	0	0	---
39	ISSR 7	0	0	0	---
40	ISSR 8	0	0	0	---
41	ISSR 9	0	0	0	---

Table 11. Sequence data of the ISSR primers selected for coconut genotypes

Sl No	Primer	Sequence
1	UBC 354	5’CTAGAGGCCGGCCGC3’
2	UBC 811	5’GAGAGAGAGAGAGAGAC3’
3	UBC 825	5’ACACACACACACACACT3’
4	UBC 834	5’AGAGAGAGAGAGAGAGYT3’
5	UBC 836	5’AGAGAGAGAGAGAGAGYA3’
6	UBC 846	5’CACACACACACACACART3’
7	UBC 854	5’TCTCTCTCTCTCTCTCRG3’
8	UBC 855	5’ACACACACACACACACYT3’
9	UBC 857	5’ACACACACACACACACYG3’
10	UBC890	5’VHVGTGTGTGTGTGTGT3’
11	UBC S2	5’CTCTCTCTCGTGTGTGTG3’

absence of one amplicon. In Kasaragode, palm nos 3, 4 and 5 were similar and varied from palm no 1 and 2 by the absence of single amplicon. In Chowghat Orange Dwarf, palm no 2 differed from the rest by the presence of single polymorphic band. In Chowghat Green Dwarf, palm no 2 and 5 were similar and differed from others by the absence of one polymorphic band.

UBC 857 produced 8 amplicons in Laccadive Ordinary (Plate 12). The palms Laccadive Ordinary 3 and 4 produced same banding pattern to that of others except for the absence of a single polymorphic band. All the five palms belonging to the group, Tiptur Tall, Kuttiadi and Kasaragode produced identical amplification pattern. Palm no 3 of Komadan differed from others by the absence of single polymorphic amplicon. Similarly, in Malappuram, palm no 1 is different from others by the absence of single polymorphic band. In Chowghat Orange Dwarf, palm no 2 differed from the others by the presence of two polymorphic bands. Palm no 2 in Chowghat Green Dwarf is different from others by the absence of single polymorphic band. In Malayan Yellow Dwarf, palm nos 1and 3 gave similar amplification pattern and differed from the others by the absence of one polymorphic amplicon. In Gangabondam, all palms produced identical banding pattern.

With the two selected ISSR primers (UBC 846 and UBC 857) each cultivar (represented by five palms) produced amplicons ranging from 14-16 (Laccadive Ordinary-16, Tiptur Tall-16, Komadan-14, Kuttiadi-18, Malappuram-17, Kasaragode-15, Chowghat Orange Dwarf-18, Chowghat Green Dwarf-15, Malayan Yellow Dwarf-16 and Gangabondam-16). The polymorphism percentage calculated was less than 10 among the cultivars, Kasaragode, Chowghat Green Dwarf and Malayan Yellow Dwarf and less than 20 per cent in case of Komadan, Malappuram and Chowghat Orange Dwarf. The four cultivars Laccadive Ordinary, Tiptur Tall, Kuttiadi and Gangabondam could not produce any polymorphic band.

Since ISSR marker system failed to bring out the variability between palms within a cultivar, the DNA from the five palms in each cultivar was pooled to study

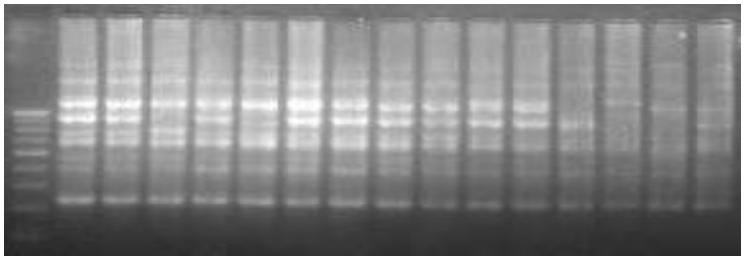


Plate 11 (a)

M 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30

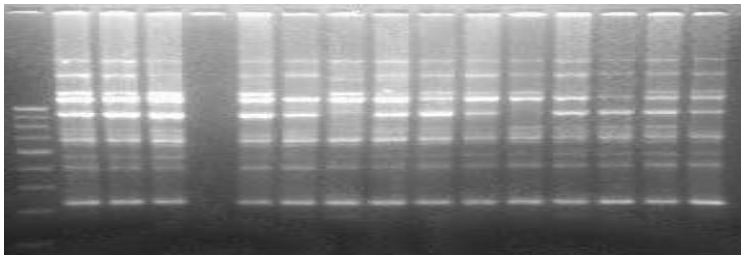


Plate 11 (b)

M 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45

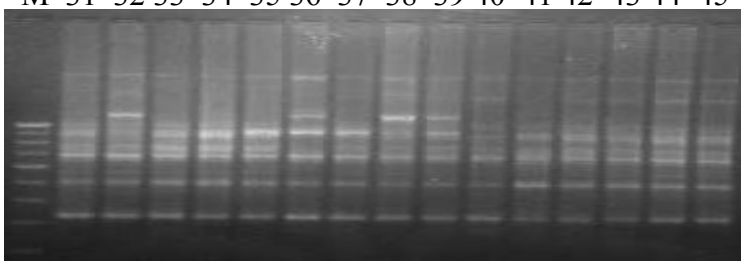


Plate 11 (c)

M 46 47 48 49 50

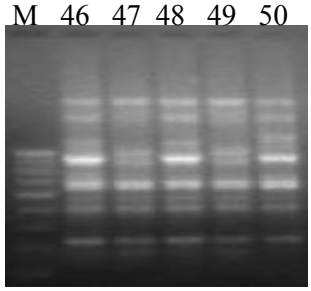


Plate 11 (d)

M : 100 bp ladder

1-5 : Laccadive Ordinary Tall 1-5 6-10 : Tiptur Tall 1-5 11-15 : Komadan1-5
16-20 : Kuttiadi 1-5 21-25 : Malappuram1-5 26-30 : Kasaragode1-5

Plate 11. Amplification (ISSR) of fifty (a) 1-15 (b) 16-30 (c) 31-45 (d) 46-50 coconut genotypes with selected ISSR primer UBC 846

Plate 12 (a)

M 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30

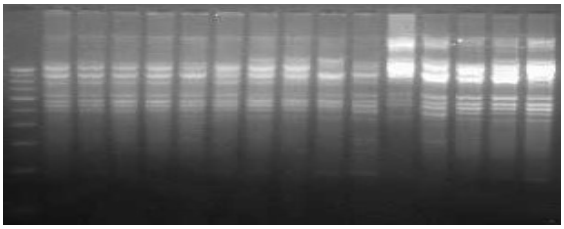


Plate 12 (b)

M 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45

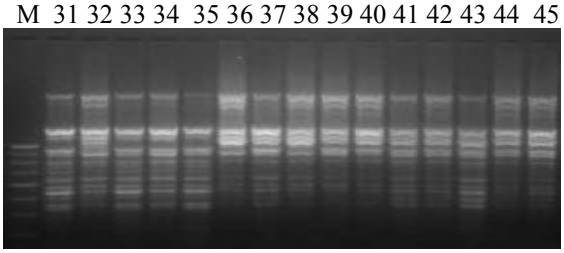


Plate 12 (c)

M 46 47 48 49 50

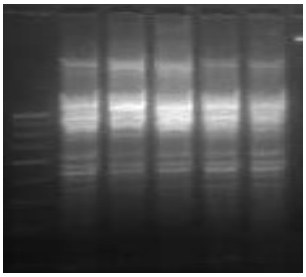


Plate 12 (d)

M : 100 bp ladder

1-5 : Laccadive Ordinary Tall 1-5 6-10 : Tiptur Tall 1-5 11-15 : Komadan1-5
16-20 : Kuttiadi 1-5 21-25 : Malappuram1-5 26-30 : Kasaragode1-5

Plate 12. Amplification (ISSR) of fifty (a) 1-15 (b) 16-30 (c) 31-45 (d) 46-50 coconut genotypes with selected ISSR primer UBC 857

the diversity between the ten cultivars. The ten coconut genotypes are amplified with the selected ISSR primers.

4.3.2.4. Diversity and population structure analysis of ten coconut cultivars using selected ISSR primers

The gel profile for amplification of ten coconut cultivars with ISSR primers is provided in Plate 13 a and b. Percent of polymorphism was worked out for each primer. The details of amplification are provided in Table 12. The observations are as follows.

UBC 354

The primer UBC 354 could generate a total of eight clear, distinct and reproducible bands and it could generate four polymorphic bands (Plate 13 a. 1) giving a polymorphism percentage of 50.

UBC 811

A total of eight amplicons were produced by the primer UBC 811. They were clear, distinct and reproducible (Plate 13 a. 2). It could generate four polymorphic bands out of eight amplicons and the per cent of polymorphism was 50.

UBC 825

The ISSR primer UBC 825 was able to generate eight amplicons. They were clear, distinct and reproducible. It could generate four polymorphic bands out of eight amplicons (Plate 13 a. 3) and the per cent of polymorphism was 50.

UBC 834

UBC 834 generated twelve clear, distinct and reproducible amplicons and five of them were polymorphic (Plate 13 a. 4). The per cent of polymorphism was less and was 41.6.

UBC 836

Seven amplicons were generated by UBC 836. All bands were very clear, distinct and reproducible. It could generate only one polymorphic band (Plate 13 a. 5). The polymorphism percentage was very less (14.28) since it could detect only one polymorphic band.

UBC 846

Amplification of ten coconut cultivars with the selected primer UBC 846 produced nine clear, distinct and reproducible amplicons (Plate 13 b. 1). Five amplicons were polymorphic giving 55.5 per cent polymorphism.

UBC 854

The primer UBC 854 generated a total of seven clear, distinct and reproducible bands and it could detect four polymorphic bands (Plate 13 b. 2). The per cent of polymorphism was 57.14.

UBC 855

The gel profile with the amplification of UBC 855 gave a total of five amplicons and all amplicons were clear and distinct. It could generate only two polymorphic bands (Plate 13 b. 3), giving a polymorphism percentage of 40.

UBC 857

A total of 11 amplicons were produced by the primer UBC 857. They were clear, distinct and reproducible. It could detect six polymorphic bands out of 11 amplicons (Plate 13 b. 4) and the per cent of polymorphism was 54.54.

UBC 890

The ISSR primer UBC 890 was able to generate a total of ten amplicons, it could detect only two polymorphic amplicons (Plate 13 b. 5) giving a polymorphism percentage of 20. The polymorphism percentage was very low.

UBC S2

UBC S2 produced five clear, distinct and reproducible amplicons (Plate 13. b. 6). Three bands were polymorphic giving a polymorphism percentage of sixty. The bands were very well separated.

ISSR analysis using ten selected primers produced a total of 90 markers among the ten coconut genotypes (Table 12). The number of scorable markers produced by each primer ranged from five (UBCS2) to twelve (UBC834) with an average of 8.2 markers per primer. The molecular weight of these markers ranged from 190 bp to 2600 bp, with an average molecular weight of 810 bp. The polymorphic bands were 44.44 per cent of the total, each primer detecting on an average 3.6 polymorphic bands.

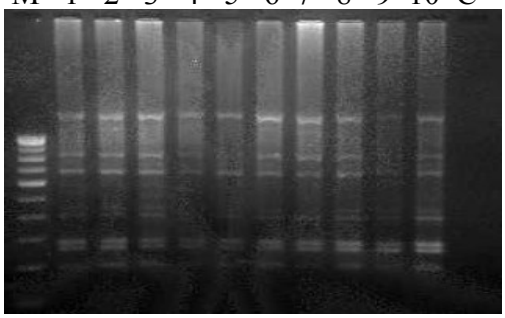
An amplicon of 476 bp with the primer UBC 811 was found only in Malayan Yellow Dwarf and Gangabondam. Another amplicon of 1752 bp produced by the primer UBC 825 could differentiate Dwarfs from the Talls as this was specific to the Talls and the Dwarfs and intermediate cultivars lacked this amplicon. Another amplicon by the same primer could differentiate Talls from Dwarfs as this was found only in Dwarfs and intermediate type. An amplicon of 420 bp amplified with the primer UBC 834 by the ten coconut genotypes was only present in Tiptur Tall and Kuttiadi.

Data scored as presence (1) or absence (0) with respect to the 10 genotypes was subjected to diversity analysis.

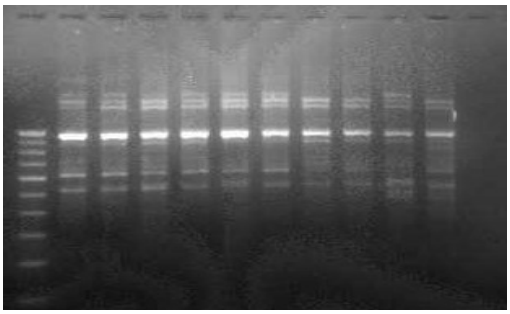
Computing the genetic distances using Dice coefficients, the Neighbour Joining tree (Saitou and Nei, 1987) and bootstrap analysis (5000) were executed using DARwin ver.5.0. The dendrogram generated using ISSR data is given in Fig 2.a. The details of clusters are given in Table 13.a.

The dendrogram grouped the cultivars into three major clusters. Cluster I included the tall cultivars, Kuttiadi, Tiptur Tall, Laccadive Ordinary and

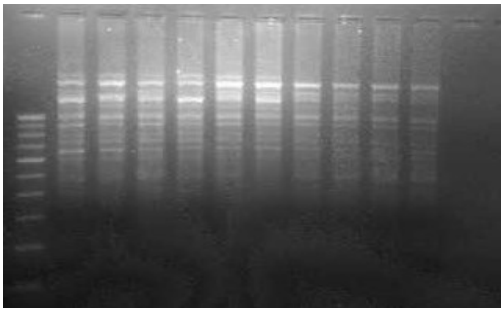
1. UBC 354



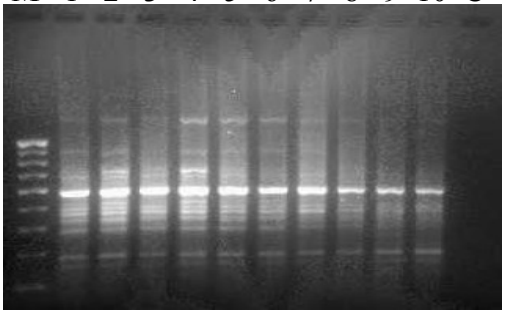
2. UBC 811



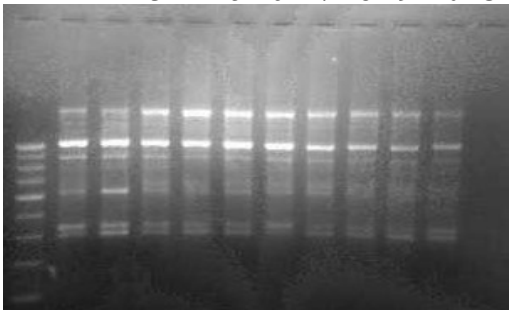
3. UBC 825



4. UBC834



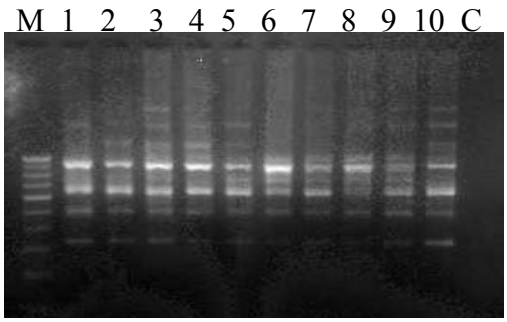
5. UBC836



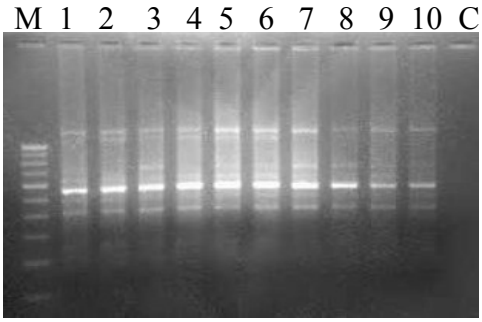
M : 100 bp ladder
1 : Laccadive Ordinary Tall
2 : Tiptur Tall
3 : Komadan
4 : Kuttiadi
5 : Kasaragode
C : Control
6 : Chowghat Orange Dwarf
7 : Chowghat Green Dwarf
8 : Malayan Yellow Dwarf
9 : Gangabondam
10 : Malappuram

Plate 13 a. Amplification of coconut genotypes with selected ISSR primers

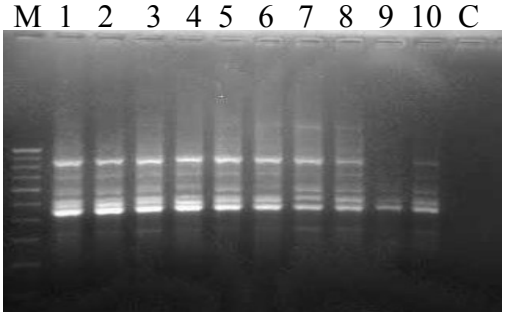
1. UBC846



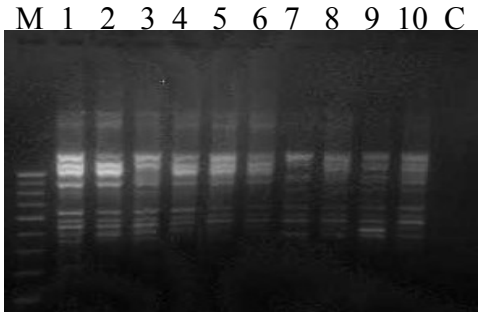
2. UBC854



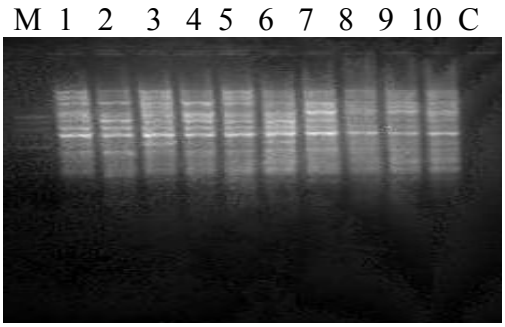
3. UBC855



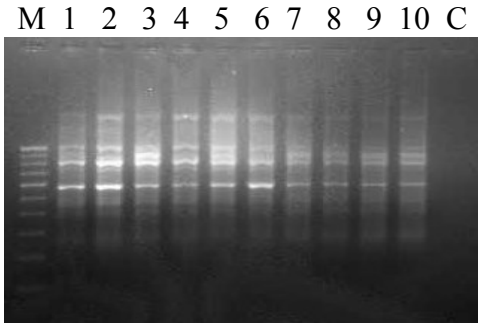
4. UBC857



5. UBC 890



6. UBCS2



M : 100 bp ladder C : Control

1 : Laccadive Ordinary Tall
2 : Tiptur Tall
3 : Komadan
4 : Kuttiadi
5 : Kasaragode
6 : Chowghat Orange Dwarf
7 : Chowghat Green Dwarf
8 : Malayan Yellow Dwarf
9 : Gangabondam
10 : Malappuram

Plate 13 b. Amplification of coconut genotypes with selected ISSR primers

Table 12. Details of amplification with selected primers for ISSR assay in coconut

Sl No	Primer	Total no of amplicons	No of polymorphic amplicons	No of monomorphic amplicons	Polymorphism (%)
1	UBC 354	8	4	4	50.00
2	UBC 811	8	4	4	50.00
3	UBC 825	8	4	4	50.00
4	UBC 834	12	5	7	41.66
5	UBC 836	7	1	6	14.28
6	UBC 846	9	5	4	55.55
7	UBC 854	7	4	3	57.14
8	UBC 855	5	2	3	40.00
9	UBC 857	11	6	5	54.54
10	UBC 890	10	2	8	20.00
11	UBC S2	5	3	2	60.00
Total		90	40	50	44.44
Average		8.2	3.6	4.5	

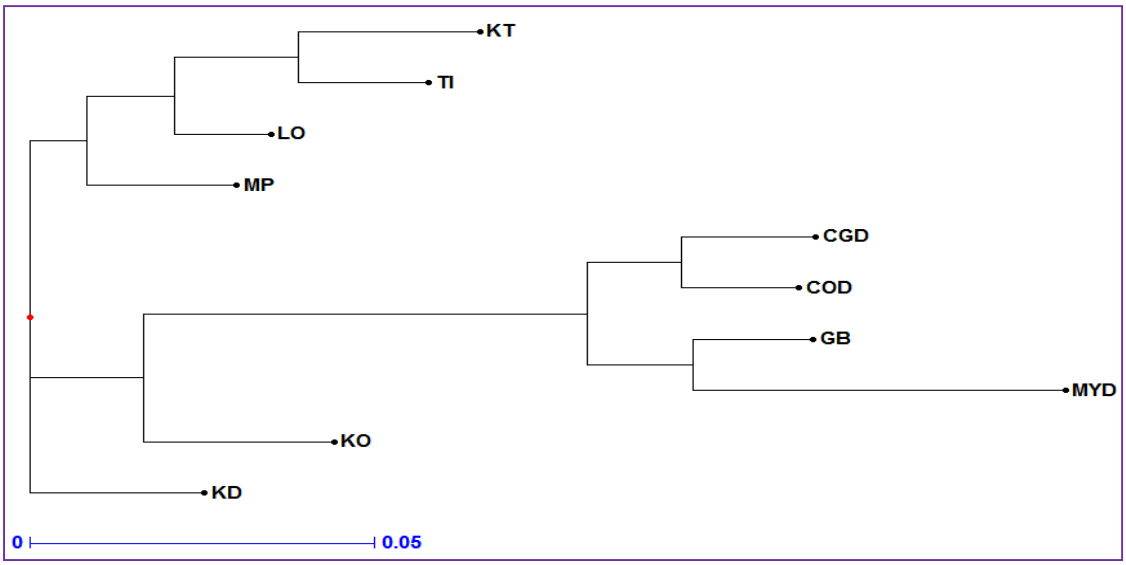
Malappuram. Cluster II included all the Dwarf cultivars (Chowghat Orange Dwarf, Chowghat Green Dwarf, Malayan Yellow Dwarf), intermediate (Gangabondam) and one tall cultivar, Komadan. The third cluster included only the tall cultivar, Kasaragode.

The same binary data matrix was used for calculating the similarity coefficient using Jaccard's coefficient (Jaccard, 1908). The mean similarity coefficient for each accession pair was calculated and used for cluster analysis using the UPGMA method and a dendrogram was constructed using the software package NTSYS-PC (Rohlf, 1993). The dendrogram generated using NTSYS is given in Fig 2.b and details of clusters are given in Table 13.b. The dissimilarity matrix of different coconut genotypes based on the proportion of shared ISSR fragments (Table 14) was also generated.

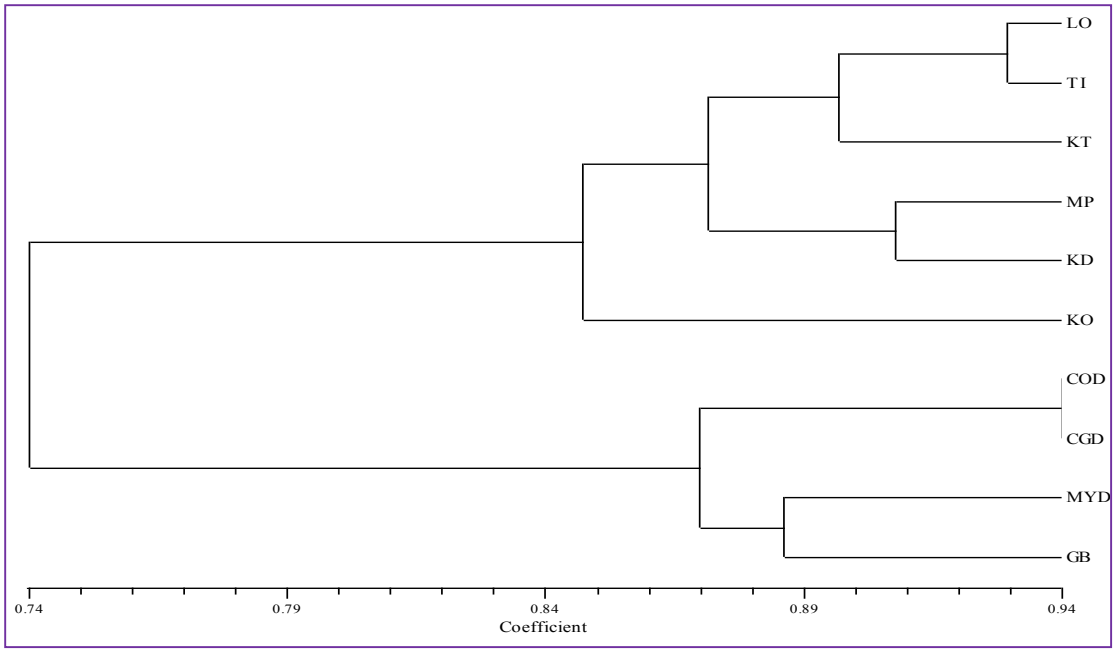
The dendrogram generated using NTSYS grouped the cultivars into two major clusters. The cluster I included all the tall cultivars (Laccadive Ordinary, Tiptur Tall, Kuttiadi, Malappuram, Kasaragode and Komadan) and cluster II included the Dwarfs (Chowghat Orange Dwarf, Chowghat Green Dwarf, Malayan Yellow Dwarf) and the intermediate (Gangabondam).

ISSR markers successfully classified the Talls and Dwarfs in separate clusters. Dissimilarity values ranged from 0.0365 (Chowghat Orange Dwarf and Chowghat Green Dwarf) to 0.2276 (Tiptur Tall and Malayan Yellow Dwarf) as inferred from the dissimilarity matrix (Table 14). The cultivar Malayan Yellow Dwarf was distantly related to the rest of the varieties with an average dissimilarity value of 0.14.

In the cluster for Talls, cv. Kasaragode showed more difference from the rest of the group and formed a separate clade. Within this cluster, Kuttiadi was genetically very close to Tiptur Tall. Malappuram and Laccadive Ordinary were more distinct among the cluster for Talls.



a. DARwin ver.5.0



b. NTSYS

LO: Laccadive Ordinary, TI: Tiptur Tall, KO: Komadan, KT: Kuttiadi, MP: Malappuram, KD: Kasaragode,

COD: Chowghat Orange Dwarf, CGD: Chowghat Green Dwarf, MYD: Malayan Yellow Dwarf, GB: Gangabondam

Fig. 2. Dendrogram generated for coconut cultivars out of ISSR data using the softwares, DARwin and NTSYS

Table 13. Grouping of accessions based on ISSR data using the softwares, DARwin ver.5.0 and NTSYS

a. Grouping of accessions based on ISSR data using the software DARwin ver.5.0

Cluster No	No of cultivars in each cluster	Name of the cultivars
Cluster I	4	Kuttiadi, Tiptur Tall, Laccadive Ordinary Malappuram,
Cluster II	5	Chowghat Orange Dwarf, Chowghat Green Dwarf, Malayan Yellow Dwarf, Gangabondam, Komadan,
Cluster III	1	Kasaragode

b. Grouping of accessions based on ISSR data using the software NTSYS

Cluster No	No of cultivars in each cluster	Name of the cultivars
Cluster I	6	Laccadive Ordinary, Tiptur Tall, Kuttiadi, Malappuram, Kasaragode, Komadan
Cluster II	4	Chowghat Orange Dwarf, Chowghat Green Dwarf, Malayan Yellow Dwarf, Gangabondam

Table 14. Dissimilarity matrix of different coconut cultivars based on the proportion of shared ISSR fragments

	LO	TI	KO	KT	MP	KD	COD	CGD	MYD
TI	0.0385								
KO	0.0701	0.0955							
KT	0.0709	0.0452	0.1026						
MP	0.0641	0.0769	0.0828	0.0581					
KD	0.0641	0.0769	0.0828	0.0968	0.0513				
COD	0.1429	0.1701	0.1351	0.1782	0.1293	0.1156			
CGD	0.1507	0.1781	0.1293	0.2000	0.1507	0.1233	0.0365		
MYD	0.1862	0.2276	0.1644	0.2083	0.1724	0.1862	0.1029	0.0815	
GB	0.1391	0.1656	0.1053	0.1733	0.1523	0.1523	0.0845	0.0639	0.0714

LO: Laccadive Ordinary, TI: Tiptur Tall, KO: Komadan, KT: Kuttiadi, MP: Malappuram, KD: Kasaragode,

COD: Chowghat Orange Dwarf, CGD: Chowghat Green Dwarf, MYD: Malayan Yellow Dwarf, GB: Gangabondam

Among the Dwarfs, Chowghat Green Dwarf was found more similar to Chowghat Orange Dwarf as well as Malayan Yellow Dwarf to Gangabondam. The intermediate type Gangabondam showed more similarity with Malayan Yellow Dwarf than with Chowghat Orange Dwarf and Chowghat Green Dwarf. The tall cultivar Komadan got clustered along with Dwarf group.

Among all the cultivars, Tiptur Tall and Malayan Yellow Dwarf were found to be most distant ones from the rest of the members in the group.

The number of markers varied in different cultivars. In general, more markers were present in Tall accessions than in Dwarfs. Based on the dissimilarity matrix it was found that within the Tall (involving six cultivars) and within the Dwarf (involving three cultivars) group showed the same genetic diversity (GD) of 0.07. Between Talls and Dwarfs the average dissimilarity was 0.16. Between Talls and intermediate type the genetic diversity observed was 0.14, between Dwarfs and intermediate type the genetic diversity was 0.07. The genetic diversity calculated for all cultivars studied was 0.16.

4.3.3. Simple Sequence Repeats (SSR) assay of coconut genotypes

4.3.3.1. Standardization of PCR conditions

Both the proportion of components in the reaction mixture and the thermal profile (annealing temperature of primers) were standardized for good amplification of coconut samples.

4.3.3.2. Screening of SSR primers

Forty three primers- 33 from SSR series and 10 from CnCir series were screened for the amplification of coconut DNA from the genotypes, Laccadive Ordinary and Chowghat Green Dwarf using the standardized protocol for SSR assay.

The SSR primers SSR 72, 74, 85, 122, 188, 214, 218, 248, 276, 286, 296, 301, 304, 318, 326, 350, 360, 479, 526, 565, 578, 593, 603, 60800 and SSR PT

30452 could not amplify coconut DNA. The primers *viz*., SSR 43, SSR 94 and SSR 378 were unable to produce distinct amplicons. The primers SSR 34, SSR 47, SSR 108, SSR 223 and SSR 555 could not produce amplicons belong to the SSR regime (Plate 14 a and b; Table 15). Hence these primers were not selected for final amplification of coconut genotypes. The primers from CnCir series (Plate 14 b) gave good amplification pattern with markers in the SSR regime and they were selected (Table 16) for final amplification of fifty coconut genotypes and their annealing temperatures for amplification except for the primer CnCir F2 were standardized by providing different annealing temperatures (Plate 15a, b and c) using gradient thermo cycler (Eppendorf, USA). CnCir F2 gave good amplification with the annealing temperature of 57.3. The details of standardization of annealing temperatures are given (Table 17). After that the amplification of coconut genotypes using these primers with standardized annealing temperatures was done.

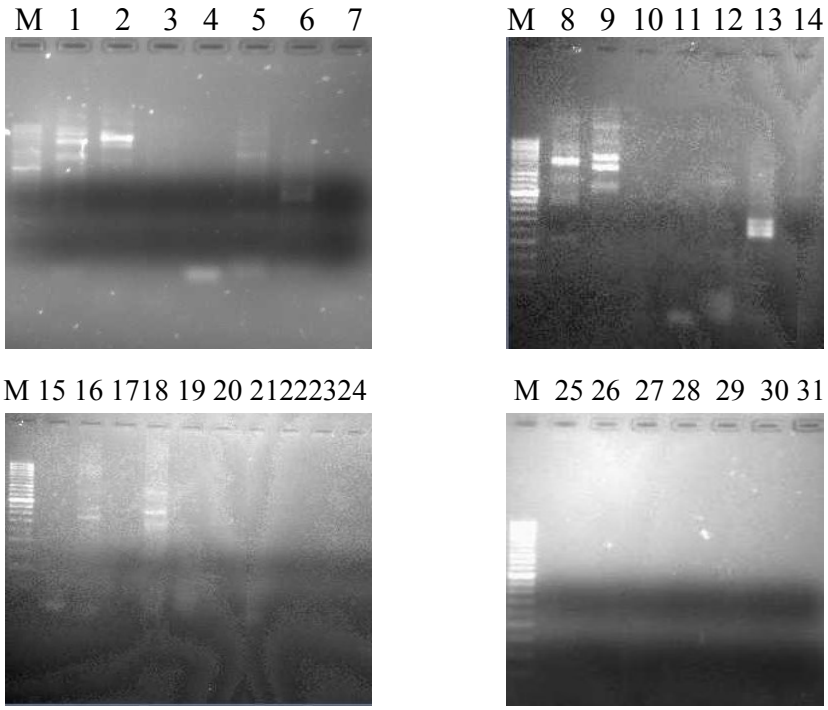
4.3.3.2.1. Standardization of annealing temperatures for the selected SSR primers

CnCir B6

CnCir B6 was selected for final amplification since the amplicons were in the SSR marker regime. Different annealing temperatures were provided for PCR amplification of coconut DNA and it ranged 53.5-65.0^{0}C. Two amplicons were produced and the annealing temperature of 61^{0}C was selected for final amplification since it gave the clear and distinct band (Plate 15 a.1).

CnCir B12

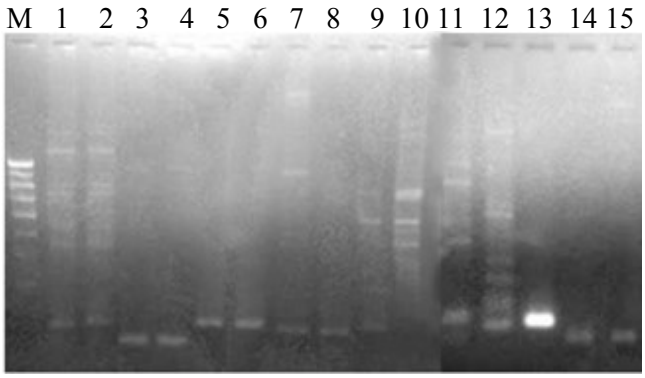
CnCir B12 was selected for final amplification since the amplicon was in the SSR marker regime. Different annealing temperatures were provided for PCR amplification of coconut DNA by this primer and it ranged 45-63.6^{0}C. Two amplicons were produced and an annealing temperature of 59.8^{0}C was selected for final amplification since it gave the clear and distinct band. The gel profile is depicted in (Plate 15 a. 2).

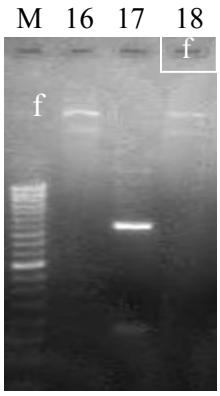


M : 50 bp ladder

1 : SSR 43	11 : SSR 304	21 : SSR 593
2 : SSR 47	12 : SSR 318	22 : SSR 565
3 : SSR 74	13 : SSR 555	23 : SSR PT30452
4 : SSR 85	14 : SSR 603	24 : SSR 60800
5 : SSR 214	15 : SSR 318	25 : SSR 72
6 : SSR 479	16 : SSR 578	26 : SSR 218
7 : SSR 526	17 : SSR 350	27 : SSR 286
8 : SSR 34	18 : SSR 122	28 : SSR 360
9 : SSR 223	19 : SSR 301	29 : SSR 247
10 : SSR 296	20 : SSR 326	30 : SSR 188
		31 : SSR 276

Plate 14 a. Screening of SSR primers for amplification of coconut





1: CnCir B12
2: CnCir B12
3: CnCir C7
4: CnCir C7
5: CnCir C12
6: CnCir C12
7: CnCir G11
8: CnCir G11
9: CnCir E10
10: CnCir E10
11: CnCir B6
12: CnCir C3
13: CnCir F2
14: CnCir E2
15: CnCir H4
16: SSR 94
17: SSR 108
18: SSR 378

Plate 14 b. Screening of SSR primers for amplification of coconut DNA

Table 15. Screening of 43 primers for SSR assay in coconut

Sl No	Primer	Amplification pattern			
		No of amplicons	Type of amplicons		Remarks
			Distinct	faint	
1	SSR 34	1	1	0	-
2	SSR 43	1	0	1	-
3	SSR 47	1	1	0	-
4	SSR 72	0	0	0	-
5	SSR 74	0	0	0	-
6	SSR 85	0	0	0	-
7	SSR 94	2	0	2	-
8	SSR 108	1	1	0	-
9	SSR 122	0	0	0	-
10	SSR 188	0	0	0	-
11	SSR 214	0	0	0	-
12	SSR 218	0	0	0	-
13	SSR 223	2	2	0	-
14	SSR 248	0	0	0	-
15	SSR 276	0	0	0	-
16	SSR 286	0	0	0	-
17	SSR 296	0	0	0	-
18	SSR 301	0	0	0	-
19	SSR 304	0	0	0	-
20	SSR 318	0	0	0	-
21	SSR 326	0	0	0	-
22	SSR 350	0	0	0	-

Table 15 continued. Screening of 43 primers for SSR assay in coconut

Sl No	Primer	Amplification pattern			
		No of amplicons	Type of amplicons		Remarks
			Distinct	faint	
23	SSR 360	0	0	0	-
24	SSR 378	2	0	2	-
25	SSR 479	0	0	0	-
26	SSR 526	0	0	0	-
27	SSR 555	2	2	0	-
28	SSR 565	0	0	0	-
29	SSR 578	0	0	0	-
30	SSR 593	0	0	0	-
23	SSR 360	0	0	0	-
24	SSR 378	2	0	2	-
25	SSR 479	0	0	0	-
26	SSR 526	0	0	0	-
27	SSR 555	2	2	0	-
28	SSR 565	0	0	0	-
29	SSR 578	0	0	0	-
30	SSR 593	0	0	0	-
31	SSR 603	0	0	0	-
32	SSR 60800	0	0	0	-
33	SSR PT 30452	0	0	0	-
34	CnCir B6	3	1	2	selected
35	CnCir B12	5	1	4	selected
36	CnCir C3	4	1	3	selected
37	CnCir C7	1	0	1	selected
38	CnCir C12	1	1	0	selected
39	CnCir E2	1	1	0	selected
40	CnCir E10	3	1	2	selected
41	CnCir F2	1	1	0	Selected
42	CnCir G11	1	1	0	selected
43	CnCir H4	1	1	0	selected

Table 16. Sequence data of the SSR primers selected for coconut genotypes

Sl No	Primer	Sequence
1	CnCir B6	F 5'GAGTGTGTGAGCCAGCAT3' R 5'ATTGTTCACAGTCCTTCCA3'
2	CnCir B12	F 5'GCTCTTCAGTCTTTCTCAA3' R 5'CTGTATGCCAATTTTTCTA3'
3	CnCir C3	F 5'AGAAAGCTGAGAGGGAGATT3' R 5'GTGGGGCATGAAAAGTAAC3'
4	CnCir C7	F 5'ATAGCATATTGGTTTTCCT3' R 5'TGCTCCAGCGTTCATTCA3'
5	CnCir C12	F 5'ATACCACAGGCTAACAT3' R 5'AACCAGAGACATTTGAA3'
6	CnCir E2	F 5'TCGCTGATGAATGCTTGCT3' R 5'GGGGCTGAGGGATAAACC3'
7	CnCir E10	F5'TGGGTTCCATTTCTTCTCTCATC3' R5'GCTCTTTAGGGTTCGCTTTCTTG'
8	CnCir F2	F5'GGTCTCCTCTCCCTCCTTATCTA3' R 5'CGACGACCCAAAACTGAACAC3'
9	CnCir G11	F5'AATATCTCCAAAAATCATCGAA3' R 5'TCATCCCACACCCTCCTCT3'
10	CnCir H4	F 5'TTAGATCTCCTCCCAAAG3' R 5'ATCGAAAGAACAGTCACG3'

CnCir C3

The SSR primer CnCir C3 was selected for final amplification since the amplicons were in the SSR marker regime. Different annealing temperatures were provided for PCR amplification of coconut DNA and it ranged from 53.5-65.0^{0}C. Two amplicons were produced and an annealing temperature of 58 ^{0}C was selected for final amplification since it gave the clear and distinct band (Plate 15 a. 3).

CnCir C7

CnCir C7 was selected for final amplification since the amplicon was in the SSR marker regime. Different annealing temperatures were provided for PCR amplification of coconut DNA and were in the range of 49-65^{0}C. One amplicon was produced and the annealing temperature of 56.3^{0}C was selected for final amplification since it gave the clear and distinct band (Plate 15 a. 4).

CnCir C12

The SSR primer CnCir C12 was selected for final amplification since the amplicons were in the SSR marker regime. Different annealing temperatures were provided for PCR amplification of coconut DNA and were in the range of 50-61.9^{0}C. Two amplicons were produced and an annealing temperature of 50.2^{0}C was selected for final amplification since it gave the clear and distinct band (Plate 15 b. 1).

CnCir E2

The SSR primer CnCir E2 was selected for final amplification since the amplicon was in the SSR marker regime. Different annealing temperatures were provided for PCR amplification of coconut DNA and were in the range of 50-63.1^{0}C. Two amplicons were produced and an annealing temperature of 63.1^{0}C was selected for final amplification since it gave the clear and distinct band (Plate 15 b. 2).

CnCir E10

CnCir E10 was selected for final amplification since the amplicons were in the SSR marker regime. Different annealing temperatures were provided for PCR

amplification of coconut DNA and were in the range of 53.5-63.9^{0}C. Two amplicons were produced and annealing temperature of 59.5^{0}C was selected for final amplification since it gave the clear and distinct band (Plate 15 b. 3).

CnCir G11

CnCir G11 was selected for final amplification since the amplicon was in the SSR marker regime. Different annealing temperatures were provided for PCR amplification of coconut DNA and were in the range of 55.3- 62.0^{0}C. One amplicon was produced and an annealing temperature of 59.1^{0}C was selected for final amplification since it gave the clear and distinct band (Plate 15 c. 1).

CnCir H4

The SSR primer CnCir H4 was selected for final amplification. Different annealing temperatures were provided for PCR amplification of coconut DNA and were in the range of 49.5-61^{0}C. Two amplicons were produced and the annealing temperature of 60.5^{0}C was selected for final amplification since it gave the clear and distinct band (Plate 15 c. 2).

4.3.3.3. Diversity analysis within each cultivar using selected SSR primers

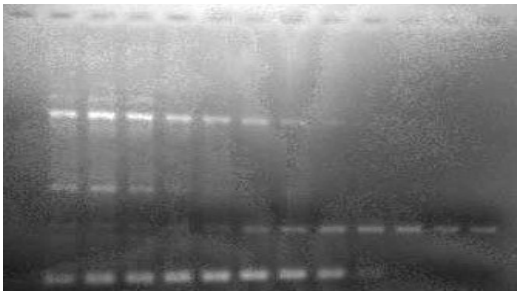
Out of forty three primer sets screened for SSR analysis, ten primer sets were selected for diversity and population structure analysis of coconut genotypes. All the selected primers were from the CnCir series. The selected primers produced clear and distinct amplification pattern with the ten coconut cultivars selected for the study.

Initially the fifty genotypes are amplified with the selected primers, CnCir B6 and CnCir F2.

All the five palms from Laccadive Ordinary, Komadan, Chowghat Orange Dwarf, Chowghat Green Dwarf and Malayan Yellow Dwarf showed similar banding pattern (Plate 16) with respect to the primer, CnCir F2. In Tiptur Tall, palm no 3 and

1. Primer CnCir C12

M 1 2 3 4 5 6 7 8 9 10 11 12

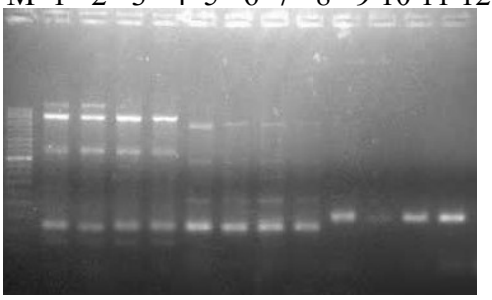


M : 50 bp ladder

1 : 53 ^{0}C	7 : 60^{0}C
2 : 54^{0}C	8 : 61^{0}C
3 : 56^{0}C	9 : 62^{0}C
4 : 57^{0}C	10 : 63^{0}C
5 : 58^{0}C	11 : 64^{0}C
6 : 59^{0}C	12 : 65^{0}C

2. Primer CnCir E2

M 1 2 3 4 5 6 7 8 9 10 11 12

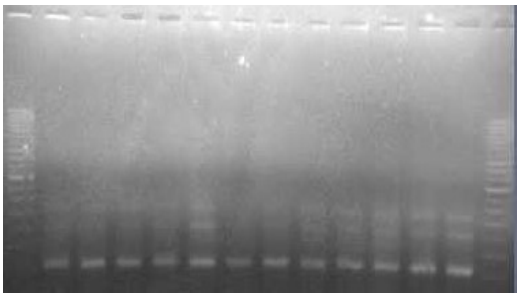


M : 50 bp ladder

1 : 45^{0}C	7 : 53.4^{0}C
2 : 46.7^{0}C	8 : 55.5^{0}C
3 : 47.8^{0}C	9 : 57.7^{0}C
4 : 49.2^{0}C	10 : 59.8^{0}C
5 : 50.1^{0}C	11 : 61.8^{0}C
6 : 51.5^{0}C	12 : 63.6^{0}C

3. Primer CnCir C3

M 1 2 3 4 5 6 7 8 9 10 11 12 M

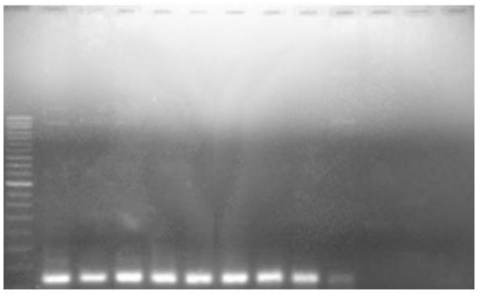


M : 50 bp ladder

1 : 53.5^{0}C	7 : 59.5^{0}C
2 : 53.7^{0}C	8 : 61.1^{0}C
3 : 54.3^{0}C	9 : 62.4^{0}C
4 : 55.6^{0}C	10 : 63.6^{0}C
5 : 56.5^{0}C	11 : 64.5^{0}C
6 : 58.0^{0}C	12 : 65.0^{0}C

4. Primer CnCir C7

M 1 2 3 4 5 6 7 8 9 10 11 12

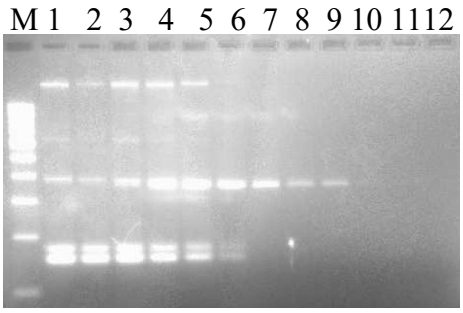


M : 50 bp ladder

1 : 49.0^{0}C	7 : 59.1^{0}C
2 : 52.8^{0}C	8 : 60.7^{0}C
3 : 55.5^{0}C	9 : 62.6^{0}C
4 : 56.3^{0}C	10 : 63.5^{0}C
5 : 57.1^{0}C	11 : 64.3^{0}C
6 : 58.3^{0}C	12 : 65.0^{0}C

Plate 15 a. Standardization of annealing temperatures of selected SSR primers

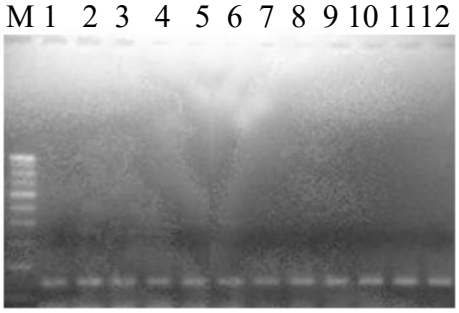
1. Primer CnCir C12



M : 100 bp ladder

1 : 50.0°C	7 : 54.8°C
2 : 50.2°C	8 : 56.5°C
3 : 50.8°C	9 : 58.1°C
4 : 51.9°C	10 : 59.7°C
5 : 52.4°C	11 : 61.0°C
6 : 53.3°C	12 : 61.9°

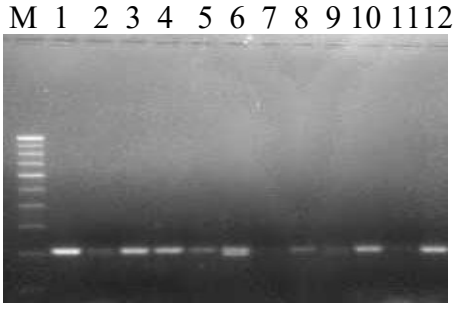
2. Primer CnCir E2



M : 100 bp ladder

1 : 50.0°C	7 : 57.8°C
2 : 51.3°C	8 : 58.7°C
3 : 52.5°C	9 : 59.1°C
4 : 55.0°C	10 : 60.5°C
5 : 55.7°C	11 : 61.8°C
6 : 56.6°C	12 : 63.1°C

3. Primer CnCir E10

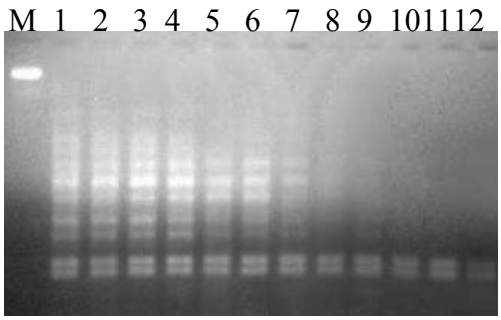


M : 100 bp ladder

1 : 53.5°C	7 : 60.0°C
2 : 54.3°C	8 : 60.7°C
3 : 55.7°C	9 : 61.2°C
4 : 57.4°C	10 : 62.0°C
5 : 58.8°C	11 : 62.8°C
6 : 59.5°C	12 : 63.9°C

Plate 15 b. Standardization of annealing temperatures of selected SSR primers

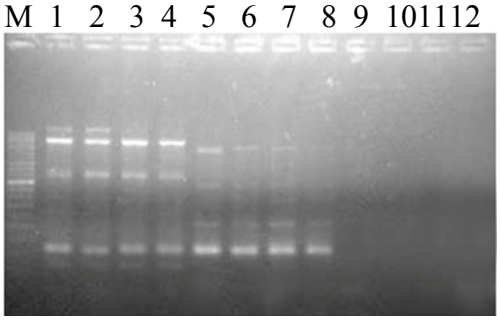
1. Primer CnCir G11



M : 100 bp ladder

1 : 55.3^{0}C	7 : 58.5^{0}C
2 : 55.9^{0}C	8 : 59.1^{0}C
3 : 56.2^{0}C	9 : 60.4^{0}C
4 : 56.7^{0}C	10 : 61.2^{0}C
5 : 57.0^{0}C	11 : 61.9^{0}C
6 : 57.9^{0}C	12 : 62.0

2. Primer CnCir H4



M : 100 bp ladder

1 : 49.5^{0}C	7 : 55.5^{0}C
2 : 49.7^{0}C	8 : 57.0^{0}C
3 : 50.3^{0}C	9 : 58.4^{0}C
4 : 51.2^{0}C	10 : 59.6^{0}C
5 : 52.5^{0}C	11 : 60.5^{0}C
6 : 53.4^{0}C	12 : 61.0^{0}C

Plate 15 c. Standardization of annealing temperatures of selected SSR primers

four gave similar banding profile and differed from others by the presence of one amplicon.

In Kuttiadi, palm no 1 and 5 gave similar amplification profile and different from others with respect to the presence of one polymorphic band. In Malappuram, only palm no 4 is different from others by the presence of single amplicon. In Kasaragode, palm no 2 and four were not amplified. Palm no 1 is different from others with respect to the presence of one polymorphic band. Palm no 3 and five in Gangabondam showed identical gel profile of amplification and different from others with respect to the presence of one polymorphic band.

5. CnCir C12 6. CnCir E2

Five palms each from Tiptur Tall, Kuttiadi, Kasaragode, Chowghat Orange Dwarf, Malayan Yellow Dwarf and Gangabondam showed similar banding pattern with the amplification of fifty coconut genotypes with the SSR primer CnCir B6 (Plate 17). In Laccadive Ordinary and Komadan, palm no 1 is differed from the rest of the palms by the absence of single polymorphic amplicon. Three palms (palm no1, 2 and 3) from the Dwarf genotype Chowghat Green Dwarf gave similar banding pattern. The primer CnCir B6 could not amplify palm no 4 and 5 from the Chowghat Green Dwarf genotypes.

With the two selected SSR primers (CnCir B6 and CnCir F2), each cultivar (represented by five palms) produced amplicons ranging from 2-4 (Laccadive Ordinary-4, Tiptur Tall-3, Komadan-4, Kuttiadi-2, Malappuram-4, Kasaragode-2, Chowghat Orange Dwarf-3, Chowghat Green Dwarf-3, Malayan Yellow Dwarf-1 and Gangabondam-4). The polymorphism percentage calculated was less than 20 among the cultivars Laccadive Ordinary, Tiptur Tall, Komadan, Malappuram and Gangabondam. The five cultivars Kuttiadi, Kasaragode Chowghat Orange Dwarf, Chowghat Green Dwarf and Malayan Yellow Dwarf could not produce any polymorphic band.

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

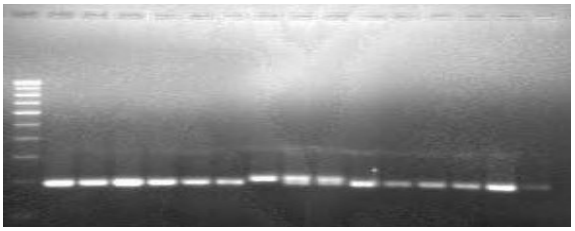


Plate 16 (a)

M 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30

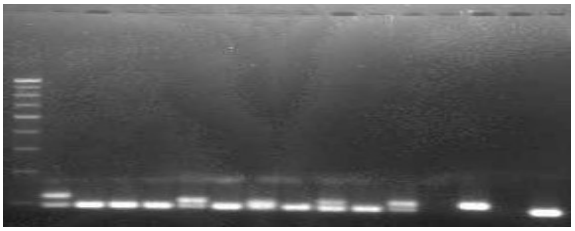


Plate 16 (b)

M 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45

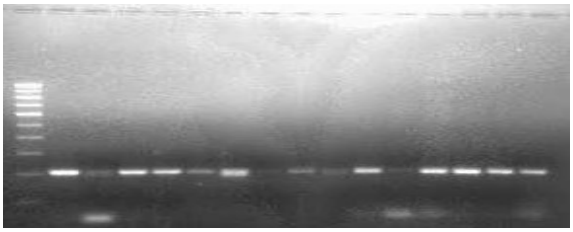


Plate 16 (c)

M 46 47 48 49 50

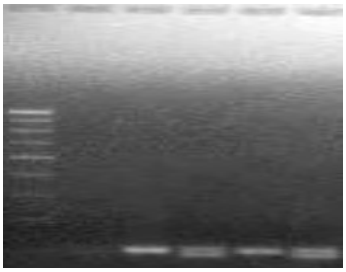


Plate 16 (d)

M : 100 bp ladder

1-5 : Laccadive Ordinary Tall 1-5 6-10 : Tiptur Tall 1-5 11-15 : Komadan1-5
16-20 : Kuttiadi 1-5 21-25 : Malappuram1-5 26-30 : Kasaragode1-5

Plate 16. Amplification (SSR) of fifty (a) 1-15 (b) 16-30 (c) 31-45 (d) 46-50 coconut genotypes with selected SSR primer CnCir F2

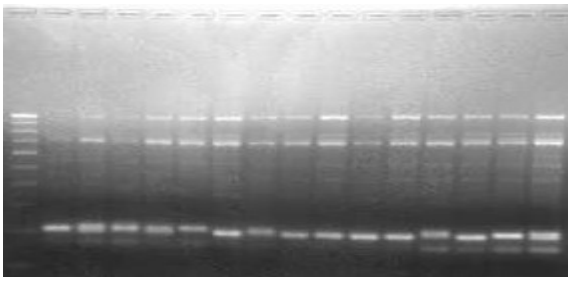


Plate 17 (a)

M 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30

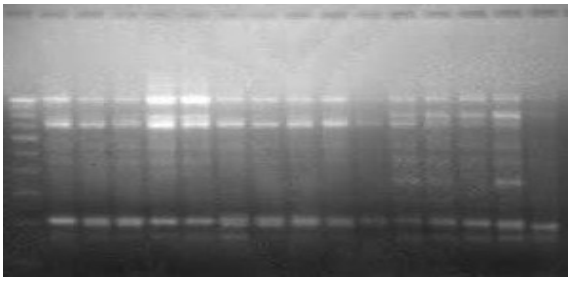


Plate 17 (b)

M 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45

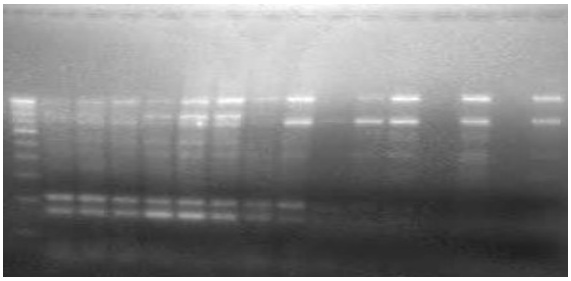


Plate 17 (c)

M 46 47 48 49 50

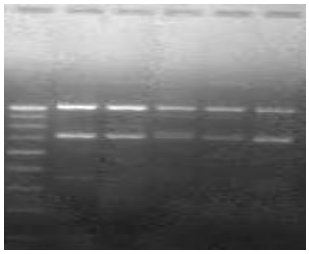


Plate 17 (d)

M : 100 bp ladder

1-5 : Laccadive Ordinary Tall 1-5 6-10 : Tiptur Tall 1-5 11-15 : Komadan1-5
16-20 : Kuttiadi 1-5 21-25 : Malappuram1-5 26-30 : Kasaragode1-5

Plate 17. Amplification (SSR) of fifty (a) 1-15 (b) 16-30 (c) 31-45 (d) 46-50 coconut genotypes with selected SSR primer CnCir B6

Table 17. Standardization of annealing temperatures of selected SSR primers for effective amplification

Sl No	Primer	Attended temperature range (^{0}C)	Selected temperature (^{0}C)
1	CnCir B6	53.5-65.0	61.0
2	CnCir B12	45-63.6	59.8
3	CnCir C3	53.5-65.0	58.0
4	CnCir C7	49-65	56.3
5	CnCir C12	50-61.9	50.2
6	CnCir E2	50-63.1	63.1
7	CnCir E10	53.5-63.9	59.5
8	CnCir G11	55.3- 62.0	59.1
9	CnCir H4	49.5-61.0	60.5

Since SSR marker system failed to bring out the variability between palms within a cultivar, the DNA from the five palms in each cultivar was pooled to study the diversity between the ten cultivars.

4.3.3.4. Diversity and population structure analysis of ten coconut cultivars using selected SSR primers

The gel profile for amplification of ten coconut cultivars with SSR primers is provided in Plate 18 a and 18 b. Percent of polymorphism was worked out for each primer. The details of the amplification are provided in Table 18. Observations are as follows.

CnCir B6

The primer CnCir B6 could generate two clear, distinct and reproducible bands (Plate 18 a. 1). It couldn't generate any polymorphic band.

CnCir B12

Only one amplicon was produced by the primer CnCir B12. The band was clear, distinct and reproducible (Plate 18 a. 2). It couldn't generate any polymorphism.

CnCir C3

Amplification of coconut genotypes by the primer CnCir B12 produced only one amplicon. The band was clear, distinct and reproducible (Plate 18 a. 3). It couldn't generate any polymorphism.

CnCir C7

CnCir C7 produced only single amplicon. The band was clear, distinct and reproducible (Plate 18 a. 4). It couldn't generate any polymorphism.

CnCir C12

The primer CnCir C12 generated two clear, distinct and reproducible amplicons and none of them were polymorphic (Plate 18 a. 5).

CnCir E2

The SSR primer CnCir E2 could generate only one amplicon by the amplification of coconut genotypes (Plate 18 a. 6) and it showed cent per cent polymorphism.

CnCir E10

CnCir E10 could generate only one amplicon by the amplification of coconut genotypes and it showed cent per cent polymorphism (Plate 18 b.1).

CnCir F2

Amplification of ten coconut cultivars with the selected primer CnCir F2 produced one clear, distinct and reproducible amplicon. It couldn't produce any polymorphic band.

The gel profile showing the amplification pattern of CnCir F2 with the ten coconut cultivars was presented in Plate 18 b. 2.

CnCir G11

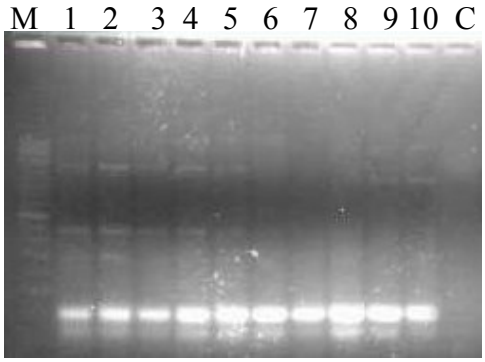
CnCir G11 was able to amplify two amplicons and the two were polymorphic giving cent per cent polymorphism. The bands were clear, distinct and reproducible (Plate 18 b. 3).

CnCir H4

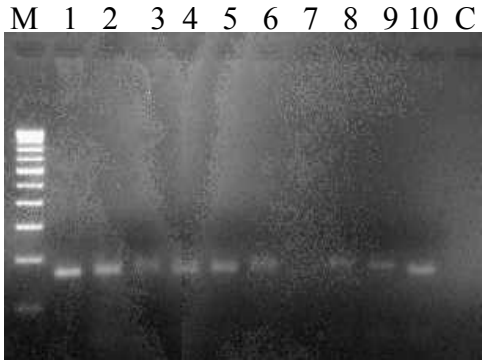
Only one amplicon was produced by the primer CnCir H4. The band was clear, distinct and reproducible (Plate 18 b.4). It couldn't generate any polymorphism.

SSR analysis using ten selected primer sets produced a total of 13 markers (Table 18) between the ten coconut genotypes. The number of scorable markers produced by each primer ranged from one (CnCir B12, CnCir C3, CnCir C7, CnCir E2, CnCir E10, CnCir F2 and CnCir H4) to two (CnCir B6, CnCir C12 and CnCir G11) with an average of 1.3 marker per primer. The molecular weight of these

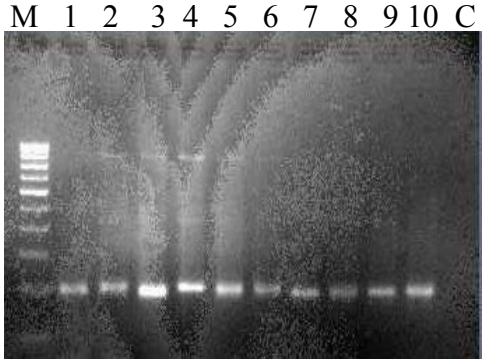
1. Primer CnCir B6



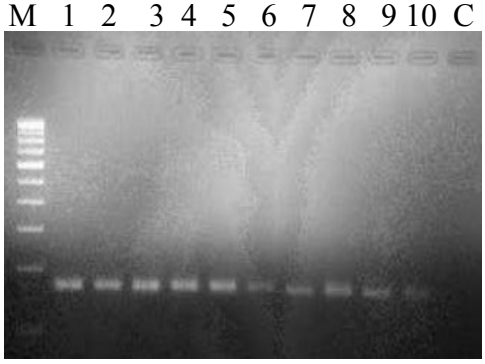
2. Primer CnCir B12



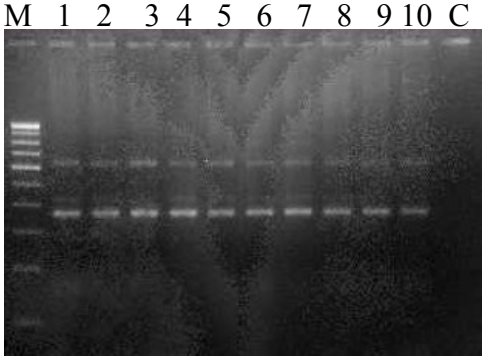
3. Primer CnCir C3



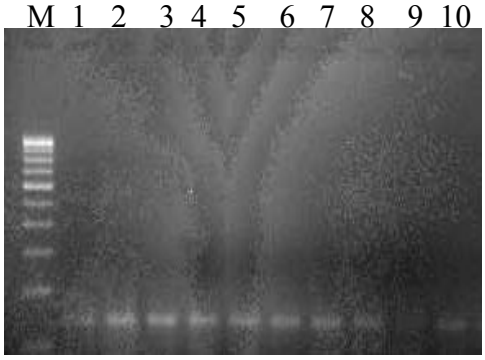
4. Primer CnCir C7



5. Primer CnCir C12



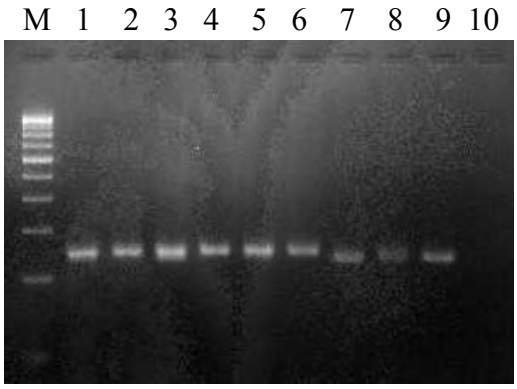
6. Primer CnCir E2



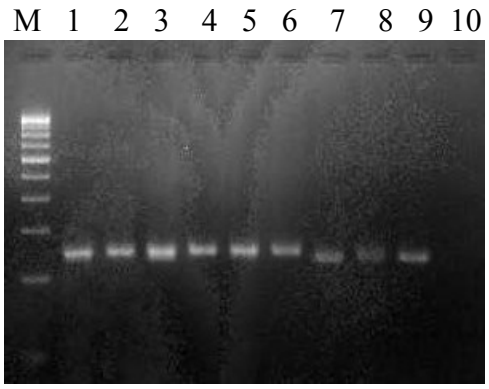
M : 100 bp ladder
1 : Laccadive Ordinary Tall
2 : Tiptur Tall
3 : Komadan
4 : Kuttiadi
5 : Kasaragod
6 : Chowghat Orange Dwarf
7 : Chowghat Green Dwarf
8 : Malayan Yellow Dwarf
9 : Gangabondam
10 : Malappuram
C : Control

Plate18 a. Amplification of coconut genotypes with selected SSR primers

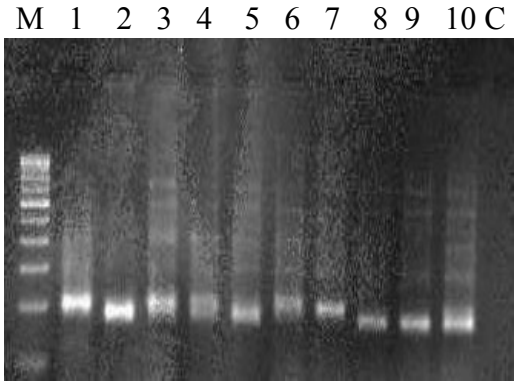
1. Primer CnCir E10



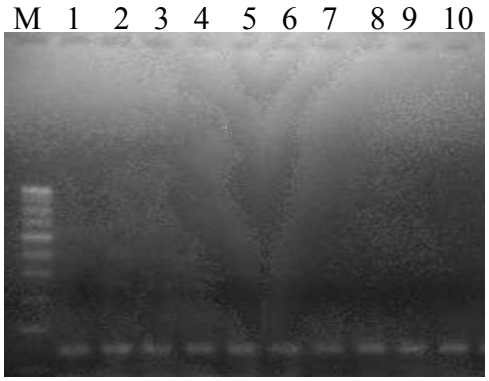
2. Primer CnCir F2



3. Primer CnCir G11



4. Primer CnCir H4



M : 100 bp ladder
1 : Laccadive Ordinary Tall
2 : Tiptur Tall
3 : Komadan
4 : Kuttiadi
5 : Kasaragod
6 : Chowghat Orange Dwarf
7 : Chowghat Green Dwarf
8 : Malayan Yellow Dwarf
9 : Gangabondam
10 : Malappuram
C : Control

Plate18 b. Amplification of coconut genotypes with selected SSR primers

Table 18. Details of amplification with selected primers for SSR assay in coconut

Sl No	Primer	Total no of amplicons	No of polymorphic amplicons	No of monomorphic amplicons	Polymorphism (%)
1	CnCir B6	2	0	2	0
2	CnCir B12	1	0	1	0
3	CnCir C3	1	0	1	0
4	CnCir C7	1	0	1	0
5	CnCir C12	2	0	2	0
6	CnCir E2	1	1	0	100
7	CnCir E10	1	1	0	100
8	CnCir F2	1	0	1	0
9	CnCir G11	2	2	0	100
10	CnCir H4	1	0	1	0
Total		13	4	9	30.76
Average		1.3	0.4	0.9	

markers was in between the range of 200 bp to 400 bp. The polymorphic bands were 30.76 per cent of the total.

4.3.4. Diversity and population structure analysis of ten coconut cultivars using combined (RAPD, ISSR and SSR) data

A total of 168 markers were produced by the amplification of ten coconut genotypes by RAPD, ISSR and SSR assay with an average of 5.4 markers per primer. The polymorphic bands were 41.66 per cent of the total, each primer detecting on an average 2.26 polymorphic bands per primer.

Data scored as presence (1) or absence (0) with respect to the 10 genotypes was subjected to diversity analysis.

Computing the genetic distances using Dice coefficients, the Neighbour Joining tree (Saitou and Nei, 1987) and bootstrap analysis (5000) were executed using DARwin ver.5.0. Cluster analysis was done using the UPGMA method and dendrogram was constructed by neighbor joining (Saitou and Nei, 1987). The dendrogram generated using combined data is given in Fig 3.a. The details of clusters are given in Table 19.a.

The dendrogram showed grouping the cultivars into three major clusters. ClusterI included four tall cultivars namely, Kuttiadi, Tiptur Tall, Malappuram and Kasaragode. ClusterII included three Dwarf cultivars (Chowghat Orange Dwarf, Chowghat Green Dwarf and Malayan Yellow Dwarf) and one intermediate (Gangabondam). ClusterIII included two tall cultivars-Laccadive Ordinary and Komadan.

The same binary data matrix was used for calculating the similarity coefficient using Jaccard's coefficient (Jaccard, 1908). The mean similarity coefficient for each accession pair was calculated and used for cluster analysis using the UPGMA method and a dendrogram was constructed using the software package

NTSYS-PC (Rohlf, 1993). The dendrogram generated using NTSYS is given in Fig. 3.b and details of clusters are given in table 19.b.

Using NTSYS, the tree was grouped into two major clusters including sub clusters. ClusterI for Talls (six cultivars) and cluster II for Dwarfs and intermediate (three Dwarfs and one intermediate).

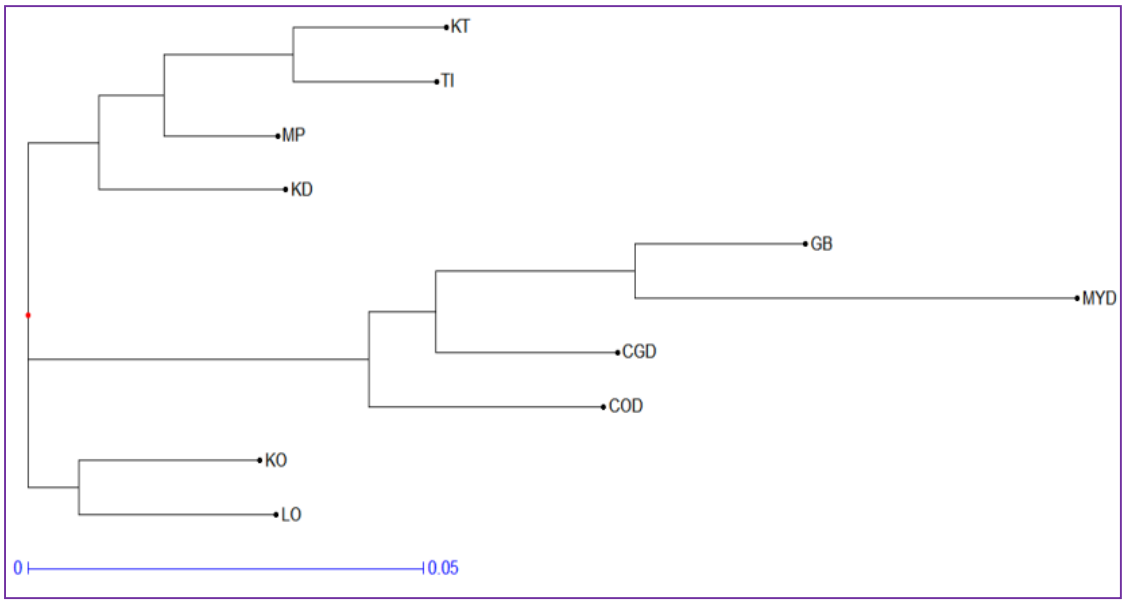
In the cluster for Talls, Kuttiadi was found genetically very close to Tiptur Tall. Komadan and Laccadive Ordinary showed more similarity as well as Malappuram to Kasaragode.

Among the Dwarfs, Chowghat Green Dwarf was found more similar to Chowghat Orange Dwarf than with Malayan Yellow Dwarf. The intermediate type Gangabondam showed more similarity with Malayan Yellow Dwarf than with Chowghat Orange Dwarf and Chowghat Green Dwarf.

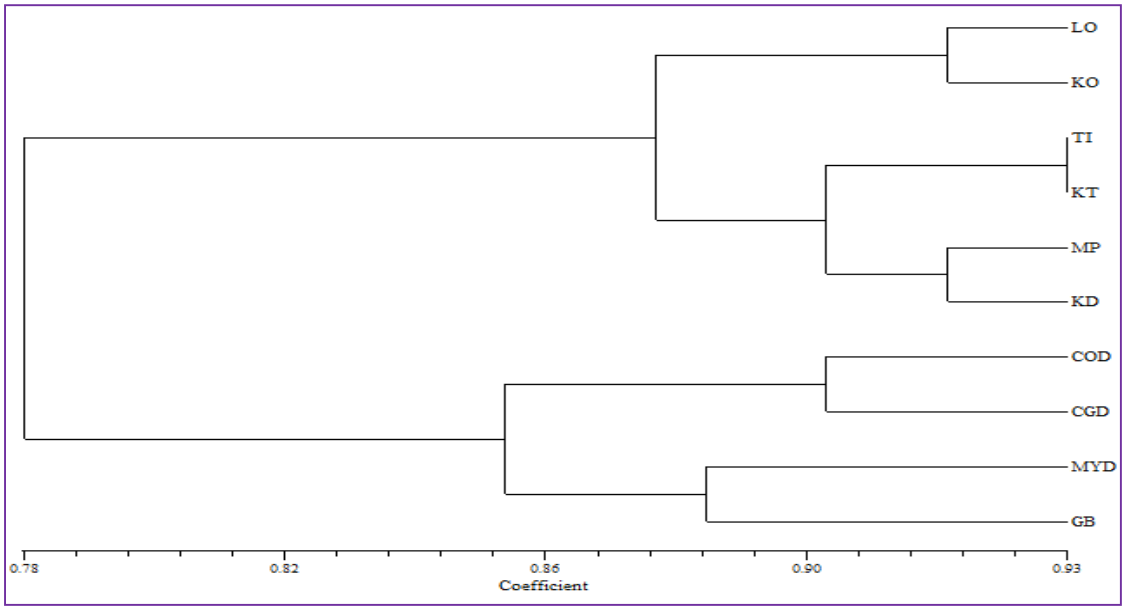
The dissimilarity matrix of different coconut genotypes based on the proportion of shared (RAPD, ISSR and SSR) fragments was also generated.

Combined (RAPD, ISSR and SSR) markers successfully classified the Talls and Dwarfs in separate clusters. Dissimilarity values ranged from 0.0375 (Laccadive Ordinary and Komadan) to 0.1956 (Tiptur Tall and Malayan Yellow Dwarf) as inferred from the dissimilarity matrix (Table 20). The cultivar Malayan Yellow Dwarf was distantly related to the rest of the varieties with an average genetic diversity (GD) value of 0.13.

Within tall population, the observed diversity is less (GD=0.06). In the cluster for Talls, cv. Kasaragode showed more difference from the rest of the group. Within this cluster, Kuttiadi was genetically very close to Tiptur Tall (GD=0.03) as well as Komadan to Laccadive Ordinary (GD=0.04) and they got clustered together seperately in same cluster.



a. DARwin ver.5.0



c. NTSYS

LO: Laccadive Ordinary, TI: Tiptur Tall, KO: Komadan, KT: Kuttiadi, MP: Malappuram, KD: Kasaragode, COD: Chowghat Orange Dwarf, CGD: Chowghat Green Dwarf, MYD: Malayan Yellow Dwarf, GB: Gangabondam

Fig. 3. Dendrogram generated for coconut cultivars out of combined (RAPD, ISSR and SSR) data using the softwares, DARwin and NTSYS

Table 19. Grouping of accessions based on combined (RAPD, ISSR and SSR) data using the softwares DARwin ver.5.0 and NTSYS

a. Grouping of accessions based on combined (RAPD, ISSR and SSR) data using the software DARwin ver.5.0

Cluster No	No of cultivars in each cluster	Name of the cultivars
Cluster I	4	Kuttiadi, Tiptur Tall, Laccadive Ordinary Malappuram,
Cluster II	5	Chowghat Orange Dwarf, Chowghat Green Dwarf, Malayan Yellow Dwarf, Gangabondam, Komadan,
Cluster III	1	Kasaragode

b. Grouping of accessions based on combined (RAPD, ISSR and SSR) data using the software NTSYS

Cluster No	No of cultivars in each cluster	Name of the cultivars
Cluster I	6	Laccadive Ordinary, Komadan, Kuttiadi, Tiptur Tall Malappuram, Kasaragode
Cluster II	4	Chowghat Orange Dwarf, Chowghat Green Dwarf, Malayan Yellow Dwarf, Gangabondam, ,

Table 20. Dissimilarity matrix of different coconut cultivars based on the proportion of shared RAPD, ISSR and SSR fragments

	LO	TI	KO	KT	MP	KD	COD	CGD	MYD
TI	0.0580								
KO	0.0479	0.0785							
KT	0.0753	0.0375	0.0821						
MP	0.0717	0.0544	0.0717	0.0443					
KD	0.0648	0.0680	0.0785	0.0648	0.0476				
COD	0.1095	0.1338	0.1095	0.1307	0.0986	0.0845			
CGD	0.1206	0.1237	0.0993	0.1348	0.0954	0.1025	0.0623		
MYD	0.1704	0.1956	0.1556	0.1852	0.1587	0.1661	0.1188	0.1000	
GB	0.1286	0.1530	0.1071	0.1571	0.1317	0.1388	0.0849	0.0741	0.0775

LO: Laccadive Ordinary, TI: Tiptur Tall, KO: Komadan, KT: Kuttiadi, MP: Malappuram, KD: Kasaragode,

COD: Chowghat Orange Dwarf, CGD: Chowghat Green Dwarf, MYD: Malayan Yellow Dwarf, GB: Gangabondam

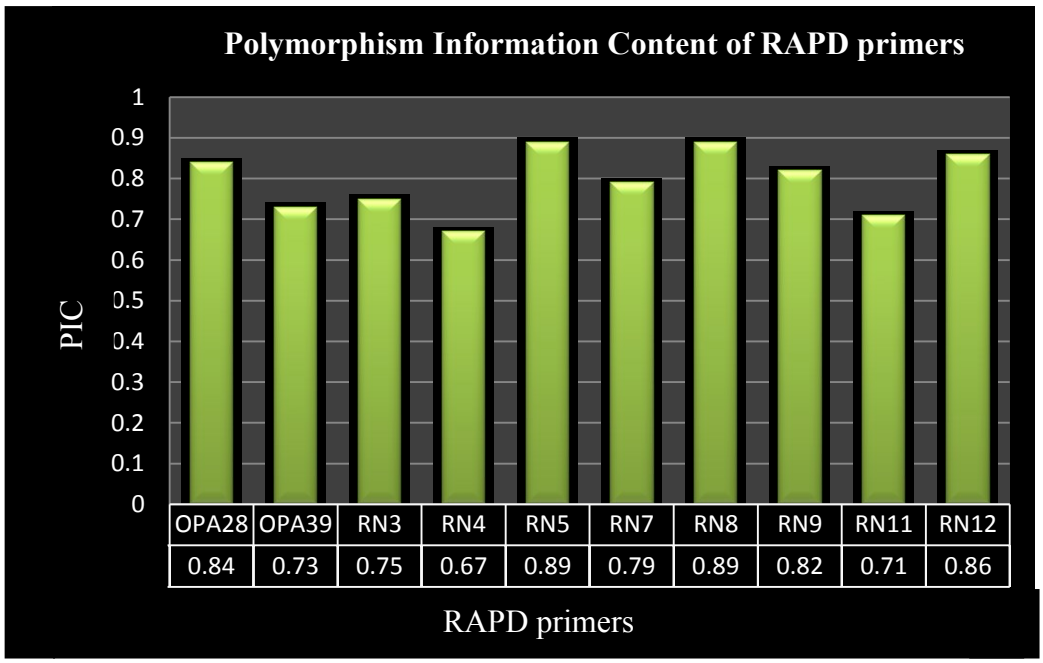
Among the Dwarfs, Chowghat Green Dwarf was found distinct from Chowghat Orange Dwarf (GD=0.06) and Malayan Yellow Dwarf (GD=0.10). The intermediate type Gangabondam showed more similarity with Malayan Yellow Dwarf than with Chowghat Orange Dwarf and Chowghat Green Dwarf.

Among all the cultivars, Tiptur Tall and Malayan Yellow Dwarf (GD=0.19) were found to be most distant ones from the rest of the members in the group and Tiptur Tall and Kuttiadi (GD=0.03) were found more similar.

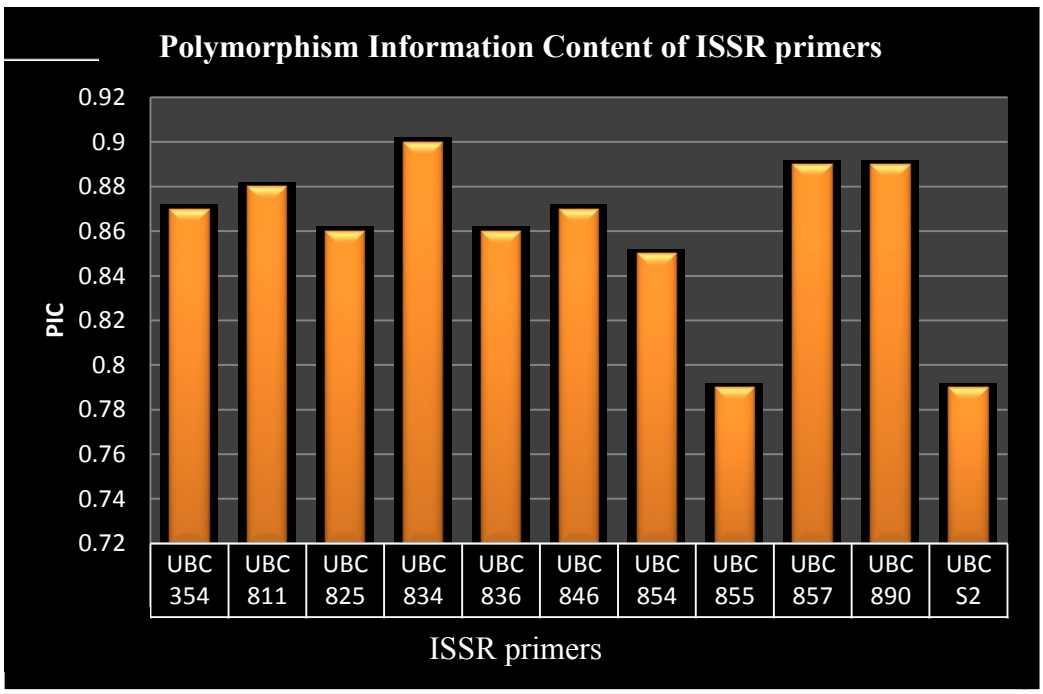
The number of markers varied in different cultivars. In general, more markers were present in tall accessions than in Dwarfs. Based on 168 markers from three marker systems the overall genetic diversity observed among the three morphological groups viz., Talls, Dwarfs and intermediate type palms in the selected population of coconut seed farm at Vellanikkara is comparatively less (GD=0.13). The highest genetic diversity was between Talls and Dwarfs (GD=0.14). The tall population consisting of six tall cultivars among which four are ecotypes of West Coast Tall showed the lowest diversity (GD-0.06). Within Talls, Kuttiadi is found to be similar to Tiptur Tall as well as Malappuram with least diversity (GD=0.04). The diversity observed within the Dwarf population of present study showed a higher diversity index (GD=0.09). This is mainly contributed by the Dwarf cultivar Malayan Yellow Dwarf collected from Regional Agricultural Research Station, Pilicode which is found to be genetically most distant from the rest of the nine cultivars (average GD=0.13). The intermediate type Gangabondam is genetically diverse from Talls and is grouped under the cluster for Dwarfs. Within this cluster it was found genetically closer to Malayan Yellow Dwarf.

4.3.5. Polymorphism Information Content (PIC) value for the 10 selected RAPD and ISSR primers

The polymorphism Information Content (PIC) value calculated for the 10 selected RAPD primers (Fig. 4a) varied from 0.67 (RN 4) to 0.89 (RN 5, RN 8). The 10 selected ISSR primers (Fig. 4b) recorded PIC values ranging from 0.79 (UBC 855, UBC S2) to 0.90 (UBC 834).



a. 10 selected RAPD primers



b. 10 selected ISSR primers

Fig.4. Polymorphism Information Content (PIC) value for 10 selected RAPD and ISSR primers

Discussion

5. DISCUSSION

The coconut palm (*Cocos nucifera* L.) is one of the major perennial oil crops in the wet tropics and it plays an important role in the rural and national economies of many coconut-growing countries. It is grown both as a cash and subsistence crop. Its multiple uses for food, oil, cosmetics and fuel, coupled with its tolerance of primitive husbandry and its role as provider of year-round employment for rural households, makes it one of the most useful plants among tropical cultivated crops.

Cocos nucifera (2n = 2x = 32) is a member of the monocotyledonous family Arecaceae (Palmae), subfamily Cocoideae and the monospecific genus *Cocos*. Traditionally, based on the growth habits, breeding behavior and other morphological characters, coconut is classified as Tall, Dwarf and intermediate type. In India, Talls are the predominant cultivated form, although Dwarfs occupy a significant area. The Talls are generally cross fertilized and the Dwarfs are self fertilized. The variability among tall varieties is higher compared to Dwarfs which is attributed by the cross pollinating nature of the tall palms.

Varietal diversity is an important criterion for crop improvement programmes. Coconut is blessed with a large number of varieties both in tall and dwarf forms. A wide range of variations occur within the same variety in tall palms due to their cross pollinating nature. The variations arise in the height of the palm, colour, shape and size of the nuts as well as yield and quality of copra. Conventionally, for genetic diversity studies, morphological traits have been used, but these poses several limitations such as low polymorphism, dependence on environmental conditions, stage of the crop etc. Molecular markers provide an efficient method of determining diversity among crop plants. Use of molecular markers for estimation of genetic diversity and varietal identification gives a better insight into the similarity / differences at the genetic level. Several molecular markers viz., RFLP (Lebrun *et al*., 1998), AFLP (Teulat *et al*., 2000), RAPD (Upadhyay *et al*., 2004), ISSR (Manimekalai and Nagarajan, 2006) SSR (Rajesh

et al., 2008) etc. have been extensively used for the diversity and population structure analysis in coconut.

It is essential to understand the genetic diversity within Indian coconut cultivars in order to utilize them effectively for breeding purposes. Knowledge of relatedness among different coconut cultivars will also help in identifying cultivars and thus avoiding duplication in the continuing screening programme for biotic and abiotic resistance. Knowing relationships among Indian cultivars and other major cultivars will also help when formulating future breeding strategies involving exotic cultivars. The DNA markers can be used for identification of superior but diverse parents for maximum exploitation of heterosis. The population structure and genetic diversity analysis of a crop help in the understanding of intra-specific relationships. The analysis of the relationships among genetic groups may also convey information regarding their origin and diversification. Combined with the three tier selection system used in seed production programmes of coconut, characterization at molecular level will be useful for selection programmes in identification of superior mother palms and seedlings at a very early stage.

The present study was thus designed to estimate the intra and inter population genetic variability, population structure and genetic relationship of fifty genotypes belonging to ten coconut cultivars commonly used for seed production in Kerala, based on molecular marker analysis.

5.1. Standardization of genomic DNA extraction

Isolation of good quality DNA is one of the important prerequisites in a reliable molecular biology work. Coconut is quite difficult to work on because of the high lipid, polysaccharide and polyphenol content of its leaves. During DNA isolation, the contaminants such as polysaccharides and polyphenols co-precipitate with DNA, making it unsuitable for molecular analysis. Polysaccharides are visually evident in

DNA extracted by their viscous glue-like texture and make the DNA unmanageable in pipetting and unamplifiable in polymerase chain reaction by inhibiting Taq polymerase activity.

For the present study, DNA was isolated from the first emerged spindle leaflets as the polysaccharide and polyphenol contaminants were the least in DNA isolated from this part. Angeles *et al*. (2005) confirmed that the young leaves of the first emergent frond provided enzyme digestible, good-quality DNA. Several methods and their modifications were tested for the extraction of good quality DNA with sufficient quantity. The protocols reported by Rogers and Bendich, (1994), Porebski *et al*. (1997) and Upadhyay *et al*. (1999) with modifications were tried for the isolation of good quality DNA. In all the methods tested except modified Porebsk*i et al*. (1997) gave DNA contaminated with polysaccharides. Polysaccharides co-precipitated with DNA and it was seen as a chalky white or glue- like precipitate along with DNA which made the DNA unamenable for PCR reactions. Method standardized by Porebski *et al*. (1997) was modified by avoiding the use of phenol and sodium acetate (2M) during extraction and chloroform:isoamyl alcohol (24:1) was used instead of phenol:chloroform reported by Porebsk*i et al*. (1997). DNA extracted by modified Cheung *et al*. (1993) method confirmed the use of 2.0 M NaCl in removing polysaccharides from young coconut leaves. Modified Porebski *et al*. (1997) protocol gave good quality DNA with sufficient quantity. It differed from the CTAB extraction protocol by the ethanol (95%) precipitation of DNA in presence of 5M NaCl.

5.2. Determination of the quality and quantity of DNA

The quality and quantity of DNA isolated was determined by agarose gel electrophoresis and Nanodrop ND-1000 spectrophotometer. In all fifty coconut samples isolated, intact, clear band was observed on agarose gel which indicated good quality DNA without degradation. Spectrophotometer method showed that the ratio of UV absorbance ranged between 1.8 to 2.0 which indicates the purity of DNA. The quantity of DNA was also good and is ranged from 800 to 3800 ng/µl per gram of leaf sample.

5.3. Molecular Marker Analysis

Molecular markers have been proved to be a fundamental and reliable tool for fingerprinting varieties, establishing the fidelity of progenies, germplasm characterization, diversity and population structure analysis etc. Molecular markers provide an important technology for evaluating levels and patterns of genetic diversity and population structure studies and have been utilized in a variety of plant species. Molecular markers, which detect variation at the DNA level overcome most of the limitations of morphological and biochemical markers. Use of molecular markers gains importance for perennial and recalcitrant crops like coconut, where progress in crop improvement is often hampered by many reasons such as long generation period, tall stature, perennial nature, high heterozygosity, lack of vegetative method of propagation etc. Molecular markers are independent of developmental stages of the crop and are not influenced by the varying environmental conditions. Hence it is preferred for diversity analysis than traditional morphological markers (Mohapatra, 2007; Krishna and Singh, 2007; Spooner *et al*., 2005).

The protocols (reaction mixture and thermal profile) for different marker systems such as RAPD, ISSR and SSR were standardized with the DNA from the cultivar, Chowghat Green Dwarf. A total of hundred and nineteen primers suitable to the three markers were screened with the genomic DNA from Chowghat Green Dwarf with the standardized protocols. The protocols were standardized by altering the quantities of DNA, $MgCl_2$, primer and dNTP mix and the optimum quantity of these components was found to be 2µl, 2µl, 1.5µl and 1.5µl respectively.

5.3.1. RAPD assay of coconut genotypes

The advent of automated PCR technology made a new set of markers available to scientists interested in comparing organisms at molecular level. Williams *et al*. (1990) were the first to use RAPD markers obtained by PCR amplification of DNA segments with single arbitrary primers. The RAPD reaction performed on genomic DNA with

arbitrary oligonucleotides results in the amplification of several discrete DNA products. The polymorphism between individuals results from change in sequence in one or both of the primer binding sites and is visible as presence or absence of a particular product. Such polymorphism, in general, behaves as dominant genetic markers. The banding pattern differences existing between two species or varieties can be used for varietal identification.

The RAPD amplification generated can be classified into two types: constant (monomorphic) and variable (polymorphic) between the genotypes. These differences can be used to examine and establish systematic relationship (Hadrys *et al*.,1992).

5.3.1.1. Screening of RAPD primers

Thirty five decamer primers were screened for the amplification of DNA from the genotype Chowghat Green Dwarf using the standardized protocol for RAPD assay. Nineteen primers from Operon series, fifteen RN series and one PNK 49-R were screened for amplification of coconut DNA.

5.3.1.2. Diversity and population structure analysis of ten coconut genotypes using selected RAPD primers

Out of thirty five RAPD primers screened for DNA amplification of coconut genotypes, the best ten primers with good banding pattern were selected for the final amplification of ten coconut cultivars. Among the selected primers, eight were from the RN series and the rest two from the OPA series.

Initially, amplification of DNA from individual palms within each cultivar was carried out using selected primers from each marker system. Amplification of fifty coconut genotypes with the selected RAPD primers (RN3 and RN5) could not produce any noticeable variability among the genotypes studied. This may be due to the genotypes selected were from the progenies of same mother palm. No data was

available regarding the source of palms in the coconut farm of Vellanikkara as this is an old plantation which has been established and transferred by Coconut Development Board and being maintained by the Central nursery.

However, these two primers together produced amplicons ranging from 10-15 from each cultivar (represented by five palms) indicating the suitability of these primers to bring out the genetic difference between cultivars. Hence further analysis was carried out by pooling the DNA from the different genotypes within each cultivar inorder to analyze the diversity between cultivars.

The selected primers produced clear and distinct amplification pattern with the ten coconut cultivars selected for the study. There were 65 amplicons of which 26 were polymorphic giving a polymorphism of 40 per cent. The number of amplicons produced ranged from three to twelve with an average of 6.5 amplicons per primer and a mean of 2.6 polymorphic bands per primer. Upadhyay *et al*. (2002) reported an average of 2.2 bands per primer in the study of molecular analysis of phylogenetic relationships among coconut accessions. The average bands per primer was 6.5 in the present study where as Upadhyay *et al*. (2004) had reported 9.6 markers per primer. Ashburner *et al*. (1997b) had reported 8.7 markers per primer in the study of RAPD analysis of South Pacific coconut populations. Paul *et al*. (2008) observed 10.31 bands per primer in the analysis of genetic diversity in yellow dwarf populations of coconut assessed using RAPD markers. The number of markers detected by each primer depends on primer sequence and the extent of variation is genotype specific.

Of the ten primers studied, three gave a polymorphism of above 50 per cent. The primer OPA 39 gave the highest polymorphism of 75 per cent. The percent polymorphism was less than 10 in cultivars, Tiptur Tall, Kuttiadi, Kasaragode and Chowghat Orange Dwarf and less than 20 in case of Laccadive Ordinary, Komadan and Malappuram. The polymorphism information content (PIC) of selected primers ranged

from 0.67 (RN4) to 0.89 (RN5, RN 8). This shows the effectiveness of selected RAPD primers in diversity analysis.

In the present study, the amplicons produced by selected random primers had a molecular weight ranging from 200 bp to 1856 bp. Upadhyay *et al*. (2004) had reported amplicons of size 200 to 2750 bp where as Cardena *et al*.(2003) observed amplicons of size ranged from 350 to 1710 bp in coconut.

In the present study, the primer RN8 could produce a unique amplicon of molecular weight 400 bp which was present only in Laccadive Ordinary, others lacked this amplicon. Another amplicon of 262 bp with the OPA39 primer could differentiate Dwarfs from the Talls as this was specific to the Dwarfs and the intermediate cultivars whereas all the Tall cultivars lacked this amplicon. An amplicon (300 bp) with the primer OPA28 was found specific to the three tall cultivars namely Laccadive Ordinary, Tiptur Tall and Komadan. Another unique amplicon of 400 bp with RN5 primer was found only in the cultivar Chowghat Green Dwarf. An amplicon of 400 bp was found only in Laccadive Ordinary and Komadan. The unique primers could be further exploited for varietal identity, for which larger populations of same genotype from different locations are to be analysed with the unique primers.

5.3.1.3. Genetic distance between cultivars based on RAPD data

Computing the genetic distances using Dice coefficient (Nei and Li, 1979), dissimilarity matrix was generated (bootstrapping 5000) and Cluster analysis was done using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) cluster analysis and dendrogram was constructed by Neighbor Joining (Saitou and Nei, 1987). The dendrogram generated from sixty five RAPD markers, grouped the cultivars into three major clusters. Cluster I consisted of four tall cultivars namely Kuttiadi, Tiptur Tall, Malappuram and Kasaragode. Cluster II included three dwarf cultivars viz., Chowghat Green Dwarf, Chowghat Orange Dwarf, Malayan Yellow Dwarf and the

intermediate type, Gangabondam. Cluster III included the two tall cultivars, Laccadive Ordinary and Komadan.

The same binary data matrix was used for calculating the similarity coefficient using Jaccard's coefficient (Jaccard, 1908). The mean dissimilarity coefficient for each pair of accessions was calculated to generate the matrix based on which cluster analysis was made using the UPGMA method and a dendrogram was constructed using the software package NTSYS-PC (Rohlf, 1993). Here only two clusters were produced with first cluster comprising of all Tall cultivars, the intermediate, the dwarf cultivars except Malayan Yellow Dwarf. Also Komadan and Lacadive Ordinary was found to be clusterd as identical and separated out from the rest of the genotypes in this cluster. Second cluster consists of Malayan Yellow Dwarf alone.

Using DARwin, the cultivars were grouped into three clusters whereas NTSYS grouped the cultivars into two major clusters. ClusterI using DARwin included all tall cultivars except Komadan while clusterI using NTSYS included all cultivars except Malayan Yellow Dwarf. ClusterII using DARwin consisted all dwarf cultivars whereas in NTSYS cluster II consisted only the dwarf cultivar Malayan Yellow Dwarf which was taken from RARS, Pilicode. Komadan formed the third cluster according to DARwin.

Dissimilarity values ranged from 0.0182 (Laccadive Ordinary and Komadan) to 0.1845 (Tiptur Tall and Malayan Yellow Dwarf). The cultivar Malayan Yellow Dwarf was found to be the most distant genotype. This is because, Malayan Yellow Dwarf being an exotic cultivar showed more variability than Indian accessions.

In the cluster for Talls, cv. Kasaragode showed more difference from the rest of the group. Within this cluster, Kuttiadi was genetically very close to Tiptur Tall as well as Komadan to Laccadive Ordinary and they got clustered together seperately in the

same cluster. Kuttiadi showed more similarity to Tiptur Tall, which is a popular tall cultivar in Karnataka. It is suspected that the seed nuts of Kuttiadi may be taken to Karnataka and planted and grown in the name of Tiptur Tall. Laccadive Ordinary clustered along with the superior tall cultivar of South Kerala, Komadan and showed some similarity. This is in conformation with the previous report of Selvaraju and Jayalekshmi (2011). The study was about the morphometric diversity of popular coconut cultivars of South Travancore in which they reported that Laccadive Ordinary and Komadan recorded the same value for yield and in their study also, they clustered together.

Among the Dwarfs, Chowghat Green Dwarf was found distinct from Chowghat Orange Dwarf and Malayan Yellow Dwarf. Chowghat Orange Dwarf showed more similarity to Chowghat Green Dwarf than with Malayan Yellow Dwarf. Both Chowghat Orange Dwarf and Chowghat Green Dwarf is identified and largely grown in the Chowghat area of Thrissur district. This may be the reason Chowghat Orange Dwarf showing more similarity to Chowghat Green Dwarf than to Malayan Yellow Dwarf. The intermediate type Gangabondam showed more similarity with Malayan Yellow Dwarf than with Chowghat Orange Dwarf and Chowghat Green Dwarf.

Among all the cultivars, Tiptur Tall and Malayan Yellow Dwarf were found to be most distant ones from the rest of the members in the group. This has been attributed by the varying stature, origin and breeding system of both the palms.

In general the GD value for the Tall cultivars (GD==0.06) is found to be very low compared to previous reports (GD=0.214) of genetic diversity of 20 accessions including five exotic accessions (Upadhyay *et al.*, 2004) The GD value for Dwarfs (GD=0.11) is similar to earlier reports (GD=0.178) (Upadhyay *et al.*, 2004). The GD value between Talls and Dwarfs is 0.13, between Talls and intermediate is 0.14, between Dwarfs and intermediate is 0.08 and among all cultivars studied is 0.13.

5.3.2. Inter Simple Sequence Repeats (ISSR)

The marker system called ISSR (Inter Simple Sequence Repeats) is a PCR based method that assess variation in the numerous microsatellite regions dispersed throughout the genome. In this technique reported by Zietkiewicz *et al*. (1994), primers based on microsatellites are utilized to amplify inter simple sequence repeat sequences in the DNA. When the primer successfully locates two microsatellite regions within an amplifiable distance away on the two strands of the template DNA, the PCR reaction will generate a band of a particular molecular weight for that locus representing the intervening stretch of DNA between the microsatellites. The method uses a single oligonucleotide primer composed of 4 to 10 tri or di nucleotide repeats and ending with 3'- or 5'- anchor sequence.

ISSR is a multilocus molecular technique based on PCR that identifies insertions and deletions in DNA. It is highly sensitive, highly reproducible, provides Mendelian segregation, and has been successfully applied in genetic and evolutionary studies of many species, including coconut (Wolfe *et al*., 1998; Camacho and Liston, 2001; Zizumbo-Villarreal *et al*., 2006); Manimekalai and Nagarajan, 2006). The ISSR marker requires small amounts of DNA and does not require information on DNA sequence. ISSR primers are designed from SSR motifs and can be undertaken for any plant species containing a sufficient number and distribution of SSR motifs in the genome (Gupta *et al*., 1996; Buhulikar *et al*., 2004). ISSR targets the highly variable microsetellite regions of the nuclear genome providing a large number of polymorphic fragments (Gupta *et al.,* 1994). Therefore this technique has been used extensively to evaluate genetic diversity both within and between plant populations in angiosperms and gymnosperms (Osborn *et al*., 2005).

5.3.2.1. Standardization of PCR conditions

Both the proportion of components in the reaction mixture and the thermal profile were standardized for good amplification of coconut samples. Good quality genomic DNA (25ng) was used for ISSR analysis as per the procedure reported by

Manimekalai and Nagarajan (2006) with slight modifications of reaction conditions and thermal profile.

5.3.2.2. Screening of ISSR primers

Forty one primers belong to UBC (University of British Columbia) series were screened for the amplification of DNA from the cultivar Chowghat Green Dwarf, using the standardized protocol for ISSR assay. Eleven primers having good amplification pattern with clear and distinct bands were used for ISSR analysis.

5.3.2.3. Diversity and population structure analysis of ten coconut genotypes using selected ISSR primers

Amplification of fifty coconut genotypes with the selected ISSR primers (UBC846 and UBC857) also revealed only very low variability among the genotypes within each cultivar indicating the possibility of same parentage. Hence the pooled DNA of five palms in each cultivar was subjected to ISSR assay using the selected 11 primers.

The selected primers produced a total of 90 amplicons of which 40 were polymorphic giving a polymorphism of 44.44 per cent with mean number of 3.6 polymorphic bands per primer. Manimekalai and Nagarajan (2006) reported an average of 8.0 polymorphic bands per primer in the analysis of genetic relationships of 33 coconut accessions including exotic accessions (29 Talls, 2 intermediates and two Dwarfs). The average bands per primer is 8.2 where as Manimekalai and Nagarajan (2006) had reported 10.40 markers per primer. The amplicon size in the present study ranged from 190 bp (UBC354) to 2600 bp (UBC857). A similar range (200 to 2750 bp) was reported by Manimekalai and Nagarajan (2006b).

Primer UBC 811 and UBC 834 produced cultivar specific bands. UBC 811 produced a specific marker (476 bp) for Malayan Yellow Dwarf and Gangabondam

while UBC834 produced a band (420 bp) specific to Tiptur Tall and Kuttiadi. UBC 825 differentiated Dwarfs from the Talls by 1752 bp amplicon specific to the Talls and a 1247 bp band specific to Dwarfs and intermediate type

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5.3.2.4. Genetic distance between cultivars based on ISSR data

In this marker system also genetic distances were computed using Dice coefficient (Nei and Li, 1979), dissimilarity matrix was generated (bootstrapping 5000) and cluster analysis was done using UPGMA and dendrogram was constructed by Neighbor Joining (Saitou and Nei, 1987). The dendrogram generated from 90 ISSR markers, grouped the cultivars into three major clusters which was slightly different from that of RAPD i.e. a Dwarf group and two Tall groups.

ClusterI comprised of four tall cultivars viz., Kuttiadi, Tiptur Tall, Malappuram but instead of cv. Kasaragode in RAPD dendrogram, Laccadive Ordinary was included in first cluster of ISSR. Within this cluster, Kuttiadi was genetically very close to Tiptur Tall like in RAPD dendrogram. Malappuram and Laccadive Ordinary were more distinct among the cluster for Talls but in RAPD dendrogram both were clustered in same group.

Cluster II is similar to that in RAPD except with an anomaly that the tall cultivar, Komadan got clustered along with this dwarf group. Devakumar *et al*. (2012) reported this kind of deviation in a study about the assessment of the genetic diversity of Indian coconut accessions and their relationship to other cultivars, using microsatellite markers. They reported that, two dwarf cultivars were assembled in Tall group and similarly two tall cultivars grouped along with dwarf group. Among the Dwarfs, Chowghat Green Dwarf was found more similar to Chowghat Orange Dwarf. Similar results were observed in the study conducted by Devakumar *et al*. (2012) in which Chowghat Green Dwarf and Chowghat Orange Dwarf are homozygous at all the loci except one. The high level of similarity between these two cultivars is suspected that

either one is a derivative of the other. They reported that 'Chowghat Orange Dwarf' is a mutant form.

The cultivar Malayan Yellow Dwarf was the most distant from the rest of the varieties as in RAPD dendrogram. The possible reason for this may be that this is the only one exotic cultivar among the group. The intermediate type Gangabondam showed more similarity with Malayan Yellow Dwarf than with Chowghat Orange Dwarf and Chowghat Green Dwarf like in RAPD dendrogram.

The tall cv Kasragode was separated out as the third cluster based on ISSR data. Among all the cultivars, Tiptur Tall and Malayan Yellow Dwarf were found to be more distant ones from the rest of the members in the group. This is in conformation with the RAPD data. This may be due to the contrasting breeding behavior, stature and varying geographic origin of both cultivars.

The number of markers varied in different cultivars. In general, more markers were present in Tall accessions than in Dwarfs. Based on the dissimilarity matrix it was found that the Talls (involving six cultivars) and Dwarfs (involving three cultivars) showed the same genetic diversity value (GD) of 0.07. Between Talls and Dwarfs the average dissimilarity was 0.16. Between Talls and intermediate type the genetic diversity observed was 0.14, between Dwarfs and intermediate type the genetic diversity was 0.07. The genetic diversity calculated for all cultivars studied was 0.16.

Using NTSYS, the genotypes were grouped into two major clusters which were further divided into sub clusters. ClusterI for Talls (six cultivars) and clusterII for Dwarfs (three cultivars) and intermediate (one cultivar). In the cluster for Talls, Laccadive Ordinary and Tiptur Tall was found genetically similar as well as Malappuram and Kasaragode and tall cultivar, Komadan was distantly clustered in the same group. Among Dwarfs, Chowghat Orange Dwarf was found genetically very close

to Chowghat Green Dwarf. The intermediate type Gangabondam showed more similarity with Malayan Yellow Dwarf.

Using DARwin, the cultivars were grouped into three whereas in NTSYS, they were grouped into two. ClusterI using DARwin included the four tall cultivars (Kuttiadi, Tiptur Tall, Laccadive Ordinary and Malappuram) whereas using NTSYS, it included all the tall cultivars (Laccadive Ordinary, Tiptur Tall, Kuttiadi, Malappuram, Kasaragode and Komadan). ClusterII according to DARwin consisted of three dwarf cultivars (Chowghat Orange Dwarf, Chowghat Green Dwarf, Malayan Yellow Dwarf), one intermediate (Gangabondam) and one tall cultivar (Komadan) while clusterII using NTSYS is of Dwarfs (Chowghat Orange Dwarf, Chowghat Green Dwarf, Malayan Yellow Dwarf) and intermediate (Gangabondam). ClusterIII according to DARwin included only the tall cultivar, Kasaragode.

5.3.3. Simple Sequence Repeats (SSR)

Microsatellites, or simple sequence repeats (SSRs), are becoming increasingly attractive markers in molecular breeding and diversity assessment (Morgante and Olivieri, 1993; Powell *et al*., 1996). SSRs are short tandemly repeated sequence motifs of approximately 1-8 bp in length, which are scattered throughout the genome and can vary between individuals in repeat length. High frequencies of polymorphism have been described for SSRs in several plant species (Saghai-Maroof *et al*., 1994; Gupta *et al*., 1996; Chase *et al*., 1996). Primer pairs designed for the flanking sequences can be used in PCR reactions for site-specific amplification of the microsatellite, thereby producing sequence-tagged microsatellite markers (Powell *et al*., 1996).

Simple sequence repeats or microsatellites provide an ideal tool for diversity studies due to their high information content, ease of genotyping through PCR, co-dominant and multi allelic nature and high discriminating power (Russell *et al*., 1997). In addition only small amounts of DNA are required and the quality of the DNA need not be as high as for most of the other DNA assay methods (Rafalski *et al*., 1996).

Microsatellites have been used in both agricultural and breeding studies as well as in the analysis of natural plant populations and have previously been shown to be appropriate for evaluating and characterizing coconut germplasm (Perera *et al*., 1999).

High development cost of SSRs and generation of less number of amplicons is a major impediment to the routine application of SSRs in diversity studies of crops and identifying markers located in the chromosomal regions of interest.

5.3.3.1. Standardisation of PCR conditions

Both the proportion of components in the reaction mixture and the thermal profile were standardized for good amplification of coconut samples. Good quality genomic DNA (30-35 ng) was used for SSR analysis.

5.3.3.2. Screening of primers for SSR analysis

Ten sets of primers were selected for the SSR analysis after screening forty three, based on the amplification pattern. Selected primers showed good amplification pattern with clear and distinct bands and their annealing temperatures for amplification were standardized by providing different annealing temperatures using gradient thermo cycler (Eppendorf, USA).

5.3.3.3. Diversity analysis of ten coconut genotypes using selected SSR markers

SSR markers also did not produce any noticeable variability among the individual palms of each cultivar as in the case of RAPD and ISSR. Hence the pooled DNA of each cultivar was subjected to SSR assay.

The ten selected primer sets produced clear and distinct amplification pattern with the ten coconut cultivars used for the study. The total number of amplicons produced per primer ranged from one to two. On an average of 1.3 amplicon was produced per primer. The ability of the primers for detecting polymorphism was very poor.

SSR analysis using ten selected primer sets produced a total of 13 markers among the ten coconut genotypes. The number of scorable markers produced by each primer ranged from one (CnCir B12, CnCir C3, CnCir C7, CnCir E2, CnCir E10, CnCir F2 and CnCir H4) to two (CnCir B6, CnCir C12 and CnCir G11) with an average of 1.3 marker per primer. The molecular weight of these markers was in between the range of 200 bp to 400 bp. The polymorphic bands were 30.76 per cent of the total. The polymorphism percentage recorded was very low to assess the genetic diversity among the ten coconut cultivars selected. One possible explanation for lower polymorphism detection in SSR is that, in the present study, fewer microsatellites were used. The number of microsatellites and the discriminating capacity of microsatellites can play a role in identifying different coconut accessions. So more primers need to be screened and used for amplification of DNA in coconut cultivars.

5.3.4. Diversity analysis based on all the three marker data

A total of 168 markers were produced by the amplification of ten coconut genotypes by RAPD, ISSR and SSR assay with an average of 5.4 markers per primer. The polymorphic bands were 41.6 per cent of the total, each primer detecting on an average 2.26 polymorphic bands per primer.

Using DARwin, the dissimilarity matrix of different coconut genotypes based on the proportion of shared RAPD, ISSR and SSR markers was generated and dendrogram showed three clusters, two for Talls and one for the Dwarfs and intermediate type. In the cluster for Talls, cv. Kasaragode showed more difference from the rest of the group. Within this cluster, Kuttiadi was genetically very close to Tiptur Tall as well as Komadan to Laccadive Ordinary. Among Dwarfs, Chowghat Green Dwarf was found distinct from Chowghat Orange Dwarf and Malayan Yellow Dwarf. The intermediate type Gangabondam showed more similarity with Malayan Yellow Dwarf as in RAPD and ISSR.

The cultivar Malayan Yellow Dwarf was the most distant from the rest of the nine genotypes. This is in conformation with the clustering pattern obtained by RAPD and ISSR separately.

Among all the cultivars, Tiptur Tall and Malayan Yellow Dwarf were found to be most distant ones from the rest of the members in the group and Tiptur Tall and Kuttiadi were found more similar. This is in accordance with the dendrogram obtained by RAPD and ISSR separately. So in future breeding programmes, Malayan Yellow Dwarf and Tiptur Tall can be considered for exploitation of heterosis and for production of a T x D hybrid as they are the most divergent among the cultivars studied. However other desirable morphological characters also should be taken into consideration.

The genetic diversity values were calculated from the dissimilarity matrix obtained based on 168 markers from three marker systems. The overall genetic diversity (GD) observed between the three morphological groups viz., Talls, Dwarfs and intermediate type palms in the selected population of coconut seed farm at Vellanikkara is comparatively less (GD=0.13). Upadhyay *et al*. (2004) reported a GD value of 0.214 for all the accessions consisting of 81 palms representing 13 Talls, 6 Dwarfs and one intermediate. They have already reported that among Indian accessions, the genetic diversity was very less compared to exotic accessions. The low genetic diversity observed in the present study may be due to the inclusion of all indigenous accessions and among tall cultivars, four are the morphotypes of West Coast Tall.

The highest genetic diversity is between Talls and Dwarfs (GD=0.14). Pair wise comparison showed that genetic distance range from 0.0375 (Laccadive Ordinary and Komadan) to 0.1956 (Tiptur Tall and Malayan Yellow Dwarf). The tall population consisting of six tall cultivars among which four are ecotypes of West Coast Tall showed the lowest diversity (GD=0.06). Upadhyay *et al*. (2004) had observed in their study a GD value of 0.214 for tall cultivars including more number of accessions (13)

than the present study. Within Talls, Kuttiadi is found to be similar to Tiptur Tall as well as Malappuram with least diversity (GD=0.04). The diversity observed within the Dwarf population of present study showed a higher diversity index (GD=0.09) than Talls (GD=0.06). The study reported by Upadhyay *et al*. (2004) also observed a genetic diversity of 0.178 for dwarf accessions. However, tall groups showed higher diversity in their study. The higher genetic diversity for Dwarfs is mainly contributed by the dwarf cultivar Malayan Yellow Dwarf collected from Regional Agricultural Research Station, Pilicode which is found to be genetically most distant from the rest of the nine cultivars (average GD=0.16). The intermediate type Gangabondam is genetically diverse from Talls and is grouped under the cluster for Dwarfs. This is in conformation with the study by Upadhyay *et al*. (2002) in which Gangabondam showed more similarity to dwarf accessions than to Talls. Within that cluster it was found genetically closer to Malayan Yellow Dwarf.

Using NTSYS, the genotypes were grouped into two major clusters which were further divided into sub clusters. ClusterI for Talls (six cultivars) and cluster II for Dwarfs and intermediate (three Dwarfs and one intermediate). In the cluster for Talls, Kuttiadi was found genetically very close to Tiptur Tall. Komadan and Laccadive Ordinary showed more similarity whereas Malappuram was similar to Kasaragode. Among the Dwarfs, Chowghat Green Dwarf was found more similar to Chowghat Orange Dwarf than with Malayan Yellow Dwarf. The intermediate type Gangabondam showed more similarity with Malayan Yellow Dwarf.

The combined markers generated almost similar dendrogram using NTSYS and DARwin, except for the following differences. The tall cultivars were grouped into three clusters based on DARwin where as they were grouped into two using NTSYS. In the cluster for Talls, Malappuram and Kasaragode were found distinct using DARwin where as using NTSYS, they both found genetically similar. Chowghat Orange Dwarf and Chowghat Green Dwarf showed more similarity in the dendrogram based on

NTSYS where as they were clusterd separately in the dendrogram using DARwin. Rest of the clustering pattern is similar with respect to the two softwares.

The differences in the dendrograms based on the softwares, DARwin and NTSYS is due to the varying approach of determining dissimilarity values. DARwin software calculate dissimilarity based on Dice coefficient (Nei and Li, 1979), whereas in NTSYS, Jaccard's similarity coefficient is used.

Values for all these coefficients range between zero and one. For a given data set, the corresponding values of Jaccard's dissimilarity are always greater than those of the Dice dissimilarity. The Dice coefficient of similarity differs quantitatively from Jaccard's similarity in that the Dice measure of similarity attaches more importance to the factors with positive response for both individuals (shared ones) than to those with positive response in only one individual or the other.

5.3.5. Comparison between the three marker systems

The average number of markers produced per primer in each marker systems such as RAPD, ISSR and SSR are 6.5, 8.2 and 1.3 respectively. The number of polymorphic amplicons detected per primer in each marker system is 2.6, 3.6 and 0.4 respectively. From this data, it is clear that IISR marker system is superior than others with respect to total number of markers and polymorphic markers detected. This is due to the ability of ISSR primers amplifying more DNA fragments in between the repeat regions. For diversity studies, large numbers of markers are needed. So for genetic diversity analysis, ISSR marker system gives better result than the other two.

The dendrograms as well as genetic diversity values calculated from the dissimilarity matrix obtained by each marker system showed certain differences between them. The dendrogram derived from combined molecular data revealed a better representation of the relationship than individual markers. The dendrogram obtained by RAPD and the combined markers were similar whereas that obtained by RAPD and

ISSR showed some deviation. This may be because RAPD and ISSR techniques use two different approaches to identify DNA sequence variations. RAPD identifies random regions of the genome, while ISSR identifies insertions and deletions in the DNA sequence.

In the present study it was found that combination of three markers produced more accurate classification of genotypes. Hence different types of molecular markers (such as AFLP and SNPs) need to be included in future studies along with the morphological data and information on phylogenic relationships in order to have comprehensive information about the population structure in coconut. Also use of more number of primers for each marker system will help in better coverage of the genome. The present study was limited to the genotypes available in a compact area planted for seed production purpose. Diverse genotypes such as Laccadive Micro, Spicata, Andaman Giant and other indigenous and exotic cultivars collected from different locations have to be used in future programmes of diversity analysis to help the breeders for identifying better parental combinations for hybrid seed production.

Summary

6. SUMMARY

Estimation of genetic diversity is a pre-requisite for utilization of germplasm in crop improvement. Morphological and biochemical markers have the disadvantage of being influenced by environment and are limited in number. Molecular markers, which detect variation at the DNA level overcome most of the limitations of morphological and biochemical markers. Use of molecular markers gains importance for perennial crops like coconut, where progress in crop improvement is often hampered by its long pre bearing period. The study entitled "Diversity and population structure analysis in coconut (*Cocos nucifera* L.) using molecular markers" was carried out at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture Vellanikkara during 2010-2012, to determine the intra and inter population genetic variability, population structure and genetic relationship of fifty genotypes belonging to ten coconut cultivars commonly used for seed production in Kerala, based on molecular marker analysis.

The experiment material included six tall cultivars (Laccadive Ordinary, Tiptur Tall, Komadan, Kuttiadi, Malappuram, Kasaragode), three dwarf cultivars (Chowghat Green Dwarf, Chowghat Orange Dwarf and Malayan Yellow Dwarf) and one intermediate (Gangabondam). Five palms from each cultivar were used for the study.

The protocol for genomic DNA isolation was standardized. The protocol suggested by Porebski *et al*. (1997) with certain modifications was found to be the most appropriate for isolation of DNA from coconut genotypes. The quality and quantity of DNA was analyzed by NanoDrop® ND-1000 spectrophotometer. The absorbance ratio ranged from 1.80-2.0, which indicated the purity of DNA. The quantity of DNA was also good and ranged from 800 to 3500 ng/µl per gram of leaf sample. The DNA was suitable for RAPD, ISSR and SSR analysis.

Protocols for RAPD, ISSR and SSR assay in coconut genotypes were standardized by varying the quantities of DNA and components of PCR mixture. 35 RAPD primers, 41 ISSR primers and 43 SSR primer sets were screened for their ability to amplify DNA fragments. Out of these, 10 RAPD, 11 ISSR primers and 10 SSR primer sets were selected based on the banding pattern and number of amplicons produced with clarity, distinctness and reproducibility. Sixty five, ninety and thirteen markers were generated by RAPD, ISSR and SSR assay respectively. The Polymorphism Information content (PIC) calculated ranged between 0.67 to 0.89 for RAPD primers and 0.78 to 0.90 for ISSR primers. RAPD primers (RN5, RN 8) and ISSR primer UBC 834 reported highest PIC values. The primer OPA 39 gave the highest polymorphism of 75 per cent among RAPD primers. Among ISSR primers, UBC S2 recorded the highest polymorphism percentage of 60 and in SSR, cent per cent polymorphism was detected by the primers, CnCir E2, CnCir E10 and CnCir G11.

Data scored as presence (1) or absence (0) with respect to the 10 genotypes was subjected to diversity analysis and the genetic distance was computed using Dice coefficient using the software DARwin ver. 5.0 and Jaccard's coefficient using the software NTSYS-PC. The dendrograms were generated using RAPD, ISSR and combined (RAPD+ISSR+SSR) markers. The genetic diversity values were calculated for pairs of cultivars according to RAPD, ISSR and combined markers using the software DARwin.

The dendrograms generated using the two softwares, DARwin and NTSYS showed some differences. It is due to the varying approach of determining dissimilarity values by the softwares. DARwin software calculate dissimilarity based on Dice coefficient, whereas in NTSYS, Jaccard's similarity coefficient is used. The dendrogram generated using DARwin showed more accuracy while considering the morphological characters. Hence the genetic diversity values were calculated according to DARwin only.

Based on RAPD data, using DARwin, the cultivars were grouped into three clusters whereas NTSYS grouped the cultivars into two major clusters. ClusterI using DARwin included all tall cultivars except Komadan while clusterI using NTSYS included all cultivars except Malayan Yellow Dwarf. ClusterII using DARwin consisted all dwarf cultivars whereas in NTSYS cluster II consisted only the dwarf cultivar Malayan Yellow Dwarf which was taken from RARS, Pilicode. Komadan formed the third cluster according to DARwin.

Dissimilarity values ranged from 0.0182 (Laccadive Ordinary and Komadan) to 0.1845 (Tiptur Tall and Malayan Yellow Dwarf) according to RAPD data.

Based on the dissimilarity matrix generated out of RAPD data, it was found that within the Dwarfs (involving three cultivars), the genetic diversity (GD) was the highest (average dissimilarity 0.11) compared to that within the tall group (involving six cultivars) (average dissimilarity 0.06). Between Talls and Dwarfs the average dissimilarity was 0.10. Between Talls and intermediate type the genetic diversity observed was 0.13, between Dwarfs and intermediate type the genetic diversity was 0.08. The genetic diversity calculated for all cultivars studied was 0.11.

Based on ISSR data, using DARwin, the cultivars were grouped into three whereas in NTSYS, they were grouped into two. ClusterI using DARwin included the four tall cultivars (Kuttiadi, Tiptur Tall, Laccadive Ordinary and Malappuram) whereas using NTSYS, it included all the tall cultivars (Laccadive Ordinary, Tiptur Tall, Kuttiadi, Malappuram, Kasaragode and Komadan). ClusterII according to DARwin consisted of three dwarf cultivars (Chowghat Orange Dwarf, Chowghat Green Dwarf, Malayan Yellow Dwarf), one intermediate (Gangabondam) and one tall cultivar (Komadan) while clusterII using NTSYS is of Dwarfs (Chowghat Orange Dwarf, Chowghat Green Dwarf, Malayan Yellow Dwarf) and intermediate (Gangabondam). ClusterIII according to DARwin included only the tall cultivar, Kasaragode.

Dissimilarity values ranged from 0.0365 (Chowghat Orange Dwarf and Chowghat Green Dwarf) to 0.2276 (Tiptur Tall and Malayan Yellow Dwarf) as inferred from the dissimilarity matrix generated out of ISSR data.

Based on the dissimilarity matrix it was found that within the Tall (involving six cultivars) and within the Dwarf (involving three cultivars) group showed the same genetic diversity (GD) of 0.07. Between Talls and Dwarfs the average dissimilarity was 0.16. Between Talls and intermediate type the genetic diversity observed was 0.14, between Dwarfs and intermediate type the genetic diversity was 0.07. The genetic diversity calculated for all cultivars studied was 0.16.

The combined markers generated almost similar dendrogram using NTSYS and DARwin, except for the following differences. The tall cultivars were grouped into three clusters based on DARwin where as they were grouped into two using NTSYS. In the cluster for Talls, Malappuram and Kasaragode were found distinct based on DARwin whereas based on NTSYS, they both found genetically similar. Chowghat Orange Dwarf and Chowghat Green Dwarf showed more similarity in the dendrogram based on NTSYS where as they were clusterd separately in the dendrogram using DARwin. Rest of the clustering pattern is similar with respect to the two softwares.

Based on 168 markers from three marker systems the overall genetic diversity observed among the three morphological groups viz., Talls, Dwarfs and intermediate type palms in the selected population of coconut seed farm at Vellanikkara is comparatively less (GD=0.13). The highest genetic diversity was between Talls and Dwarfs (GD=0.14). The tall population consisting of six tall cultivars among which four are ecotypes of West Coast Tall showed the lowest diversity (GD=0.06).

Within Talls, Kuttiadi is found to be similar to Tiptur Tall as well as Malappuram with least diversity (GD=0.04). It is suspected that the seed nuts of Kuttiadi may be taken to Karnataka and planted and grown in the name of Tiptur Tall.

Laccadive Ordinary and Komadan is sharing some common features as it was clustered together.

The diversity observed within the Dwarf population of present study showed a higher diversity index (GD=0.09). This is mainly contributed by the dwarf cultivar Malayan Yellow Dwarf collected from Regional Agricultural Research Station, Pilicode which is found to be genetically most distant from the rest of the nine cultivars (average GD=0.13). The intermediate type Gangabondam is genetically diverse from Talls and is grouped under the cluster for Dwarfs. Within this cluster it was found genetically closer to Malayan Yellow Dwarf. Among all cultivars, Tiptur Tall and Malayan Yellow Dwarf were found to be most distant ones from the rest of the members in the group. This has been attributed by the varying stature, origin and breeding system of both the palms. So in future breeding programmes, Malayan Yellow Dwarf and Tiptur Tall can be considered for exploitation of heterosis and for production of a T x D hybrid as they are the most divergent among the cultivars studied. However other desirable morphological characters also should be taken into consideration.

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Appendices

APPENDIX-I

Details of laboratory equipment items used for the study

High speed refrigerated centrifuge	: Kubota, Japan
Horizontal electrophoresis system	: BIO-RAD
Thermal cycler	: Master cycler personal, Eppendorf
Gel documentation system	: BIO- RAD, USA UVP (Inc. CA)
Nanodrop^{R} ND-1000 Spectrophotometer	: Nanodrop^{R} Technologies Inc. USA

APPENDIX-II

Composition of buffers and dyes used for agarose gel electrophoresis

1. TAE Buffer 50X

- 242 g Tris base
- 57.1 ml glacial acetic acid
- 100 ml 0.5 M EDTA (pH 8.0)

2. Loading Dye (6X)

- 0.25% Bromophenol blue
- 0.25% Xylene cyanol
- Glycerol in water

3. Ethidium bromide

- The dye was prepared as a stock solution of 1mg/ml in water and was stored at room temperature in a dark bottle.

Abstract

DIVERSITY AND POPULATION STRUCTURE ANALYSIS IN COCONUT (*Cocos nucifera* L.) USING MOLECULAR MARKERS

By

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ABSTRACT OF THE THESIS

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ABSTRACT

Assessment of genetic diversity and thus understanding the population structure is the mainstay for any crop improvement programmes. With the advent of molecular markers and suitable software for statistical analysis, this became a routine work in plant breeding. The technique is especially helpful in breeding of perennial heterozygous crops such as coconut. Hence the present study on 'Diversity and population structure analysis in coconut (*Cocos nucifera* L.) using molecular markers' was undertaken with the objective to estimate the genetic diversity among the selected cultivars using three marker systems viz., Random Amplified Polymorphic DNA (RAPD), Inter Simple Sequence Repeats (ISSR) and Simple Sequence Repeats (SSR).

The experiment material included six tall cultivars (Laccadive Ordinary, Tiptur Tall, Komadan, Kuttiadi, Malappuram, Kasaragode), three dwarf cultivars (Chowghat Green Dwarf, Chowghat Orange Dwarf and Malayan Yellow Dwarf) and one intermediate (Gangabondam). Five palms from each cultivar were used for the study. The DNA isolated from each cultivar was amplified using selected primers for RAPD, ISSR and SSR.

The DNA isolation procedure for coconut was standardized as it was difficult for isolating DNA from coconut due to the high polysaccharide and polyphenol content of its leaves. Several DNA isolation procedures were tried and among them, the protocol reported by Porebski *et al.* (1997) with suitable modifications yielded good quality DNA in sufficient quantity.

The ten selected random primers for RAPD (after screening 35) generated 65 markers with an average of 40 percent polymorphism. The polymorphism information content (PIC) of selected primers ranged from 0.67 (RN4) to 0.89 (RN5, RN8). For ISSR assay, eleven primers were selected (after screening 41) which generated ninety amplicons. The average polymorphism per cent was 44.44 and the PIC value of selected

primers ranged from 0.78 (UBC 855, UBCS2) to 0.90 (UBC 834). Ten sets of primers selected for the SSR analysis (after screening forty three) produced a total of 13 amplicons with 30.76 per cent polymorphism. Altogether, there were 168 markers produced by RAPD, ISSR and SSR assay of the 10 genotypes of which 70 bands (41.66%) were polymorphic with an average of 2.29 polymorphic markers per primer.

The presence or absence of data was entered into a binary data matrix and was used for calculating the similarity coefficient using Dice coefficient (Nei and Li, 1979) using software DARwin (Version5.0) and Jaccard's coefficient (Jaccard, 1908) using software NTSYS-PC (Rohlf, 1993). Cluster analysis was done using the UPGMA method and dendrograms (using DARwin and NTSYS) were constructed by neighbor joining. The genetic diversity values were calculated using the software, DARwin as the dendrogram based on DARwin showed more accuracy according to morphological characters.

The dendrogram generated (using DARwin) from sixty five RAPD markers, grouped the cultivars into three major clusters. Cluster I consisted of four tall cultivars namely Kuttiadi, Tiptur Tall, Malappuram and Kasaragode. Cluster II included three dwarf cultivars viz., Chowghat Green Dwarf, Chowghat Orange Dwarf, Malayan Yellow Dwarf and the intermediate type, Gangabondam. Cluster III included the two tall cultivars, Laccadive Ordinary and Komadan.

The dendrogram (using DARwin) based on ISSR markers grouped the cultivars into three major clusters which was slightly different from that of RAPD. Cluster I comprised of four tall cultivars viz., Kuttiadi, Tiptur Tall, Malappuram but instead of cv. Kasaragode in RAPD dendrogram, Laccadive Ordinary was included in first cluster of ISSR. The tall cv Kasragode was separated out as the third cluster. Cluster II is similar to that in RAPD except for the presence of one tall cultivar, Komadan.

Based on all the 168 markers, dendrograms were constructed using DARwin and NTSYS software which grouped the cultivars into three clusters (Cluster I and III for Talls and Cluster II for Dwarfs and intermediate) by the former and into two (ClusterI for talls, Cluster II- Dwarfs and intermediate) by the latter. In the present study it was found that combination of three markers produced more accurate classification of genotypes.

Based on 168 markers from three marker systems the overall genetic diversity observed among the three morphological groups viz.,Talls, Dwarfs and intermediate type palms in the selected population of coconut seed farm at Vellanikkara is comparatively less (GD=0.13). The highest genetic diversity was between Talls and Dwarfs (GD=0.14). The tall population consisting of six tall cultivars among which four are ecotypes of WCT showed the lowest diversity (GD-0.06). Within Talls, Kuttiadi is found to be similar to Tiptur Tall as well as Malappuram with least diversity (GD=0.04). The diversity observed within the Dwarf population of present study showed a higher diversity index (GD=0.09). This is mainly contributed by the dwarf cultivar Malayan Yellow Dwarf collected from RARS Pilicode which is found to be genetically most distant from the rest of the nine cultivars (average GD=0.16). The intermediate type Gangabondam is genetically diverse from Talls and is grouped under the cluster for dwarfs. Within this cluster it was found genetically closer to Malayan Yellow Dwarf. The two cultivars (Tiptur Tall and Malayan Yellow Dwarf) were identified for the production of a T x D hybrid based on the study as they were the most divergent among the cultivars selected.