DEVELOPING DWARF SPECIFIC SCAR MARKERS FROM WCT INBREDS OF COCONUT (*Cocos nucifera* L.)

By

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(2016-11-127)

THESIS

Submitted in partial fulfillment of the requirement for the degree of

Master of Science in Agriculture

Faculty of Agriculture

Kerala Agricultural University, Thrissur

DEPARTMENT OF PLANT BIOTECHNOLOGY

COLLEGE OF HORTICULTURE

VELLANIKKARA, THRISSUR – 680656

KERALA, INDIA

2018

DECLARATION

I hereby declare that the thesis entitled **"Developing dwarf specific scar markers from wct inbreds of coconut (*Cocos nucifera* L.)"** is a bonafide record of research work done by me during the course of research and the thesis has not been previously formed the basis for the award to me any degree, diploma, fellowship or other similar title, of any other University or Society.

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ABBREVIATIONS

bp	**Base pairs**
BLAST	**Basic Local Alignment Search Tool**
CGD	**Chowghat Green Dwarf**
COD	**Chowghat Orange Dwarf**
CTAB	**Cetyl Trimethyl Ammonium Bromide**
^{o}C	**Degree Celcius**
DNA	**Deoxynucleic acid**
DNTPs	**Deoxyribo Nucleoside Triphosphate**
Ds	**Double stranded**
EDTA	**Ethylene Diamine Tetra Acetic Acid**
GBGD	**Ganga Bondam Green Dwarf**
Ha	**Hectare**
KAU	**Kerala Agricultural university**
Kb	**Kilo base**
LB	**Luria Bertani**
L	**Litre**
LCT	**Laccadive Ordinary Tall**
µg	**Microgram**
µl	**Microlitre**
ml	**Millilitre**
Mm	**Milli Molar**
MYD	**Malayan Yellow Dwarf**
NCBI	**National Centre for Biotechnology information**
ng	**Nanogram**
nm	**Nanometre**

OD	**Optical Density**
%	**percentage**
PCR	**Polymerase Chain Reaction**
PVP	**Poly vinyl pyrolidine**
PVPP	**Polyvinylpolypyrrolidone**
RAPD	**Random Amplified Polymorphic DNA**
RPM	**Revolutions Per Minute**
RNA	**Ribonucleic acid**
RNase	**Ribonuclease**
SCAR	**Sequence Characterized Amplified Region**
SDS	**Sodium Dodecyl Sulfate**
TAE	**Tris Acetate EDTA**
UV	**Ultra violet**
WCT	**West Coast Tall**
X-gal	**5-bromo-4-chloro-3-indoyl-β-D-galactopyranosidase**

ACKNOWLEDGEMENT

First of all, I thank God for giving me confidence, courage, strength and lots of blessings to complete my work successfully.

It is my pleasure and privilege to express my heartfelt gratitude and indebtedness to ***Dr. Sujatha R. Professor****, Dept. of Plant Biotechnology, College of Agriculture, Padannakkad and chairperson of my Advisory committee for her unforgettable guidance, constant encouragement, timely advice and care rendered during the research period, which gave me a sense of affection and care to complete thesis successfully.*

I am obliged to ***Dr Suresh P. R*** *Associate dean COA, Padanakkad whose moral support has helped to complete this venture successfully.*

My sincere thanks to Dr. ***M. R Shylaja****, Head of the Department, CPBMB, member of Advisory committee for her critical suggestions and interest during the course of study.*

I sincerely acknowledge ***Dr. Namboodiri Raji Vasudevan****, Associate Professor, Department of Plant Breeding and Genetics, for her critical suggestions during the course of this research.*

I wish to express my sincere thanks to all the non-teaching staff members and labourers of RARS, Pilicode and CPBMB, COH Thrissur for their whole hearted cooperation and timely assistance.

I express my deep sense of gratitude to Sherin chechi, Giridhari chechi, Gibbence sir, Hassain, Ajeesh, Mubarak, Shibin, Sreerag and Rajil of COA, Padanakkad who helped me in several ways for the completion of this venture.

I wish to express my thanks to my loving friends Rakesh, Arjun. P, Shibin, Niranjana, Faiza, Midhuna, Athulya, Pooja, Anushree, Abidka, Murthala, Adersh, Amal, Bennet, Fazeed, chakru, Amjad for their prompt help and cooperation who stood by me during all the hard times.

I sincerely acknowledge KAU, Thrissur for awarding me this Masters Degree.

Finally I Express my gratitude to my parents (Sri. K. p Varghese, Smt. Lissy Varghese), my sister Annmary Varghese and Greeshma for their constant encouragement, moral support and for their valuable love.

A word of apology to those I have not mentioned in person and a note of thanks to one and all who worked for the successful completion of this endeavour.

Anto Varghese

Introduction

1. INTRODUCTION

Coconut tree (*Cocos nucifera* L.) is a member of palm family *Arecaceae* and the only species coming under the genus *Cocos*. Coconut is one of the most useful palm in the world grown in about 93 countries. Coconut production plays an important role in the national economy of India. India is the third largest producer of coconut in the world, behind Philippines and Indonesia with a production of 23905 million nuts/year and an area of 20.82 lakh ha under cultivation. India leads in productivity with 11481 nuts/ha/year. Among the states, Kerala is having the top position in coconut production with 7.70 lakh ha of cultivated area and has touched 7464.25 million nuts production in 2017. However, Kerala has only fourth position in productivity with 11200 nuts/ ha/year, (Kumar, 2017).

Coconut palms are considered as an important subsistence and cash crop in the humid tropical regions of the world. Main products from coconut are copra and coconut oil which are the main source of foreign income for coconut producing -countries. The coconut palm is known as "the tree of life" because of its abundant uses as food, drink, fuel energy, building materials and so on. Coconut is an important part of human diet because it contains valuable vitamins and minerals. An average-sized nut weighing 400 g edible meat and water provide almost all daily requirements of a normal person. However, need for the crop has increased in current period due to competition from synthetic fibres and other oilseed crops. The coconut industry now lacks inputs, mostly for research and development. This condition is due to problems like absence of quality planting material, pest and disease infestation, old age of the coconut plantations etc..

Crop improvement in coconut palm by hybridization and selection is one of the most important objective in coconut breeding programmes. There are mainly two types of coconut palms based on its height- tall and dwarf. Dwarf coconut types are having smaller life span and they are short in stature when compared to the tall types. Dwarf coconut palms flower in third year after planting. It comes to regular bearing in the ninth year with an average lifespan of

lives longer when compared to dwarf palms generally to an age of about 80 to 90 years and are fairly resistant to diseases and pests.

The tall variety, West Coast Tall (WCT) is a superior coconut cultivar which is widely seen in the west coast regions of India, with productivity ranging from 40 to 100 nuts per tree and nuts of good quality (copra 176 g/nut and oil 68 %). The wide range in productivity is due to the high heterozygosity existing in WCT resulting from cross pollination. Breeding programmes were begun as early as 1924 at Coconut Research Station, Pilicode (presently Regional Agricultural Research Station) by crossing Tall x Dwarf which resulted in hybrids with increased vigour and productivity (Nair and Balakrishnan, 1991).WCT was identified as potential mother palm in many of the hybridization programmes. Simultaneously selfing programmes were also initiated to develop inbred lines in tall palms as they are highly heterozygous. From 18 West Coast Tall palms of the Coconut Research Station at Kasaragod that were selfed during 1924-26, the first and second generation selfed palms (S1 & S2) were planted at Pilicode during 1926 and 1961, respectively. The third generation selfed seedlings (S3) were planted in the field in replicated plots during 2015. Chethana(2016) characterized second generation inbred palms using vegetative, reproductive and nut characters for studying inbreeding depression and for identifying promising palms suitable for further breeding programmes. The result showed significant differences among the twelve families of second generation. The wide range of variability noticed in most of the characters confirm the heterozygosity of different groups of open pollinated WCT palms selected for the study. Among the six families in S2 generation, IIIS2-1 and VS2-1 showed lower inbreeding depression for important characters like stouter stem, average number of functional leaves, inflorescence and female flowers, medium setting percentage, high copra and oil content. However in S3 generation the inbreeding depression was lowest for seed germination in 1AS3-1 and 1BS3-1 families. These S3 sedlings were characterized based on germination, height, leaf number, girth etc. and the selected S3 seedlings from promising S2 palms were subjected to molecular

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characterization using RAPD (Random Amplified Polymorphic DNA) marker system along with tall (WCT) and dwarf palms (COD, CGD). The results revealed two dwarf specific RAPD bands in two of the S3 seedlings belonging to IAS_3-1 and IBS_3-1 family, when amplified with primers OPAU03 and OPAW15.

Molecular markers can play an important role in characterizing and distinguishing germplasm at an earlier stage and also a powerful tool for breeders to search for new sources of variation. The RAPD markers are DNA fragments amplified by PCR (Polymerase Chain Reaction) using short synthetic primers (generally 10bp) of random sequence. The main advantage of RAPDs is that they are fast and easy to assay. The major disadvantage of RAPDs is their low reproducibility. The usefulness of the amplicons will be better if they are converted to SCAR (Sequence Characterized Amplified Regions) markers. The reproducibility and reliability can be significantly improved by the conversion of RAPD to SCAR markers, by developing longer, and hence, more specific primers. Rajesh *et al.* (2013) had identified a tall specific RAPD marker in coconut and successfully converted it to SCAR marker.

Hence, the study entitled "Developing dwarf specific SCAR markers from WCT inbreds of coconut (*Cocos nucifera* L.) was undertaken during the period 2016-2018 with the objective of developing and validating dwarf specific SCAR markers for screening superior inbreds with dwarfing trait in WCT inbreds of coconut.

The investigation mainly focused on the following

1. To screen the third generation selfed seedlings (S3) of coconut obtained from six different families of S2 generation WCT palms planted in field at RARS, Pilicode along with dwarf and WCT seedlings with earlier reported RAPD primers.

2. To develop SCAR markers from dwarf specific RAPD marker for screening superior inbreds with dwarfing trait in WCT inbreds of coconut.

3. To validate SCAR marker using DNA from tall palms, dwarf palms and seedlings from different families of S3 inbreds.

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Review of literature

2. REVIEW OF LITERATURE

2.1 COCONUT

The coconut (*Cocos nucifera* L.) is widely cultivated in tropical areas and have wide applications in the field of agriculture and industry. Coconut is a small holders crop with 96 percent holdings of less than 4 ha (Batugal and Oliver, 2003). Tall and dwarf are the two types of coconut. The hybrid between these two shows promising hybrid vigour (Patel, 1938)

2.1.1 Extent of variability

The major division of coconut depends on stature and breeding habit of the two main types: tall and dwarf (Menon and Pandalai, 1958). Other than these types, some other intermediate kind of palms have been noticed in some countries. Wide range of variation occurs 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 Indian scenario, coconut is mostly cultivated in Kerala. West coast tall (WCT) is the major coconut cultivar grown in Kerala and it occupies about 95 percent area under coconut cultivation. WCT is a tall palm which gives good yield up to an age of 70 years. It has been mostly accepted that farmers' perception of coconut varieties is the ultimate resource for conserving its biodiversity. Over the years, cultivation of coconut palms in Kerala has made possible the development of many ecotypes in WCT. These ecotypes are known by the places where they are cultivated. These ecotypes which are adaptive to the local environmental conditions are good basis of precious genes in coconut breeding programmes (Remany, 2003).

Dwarf varieties of coconut are grown in many parts of the world. They might have evolved due to mutation in tall varieties or due to several generations of inbreeding occurred in tall varieties (Nair and Balakrishnan, 1991). Generally, dwarf varieties are self-pollinating, but cross pollination can also occur to some

of inbreeding occurred in tall varieties (Nair and Balakrishnan, 1991). Generally, dwarf varieties are self-pollinating, but cross pollination can also occur to some extent. They are regular or irregular bearer and are usually planted for ornamental purpose (Joseph, 2007).

The RARS, Pilicode conserves an exclusive collection of coconut germplasm consisting of 40 indigenous and 35 exotic varieties. The first ever hybrid viz. WCT x CGD, popularly known as T x D was evolved and planted at Nileshwar in 1936. Later five more hybrids viz. Lakshaganga (LCT x GBGD), Keraganga (WCT x GBGD), Anandaganga (ADT x GBGD), Kerasree (WCT x MYD), Kerasoubhagya (WCT x SSAT) were released from this station (KAU, 2016).

Balakrishnan and Namboothiri (1987) studied the genetic divergence with 24 cultivars of the tall variety (7 exotic and 17 indigenous) at Pilicode to estimate the genetic distance among the cultivars and revealed that there were wide genetic variations among them.

Chowghat Orange Dwarf (COD) is a dwarf coconut variety seen in western coastal regions of India and most commonly in Kerala. A selection from this was released by ICAR- Central Plantation Crops Research Institute (CPCRI), Kasaragod for tender coconut production. Self-pollination and cross pollination occurs in this variety and is more robust than Chowghat Green Dwarf. This cultivar is extensively used for the production of T x D and D x T hybrids in Kerala such as Chandra sankara, Kerasankara (Tall hybrids) sensitive to drought and water logging (Joseph, 2007).

Chowghat Green Dwarf (CGD) is another dwarf cultivar of coconut seen extensively in west coast region of the country and particularly in Kerala. This variety maintains purity when compared to other dwarf varieties due to the self-pollination. This is made possible due to the complete overlapping of male and female reproductive phases. However, WCT x CGD hybrids had a problem of

Malayan Yellow Dwarf (MYD) is a dwarf coconut variety which is believed to be originated in Java and later introduced to Malaysia. Yellow dwarfs are extensively used in Kerala for hybrid seed nut production.*Kerasree* is such a tall hybrid variety of coconut produced by crossing West Coast Tall with Malayan Yellow Dwarf (Joseph, 2007).

Assessment of the nature and extent of variability will be of immense value in identifying superior genotypes and in formulating breeding programmes. A reliable assessment of genetic relationships between coconut varieties and determination of genetic diversity present in coconut palms are pre-requisites for sustainable coconut breeding programmes (Pereraet *al.*, 2003).

2.1.2 Inbreeding in coconut

Dwarf coconut occurring in different countries may be the product of inbreeding in different tall varieties. In addition to the typical tall and dwarf palms, various intermediate categories occur in nature and have also been obtained by crossing the tall and dwarf strains (Swaminathan and Nambiar, 1961).

Effect of inbreeding depression on cytology of the palm was studied by Nambiar *et al,* (1970) in some distinct exotic varieties of coconut. Inbred and open pollinated progenies of Laccadives, Andaman, Philippines, New Guinea and Cochin China accessions of indigenous and Malayan origin were subjected to screening for observing meiotic behavior. The open pollinated varieties showed more chromosomal aberrations and higher percentage of pollen sterility than the self-pollinated dwarf varieties.

Pandin (2009) determined the inbreeding depression of selfed Mapanget tall coconut No. 32 (DMT-32) based on the morphological characters of second, third and fourth generations. The results showed inbreeding depression in both vegetative and fruit component characters in DMT-32 S4.

2.2 DNA EXTRACTION FROM COCONUT

The isolation of high-quality DNA is important in any molecular biology work because contaminants such as proteins, polyphenols, and polysaccharides may interfere with enzymes such as restriction enzymes (in blotting techniques) and Taq polymerase (in PCR) (Ausubel*et al.*, 1994). When the tissues taken for isolation get injured, polyphenols will be released, which will lead to the browning of leaves (Joslyn and Ponting, 1951).

The CetylTrimethyl Ammonium Bromide (CTAB) method of Doyle and Doyle (1990) modified by Kirkpatrick *et al.* (1987) has been used to extract total nucleic acids from infected coconut tissue by phytoplasma enrichment method. This method provided a good DNA template for amplification of phytoplasma.

Dellaporta (1983) developed a DNA isolation method which was adopted from a commonly used procedure for yeast DNA preparation (Davis *et al.*, 1980) and known as plant DNA mini preparation. In this procedure, leaf tissue is ground to fine powder in mortar and pestle by adding liquid nitrogen followed by adding extraction buffer and β-mercaptoethanol. SDS was added and mixed with vigorous shaking followed by addition of potassium acetate. The pellet obtained was redissolved with 50 mM TRIS and 10 mM EDTA. Supernatant obtained is transferred to centrifuge tube and, sodium acetate and isopropanol were added. Precipitation from 0.3M sodium acetate treated samples using negligible amount of isopropanol has been reported to separate high molecular DNA from polysaccharides.

Upadhyay *et al.,* (1991) found a method for plant DNA isolation which was then used by Devakumar*et al.* (2010) for isolating DNA from coconut plant. In this method, 5g of spear leaf tissue was ground in liquid nitrogen. The contents were transferred to extraction buffer containing 10% SDS. It was then incubated at 65 °C, cooled and extracted with an equal volume of 24:1 chloroform:isoamyl alcohol mixture. The supernatant was transferred to a new tube and DNA was precipitated with 70% ethanol.

Angels *et al.* (2005) used the method of Dellaporta*et al.* (1983) as per the modification by Datta*et al.* (1997) on 8-months old coconut endosperm samples. However, the report shows that the genomic DNA isolated was of poor quality after digestion with EcoRI, which can be the result of the high lipid and galactomannan content in the extract. Whitish contaminants that co-precipitated with the DNA were also reported. Inorder to minimize the presence of high level of contaminants (lipids and polysaccharides) in the DNA, tender coconut leaves from the first emergent frond of the palm were used. The modification involved the usage of a higher salt concentration (2 M instead of 0.5 M) in the extraction buffer and the use of polyvinylpolypyrrolidone (PVPP). Moreover, this modified protocol did not include the usage of organic solvents.

Manimekalai*et al.* (2015) amended phytoplasma enrichment protocol of Ahrens and Seemuller (1992) by including 2 per cent PVPP in the extraction buffer. The uniform samples were incubated at 4°C for 5min before extraction. The DNA was dissolved in TE buffer (pH8) and verified on 0.8% agarose gel. Maximum number of positive results were obtained for midrib tissue samples.

Porebski*et al.* (1997) standardized DNA isolation protocol for plant material containing large amount of polyphenols, tannins and polysaccharides. The method involved a modified CTAB extraction protocol, employing high salt concentrations to discard polysaccharides and use of polyvinyl pyrrolidone (PVP) to discard 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 was consistently amplifiable in the PCR reaction with as little as 0.5 ng DNA per 25 µl reaction.

Sharma *et al.* (2008) modified DNA extraction method in tuber crops. Genomic DNA was extracted using CTAB extraction method with some alterations. Leaves were fixed in different fixing solutions without liquid nitrogen. DNA quality and quantity obtained were comparable to those isolated with liquid nitrogen, as the λ260/λ280 ratio with liquid nitrogen was in range 1.3-1.7 and with other fixing solutions it was 1.1-1.5. Absolute alcohol showed best results as fixing solution. Good quality DNA was isolated without using liquid nitrogen from different medicinal plant species. DNA isolated by this method was suitable for various molecular biology applications.

2.3 MOLECULAR CHARACTERIZATION

Molecular marker is a DNA sequence which can be easily detected, and its inheritance can be readily monitored. DNA markers are greatly used for effective evaluation and selection of plant material for breeding programmes. DNA markers segregate as single genes and are not affected by environmental factors and thus they are different from protein markers (Kumar *et al.*, 2009)

A molecular marker should have the following qualities -

1. It should be polymorphic as it 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 find applications in germplasm characterization, genetic diagnostics, characterization of transformants, genome organization and phylogenetic analysis other than diversity and population structure analysis (Sing, 2008).

2.3.1 RAPD

Random amplified polymorphic DNA (RAPD) marker is extensively used in molecular characterization studies ranging from individual level genetic identity to studies involving closely related species. RAPDs are used in gene mapping studies inorde to fill the gaps which are not covered by other markers (Williams *et al.*, 1990; Hadryse*t al.*, 1992)

RAPD markers have many advantages over other markers such as rapidity, technical easiness, higher frequency of polymorphism, requirement of only a few nanogram of DNA, feasibility of automation and no prior information of DNA sequence is required. Molecular characterization using RAPD marker is widely used to analyze genetic relationships in several plant species (Kollere*t al.*, 1993; Shah *et al.*, 1994)

However many factors can influence the reproducibility of RAPD reactions like quantity of template DNA, PCR buffer, magnesium chloride concentration, annealing temperature, Taq DNA polymerase as well as thermal cycler (Kundane*t al.*, 2014).

2.3.1.1 RAPD assay in coconut

Upadhyaye*t al.* (2004) carried out RAPD analysis to study genetic relationship and diversity in fifteen Indian and five exotic coconut accessions using eight primers which 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 as well as genetic diversity. Exotic accessions exhibited more variation than indigenous accessions. Dwarfs from geographically distinct regions did not exhibit much variation.

Dasanayaka*et al.* (2005) carried out RAPD analysis of 17 coconut germplasm accessions and 3 coconut cultivars for determining the genetic diversity existing between them. Twenty RAPD primers were used for this study. The RAPD primers produced 129 amplicons with 6.5 bands per primer as an average.

Manimekalai*et al.* (2006) determined the effectiveness of RAPD markers to identify polymorphism among 33 coconutgermplasm accessions. Ten RAPD primers produced 86 polymorphic amplicons of which 76.7% exhibited polymorphism. The mean polymorphism information content (PIC) value for the ten RAPD primers was 0.23.

Sankaran*et al.* (2012) carried out RAPD analysis of 30 coconut accessions from Pacific Ocean Islands and, Andaman and Nicobar Islands for molecular characterization. Fourty-five primers were used for screening and only 13 primers among them showed expression and those were used for polymorphism survey. The generated amplicon size of all the 13 primers studied, ranged from 170 to 1395 bp. The polymorphism obtained in the 30 genotypes with selected RAPD markers showed distinct variation with 67.33% in all accessions. The highest number of bands was obtained in 28 accessions.

Six popular coconut cultivars were analysed by 14 RAPD primers and produced 107 amplicons within molecular range of 2 kb to less than 0.5 kb. Komadan cultivar showed 87 per cent similarity with NCD (Natural Cross Dwarfs). It also showed 77 per cent similarity with WCT cultivar. Hybridization between Komadan and WCT and between Komadan and Dwarf palms is suggested for yielding superior hybrids (Selvaraju and Jayalekshmy, 2011).

Rajesh *et al.* (2013) carried out RAPD analysis for finding tall/dwarf trait in coconut by using bulked DNA approach. Two-hundred primers were used for analysis and a RAPD primer OPBA3 was able to differentiate between tall and dwarf bulks. The polymorphisms observed between the tall and dwarf coconut cultivars were used as markers for hybrid identification in three crosses, viz., CGD x WCT, COD x KWGT (Klapawangi tall) and MYD x SNRT (San ramon tall).

2.3.1.2 RAPD in other palms

Rani *et al.*(2007) assessed genetic diversity among 40 date palm genotypes using twenty-nine RAPD primers. Average polymorphism across all the forty date palm genotypes was found to be 99.12±0.62. Three RAPD Primers OPA-09, OPB-18 and OPO-06 produced 1 unique allele (900bp, 350bp, 250bp respectively) each capable of differentiating Medjool variety female plants from male and female plants of other varieties used.

Toor*et al.*(2005) studied genetic variation in twenty cultivars of date palm using RAPD markers. Thirty-four decamer primers selected for RAPD analysis generated 191 amplicons, out of which, 159 (83.24%) were polymorphic. RAPD banding pattern was used to construct genetic similarity which ranged from 0.433 to 0.760, while the average genetic similarity was 61.8 per cent.

2.3.2 SCAR MARKER

SCAR markers are accurate, cost efficient and most practical method for screening numerous samples. They are time and labor-efficient. Hence, SCAR markers are considered as powerful tools in marker-assisted selection (Kasai *et al.*, 2000).

2.3.2.1 SCAR markers in palms

Dhavan, *et al.* (2013) worked on date palm to find a molecular diagnostic method which assures quick and reliable identification of sex types in the early stages. Genomic DNA from 45 individual plants (25 females and 20 males) belonging to different varieties of date palm was subjected to PCR amplification using 100 RAPD primers and 104 Inter Simple Sequence Repeat (ISSR) primers. Only one RAPD primer, OPA-02, amplified a fragment of ~1.0 kb in all the individual samples of male genotypes, whereas this fragment lacked in all the female genotypes. This male-specific fragment was cloned and sequenced and a

Sequence-Characterized Amplified Region (SCAR) primer pair was designed. The SCAR marker was further validated using 25 female and ten male date palm plants belonging to different varieties collected from different locations.

Premakrishnan*et al.* (2011) had done a research on oil palm to convert RAPD primers to iSCAR (*in silico* Sequence characterized amplified regions) markers. An input of 387 RAPD primers and 42,432 Expressed Sequence Tags (EST) of oil palm were used as test. In the test sequences of oil palm, covering 1.4 per cent of genome, they found that at least 60 per cent of the primers chosen using the software were sure of giving PCR amplification. They designed 641 iSCAR primers suitable for amplification of oil palm DNA.

Rajesh *et al.* (2013) had identified a tall specific RAPD marker in coconut using two samples of bulked DNA, first from five tall palms and second from five dwarf palms. After screening with 200 decamer primers, primer OPA09 revealed a unique band of around 260 bp exclusively in tall accessions. The RAPD marker OPA09 was successfully converted into a SCAR marker, which was further validated using individual samples of tall and dwarf accessions of different geographical origins

Mitra (2010) designed SCAR dpF (date-palm forward) and SCAR dpR (date-palm reverse) primers based on the sequence of male specific fragment generated after amplification by RAPD primer P50. This primer pair amplified a common 406 bp fragment in both female and male genotypes and a unique allele of 354 bp in male genotypes. This SCAR marker proved to be highly effective for the differentiation of male plants from female date-palm plants.

2.3.2.2 SCAR markers in other crops

Urasaki*et al.* (2002) used RAPD technique to determine sex of *Carica papaya L.*, with the three sex types, male, female and hermaphrodite. A 450 bp marker fragment named PSDM (Papaya Sex Determination Marker), amplified by IBRC-RP07 primer was present in all male and hermaphrodite plants but not in

female individuals. A SCAR marker was developed from PSDM to determine sex of papaya. The designed SCAR primers SDP-1 and SDP-2 amplified a fragment of 225 bp in all male and hermaphrodite individuals, but not in female individuals.

Bedoya and Nunez, (2007) screened 32 arbitrary RAPD primers and identified primer OP –Y7, which generated a 900 bp band in all the male samples analysed and absent in female plants and hermaphrodite plants of papaya. From this RAPD marker sequence, a SCAR primer was designed which amplified a fragment of size 369 bp from the genomes of male and hermaphrodite plants, but not the female ones.

Yakubov*et al.* (2005) used a combined method of SCAR primers with Touchdown-PCR for the development of female DNA marker in *Pistaciavera*L. The random decamer primer OPO-08 amplified a 905bp fragment in all female trees, also in several males. SCAR primers designed on the basis of the RAPD female molecular marker amplified a 905 bp female and a 909 bp male fragment. The diagnostic reliability of the new female specific marker was verified on 54 different genotypes.

Materials and Methods

3. MATERIALS AND METHODS

3.1 MATERIALS USED

3.1.1 Plant Materials

The plant materials for DNA isolation were collected from RARS, Pilicode and Instructional farm, College of Agriculture, Padannakkad. During 1924-26, 18 WCT palms belonging to six groups were selfed and sibmated for obtaining first generation inbred palms. These palms were classified based on coloured types and yield groups into six families. These were further selfed to produce second and third generation seedlings. Chethana (2016) studied the extend of inbreeding depression in these S2 palms and S3 seedlings and also molecular characterization of selected seedlings of S3 generation was carried out using RAPD. She reported that for most of the vegetative and reproductive characters, The S2 palms exhibited inbreeding depression which was more in selfed progenies than the sibmated progenies. In S3, the germination was low in all families (< 50%) and comparatively the seedlings from 1AS3-1 and 1BS3-1 exhibited better rate of germination. Based on the seedling characters the promising seedlings of S3 generation were subjected to molecular characterization using RAPD. The development of inbred lines at RARS, Pilicode is detailed in Fig1. Third generation seedlings belonging to the families 1AS3-1and IBS3-1, and selected plants of other families IIS3, IIIS3, VS3, along with WCT , CGD, and COD, and MYD were used for the present study.. Only the low and medium category seedlings were selected from IIS3, IIIS3 and VS3 families. The spindle leaves were taken from these coconut seedlings for the DNA extraction process. Both families1AS3-1and IBS3-1 have three replications each and each replication has six seedlings which are classified into low (L), medium (M) and high (H) based on seedling height at the time of planting during 2015. In each plot, planting was done in such a way that there were two low, two medium and two high seedlings. However, when the experiment was undertaken, several seedlings were lost due to

severe drought / pest and disease attack. The details of the plot and number of seedlings available in each are given in Table 1.

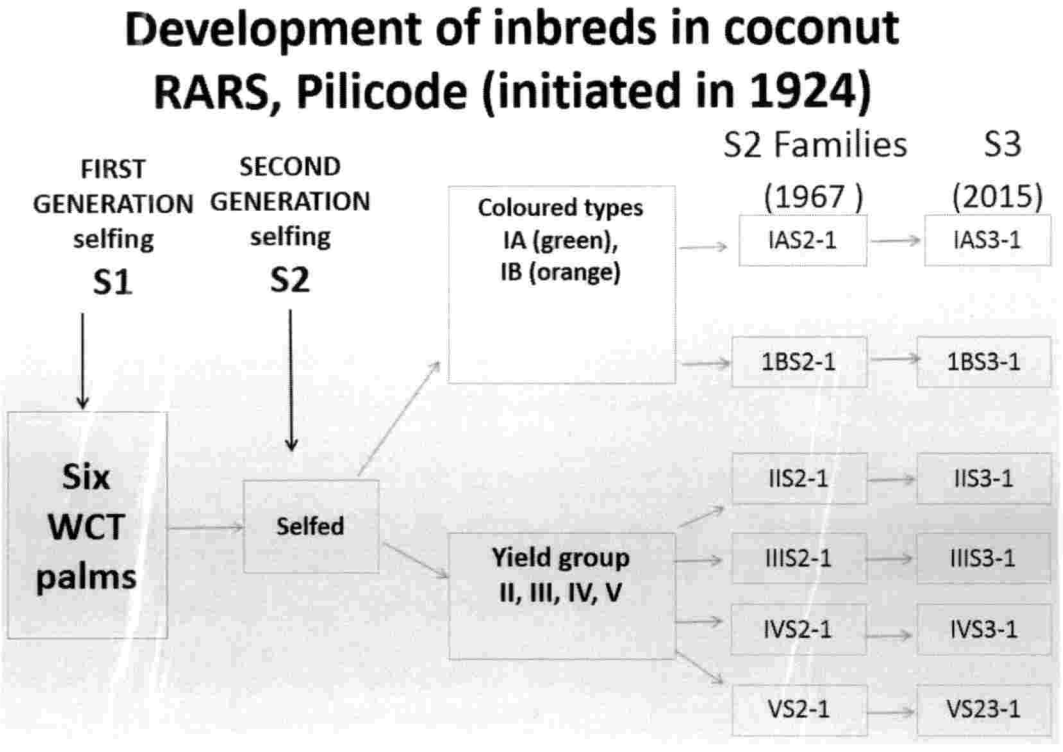

Figure 1. The development of inbred lines at RARS, pilicode

Table 1. Plot-wise details of 3rd generation coconut inbred families from WCT

Sl. No.	Name of family	Replication	Name of plot	Seedlings present during 2016-2018
1.	IAS3-1	R1	K	M1, H1, H2
		R2	V	L1, L2, M1, H2
		R3	J1	M1, M2, H1, H2
2.	IBS3-1	R1	E	H1
		R2	M	L1, L2, M1, M2, H1
		R3	C1	L2, M1, M2, H1, H2
3	IIS3-1	R1	D	M1
		R2	S	L2
4	IIIS3-1	R1	F	M1
		R2	T	L2, M1, M2
		R3	I1	L2, M1
5	VS3-1	R1	P	M1
		R2	F1	L1

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3.1.2 Laboratory Chemicals and Glasswares

The chemicals used in the study were from Merck India Ltd. and SRL laboratories. The *Taq* DNA polymerase along with buffer, dNTPs, and molecular weight markers (lambda DNA *Eco RI/ Hind III* double digest, 100 bp ladder, 1kb ladder) were from Merck-Genei, Bangalore. The random decamer primers were selected based on earlier reports and the oligos were synthesized by Integrated DNA Technolgies, New Delhi.

3.1.3 Equipment and Machinery

The equipment available in the Department of Plant Biotechnology, College of Agriculture, Padannakkad were used for the study. DNA quantification was done using biophotometer (Eppendorf, Germany) and Polymerase Chain Reaction (PCR) was done in Mastercycler (Eppendorf, Germany). Bio-Rad imaging system (Bio-Rad Laboratories, Inc., U.S.A.) was used for imaging and documenting the agarose gel.

3.2 METHODS

3.2.2 Isolation of total genomic DNA

Standardization of genomic DNA extraction

Genomic DNA was extracted from tip and middle portion of second leaf before spindle leaf from the seedlings. The CTAB method (Roger and Bendich, 1985) modified by Chethana (2016), was used for isolating DNA. Good quality DNA was obtained but had RNA contamination and hence the method was slightly modified. The quality of DNA was observed by gel electrophoresis and quantity of DNA was measured by Biophotometer.

Modified procedure:

Reagents:

1. Extraction buffer: (pH 8)
   2% CTAB
   100 Mm Tris
   200 mM
   EDTA
   1.5 M NaCl
2. β-mercaptoethanol
3. PVP
4. Chloroform: Isoamyl alcohol (24:1)
5. Isopropanol
6. Ethanol (70%)

Steps involved in DNA extraction:

1. Removed the midrib and cut the young leaves into small pieces. Pre-warmed CTAB buffer to 60-65^{0}C.
2. 1 g of leaf tissue taken and ground well in a sterilized mortar and pestle by adding a pinch of PVP in the presence of liquid nitrogen.
3. 10 ml of pre-warmed CTAB extraction buffer and 40 µl β-mercapto-ethanol added to the powdered tissue and mixed by inverting several times.
4. Incubated in water bath at 65°C for 30 minutes and the tube wasinverted occasionally.
5. 4ml of chloroform: isoamyl alcohol (24:1) added to the tube and the tube inverted for several times and later centrifuged at 10,000 rpm for 15 min.
6. Upper aqueous phase collected into the centrifuge tube and 2 µl RNase added and incubated at 37 °C for 30 minutes.

7. 4ml of chloroform: isoamyl alcohol (24:1) added to the tube and centrifuged at 10,000 rpm for 15 min.
8. Upper aqueous phase collected into the eppendorftube, half volume of 5M NaCl and 1.5 times the volume isopropanol added to it and the tube inverted slowly for several times. Then placed in -20°C for 30 mins.
9. Centrifuged at 10,000 rpm at 4°C for 10 mins.
10. Decanted the solution leaving the pellet and washed the pellet using 70% alcohol for 2 to 3 times
11. Air dried the DNA pellet at room temperature, then dissolved in sterile water.

3.2.3 Electrophoresis of DNA

The quality of DNA was examined by agarose gel electrophoresis

Reagents and equipments

1. Agarose - 0.8 per cent (for genomic DNA)
   -1.5 per cent (for RAPD analysis)
2. 50X TAE buffer (pH 8.0)
   (1X solution contains 40mM Tris, 20mM acetic acid, and 1mM EDTA)
   - 2 M Tris buffer
   - Glacial acetic acid
   - - 0.5 mM EDTA
3. Tracking/ loading dye (6X) (Himedia)
   - Bromophenol blue (Methylene blue)
4. Ethidium bromide
5. Electrophoresis unit, power pack, gel casting tray, comb
6. BIO-RAD Gel documentation and analysis system

Procedure

1. Swabbed the work area, gel tray and comb with 100% alcohol, Gel tray was taken and sealed the ends with tape. Comb should be placed in the gel tray about 1 inch from one end of the tray and comb should be positioned vertically such that the teeth are about 1mm above the surface of the tray.
2. 0.8% agarose gel (1.5% gel for RAPD analysis) prepared by dissolving 0.45 g agarose in 40 ml 1X TAE buffer and kept in microwave until the agarose dissolved fully and the solution became clear.
3. Agarose solution allowed to cool to 42 to 45°C and ethidium bromide was added at a concentration of 0.5µg/ml and mixed well taking care to avoid bubbles.
4. Poured the warm gel solution into the tray. Allowed it to solidify for about 30 – 45 minutes at room temperature.
5. Comb and tape were removed without disturbing the gel and the gel was placed in the electrophoresis tank containing 1X TAE buffer. The tray was kept in a manner that the well side was positioned towards cathode.
6. The samples were prepared by adding 5 µl of DNA sample with 2 µl of tracking dye, (20µl of PCR product with 4µl of tracking dye for RAPD samples) mixed well and was loaded into wells using micro pipette.
7. Loaded suitable molecular weight marker (λDNA Eco RI/ Hind III or DNA ladders) in one well.
8. Electrophoresis was done at 100 volts until dye has migrated two-third the length of the gel and intact DNA was observed as orange fluorescent bands under UV illumination.

3.2.4 Gel documentation

The gel documentation was carried out using BIO-RAD imagingsystem, a software package used for imaging, analysing and databasing electrophoresed DNA gels. 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 spectrophotometric method

Spectrophotometer measures the absorbance of DNA, which is used for detecting its quantity and quality. It measures the concentration of nucleic acids in the samples based on Beer-Lambert's law. Nucleic acid has absorption maxima at 260 nm whereas proteins have peak absorbance at 280 nm. Absorbance recorded at both wavelengths is indicated by the ratio OD_{260}/OD_{280}.A pure solution of double stranded DNA has an optical density of 1.0 at 260 nm and OD_{260}/OD_{280} ratio of 1.8. Contamination with protein or polyphenol will show OD_{260}/OD_{280} values significantly less than 1.8 and contamination with RNA shows a ratio greater than 1.8 (for pure RNA, OD_{260}/OD_{280} is 2.0). The value from 1.8 to 2.0 indicates that the DNA is pure which is free from proteins and other contaminants. The quantity of DNA in the pure sample was calculated using OD_{260} equivalent to 50 µg double stranded DNA/ml sample.

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

Therefore, OD_{260} x 50 gives the quantity of DNA in µg/ml.

3.2.6 RAPD assay

PCR reaction mixture and thermal profile tried

Two PCR reaction mixture were tried by making change in the quantity of DNA taken. Also two different thermal profiles were tested by making changes in annealing temperature and number of cycles as detailed in Table 2.

Table 2. PCR conditions for RAPD analysis using selected primers in coconut

PCR reaction mixture (RM) tried	Thermal profile (TP) tried
RM-1 1X buffer 200 µM dNTPs 0.75 µM primer 0.9 U Taq DNA polymerase 40 ng / µl DNA	TP-1 Initial denaturation: 94°C – 5 min Denaturation: 94°C – 1min Annealing: 37°C – 1.2min Extension: 72°C -2 min Final extension: 72°C – 10 min Hold: 4°C 40 CYCLES
RM-2 1X buffer 200 µM dNTPs 0.5 µM primer 0.9 U Taq DNA polymerase 100 ng / µl DNA	TP-2 Initial denaturation: 94°C – 5min Denaturation: 92°C – 1min Annealing: 34°C – 1min Extension: 72°C -2 min Final extension: 72°C – 10 min Hold: 4°C 35 CYCLES

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Table 3. Composition of PCR reaction mixture for DNA amplification in coconut

Component	Quantity
Autoclaved distilled water	14.2µl
*Taq*buffer with $MgCl_2$(10X)	2 µl
dNTPs (10mM)	1.6 µl
Primer (10 µM)	1.5 µl
*Taq*DNA polymerase	0.3 µl
DNA (40 ng)	0.4 µl
Total volume	20µl

RAPD primers selected

Primers were selected based on earlier reports wherein at least one or two fragments amplified were specific either to the dwarf palms (Chethana, 2016) or to tall palms (Rajesh *et al.*, 2013) of coconut. Chethana has done molecular analysis in promising third generation inbreds with 10 RAPD markers and dwarf specific bands were seen when amplified with markers OPAU03 and OPAW15. These two primers were taken for initial screening along with the early reported tall specific RAPD primer OPA09.

Table 4. Primers used for amplification of coconut DNA

Primer	Sequence (5'-3')	Size of the specific amplicon
OPAU03 (Chethana, 2016)	ACGAAACGGG	Dwarf - 1300 bp 550 bp
OPAW15 (Chethana, 2016)	CCAGTCCCAA	Dwarf - 1200 bp
OPA09 (Rajesh *et al.*, 2013)	GGGTAACGCC	Tall – 260 bp

3.2.6 DNA elution for dwarf specific RAPD band from agarose gels

The bands specific character was identified by analysis of agarose gels and the desired bands were eluted using PCR clean up gel extraction kit (Macherey-Nagel) according to the manufacturer's protocol.

Procedure

1. DNA fragment was excised from agarose gel by using a clean scalpel. Removed all excess agarose. Measured the weight of gel slice taken. Transferred it to a clean tube.
2. Added 2 volumes of Buffer NTI (provided in the kit) corresponding to the weight of excised gel (< 2 % gels)
3. Samples were incubated at 50 °C for 5-10 minutes. Vortexed the sample every 2-3 minutes in order to completely dissolve the gel slice in the buffer solution.
4. Nucleospin gel and PCR clean-up column (provided in the kit) was placed into a 2 ml collection tube. 700 µl sample solution was added to it and centrifuged at 11,000 rpm for 30 seconds. Added remaining sample by repeating the centrifugation step if required.
5. 700 µl Buffer NT3 (provided in the kit) was loaded to the Nucleospin gel and PCR clean-up column. Centrifuged at 11,000 rpm for 30 seconds. Discarded sample in the collection tube and place column back.
6. Centrifuged for 1 minute at 11,000 rpm for completely removing Buffer NT3.
7. Nucleospin gel and PCR clean-up column was placed into a new micro centrifuge tube and 15-30 µl Buffer NE was added and incubated at room temperature for one minute. Centrifuged at 11,000 rpm for one minute.
8. The microfuge tube containing eluted DNA was labeled and stored at -20°C.

3.2.7 Molecular Cloning

Cloning of the PCR product was done by using The InsTAclone PCR Cloning Kit provided by Thermo scientific. The Thermo Scientific™ InsTAclone™ PCR Cloning Kit is a TA system for direct one-step cloning of PCR products with 3'-dA overhangs (1). The high quality TA cloning vector pTZ57R/T is ready to use for efficient ligation with PCR products providing high cloning yields and low background.

3.2.7.1 Ligation

1. Set up the ligation reaction. Vortex briefly and centrifuge for 3-5 s.

Table 5. Ligation mix components

Component	**Volume**
Vector pTZ57R/T, (0.17 pmol ends)	3 µL
5X Ligation Buffer	6 µL
PCR product (0.52 pmol ends)	50 ng
Water, nuclease-free	(make upto 29 µL)
T4 DNA Ligase	1 µL
Total volume	30 µL

2. Ligation mixture was incubated at room temperature (22 °C) for 1 hour. For obtaining maximal number of transformants, incubate overnight at 4 °C
3. Used 2.5 µL of the ligation mixture directly for bacterial transformation.

3.2.6.2 Transformation

The TransformAid Bacterial Transformation Kit provided by Thermo scientific is used for transformation. Competent cells were not provided in the kit and common *E. coli* laboratory strains (DH5 alpha) was used for the transformation. The overnight kept bacterial culture was used for transformation.

Procedure

1. The day beforetransformation, inoculated with 2 ml of C-medium with a single bacterial colony and incubated the culture overnight at 37 °C in a shaker.
2. On the day of transformation, culture tubes containing the required amount of C-medium (1.5 mL for each 2 transformations) was prewarmed at 37 °C for at least 20 min. LB agar plates, supplemented with ampicillin, X-Gal and IPTG were also prewarmed.
3. T-solution: thawed T-solution A and T-solution B were prepared (provided in the kit) and contents were mixed thoroughly. Combined 250 µL of T-solution (A) and 250 µL of T-solution (B) in a separate tube and kept on ice.
4. 150 µL of the overnight bacterial culture was added to 1.5 mL of pre-warmed C-medium and was Incubated 20 min at 37 °C in a shaker.
5. Pelleted bacterial cells by 1 min centrifugation, discarded the supernatant. Resuspended cells in 300 µL of T-solution. Incubated on ice for 5 min.
6. Centrifuged for 1 min in a microcentrifuge, discarded the supernatant.
7. Resuspend pelleted cells in 120 µL of T-solution. Incubated 5 min on ice.
8. 2.5 µL of ligation mixture (containing 14 ng vector DNA) or 1 µL of supercoiled Control DNA (10-100 pg) was added into new microcentrifuge tubes. Chilled on ice for 2 min.
9. 50 µL of the prepared cells was added to each tube containing DNA, mix and incubate on ice for 5 min.
10. Plated immediately on pre-warmed LB-ampicillin X-Gal/IPTG agar plates. Incubated overnight at 37 °C.

3.2.7.3 Analysis of recombinant clones

Presence of insert in the plasmid was confirmed by performing colony PCR with the RAPD primer OPAW15.

1. Prepared enough PCR master mix for the number of colonies analyzed plus one extra. For each 20 µL reaction, mix the following reagents.

Table 6.Colony PCR reaction mix components

Component	**Quantity**
Autoclaved distilled water	14.6µl
*Taq*buffer with $MgCl_2$(10X)	2 µl
dNTPs (10mM)	1.6 µl
Primer (10 µM)	1.5 µl
*Taq*DNA polymerase	0.3 µl
Total volume	20µl

2. Mixed well. Aliquot 20 µL of the mix into the PCR tubes on ice.
3. Picked an individual white colony and resuspended in 20 µL of the PCR master mix. Made a short strike over culture plate to save the clone for repropagation.
4. The amplification was done using the following thermal profile

Thermal profile for RAPD assay:

- Initial denaturation - 94^0C for 5 min
- Final denaturation - 94^0C for 1 min
- Primer annealing - 37^0C for 1.2 min
- Primer extension - 72^0C for 2 min

} 40 Cycles

- Final extension - 72^0C for 10 min
- 4^0C - hold the sample

3.2.7.4 Plasmid isolation

Plasmid isolation was done using PureLink® Quick Plasmid Miniprep Kits provided by Invitrogen

Components

- Resuspension Buffer (R3; 50 mMTris-HCl, pH 8.0; 10 mM EDTA)
- RNase A (20 mg/mL in Resuspension Buffer R3)
- Lysis Buffer (L7; 200 mMNaOH, 1% w/v SDS)
- Precipitation Buffer (N4)
- Wash Buffer (W9)
- Wash Buffer (W10)
- TE Buffer (10 mMTris-HCl, pH 8.0; 0.1 mM EDTA)
- Wash and recovery tubes
- Spin columns

Procedure

1. 1–5 mL of the overnight LB-culture was centrifuged. Removed all medium.
2. 250 µLResuspension Buffer (R3) was added with RNase to the cell pellet and resuspended the pellet until it is homogeneous.
3. 250 µLLysis Buffer (L7) was added. Mixed gently by inverting the capped tube until the mixture is homogeneous. Incubated the tube at room temperature for 5 minutes.
4. 350 µL Precipitation Buffer (N4) was added. Mixed immediately by inverting the tube, or for large pellets, vigorously shaking the tube, until the mixture became homogeneous. Lysate was centrifuged at >12,000 × g for 10 minutes.
5. Supernatant from step 4 was loaded onto a spin column in a 2-mL wash tube. Centrifuged the column at 12,000 × g for 1 minute. Discarded the flow-through and placed the column back into the wash tube.

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6. 700 µL Wash Buffer (W9) was added with ethanol to the column. Centrifuged the column at 12,000 × g for 1 minute. Discarded the flowthrough and placed the column into the wash tube. Centrifuged the column at 12,000 × g for 1 minute. Discarded the wash tube with the flow-through.
7. Placed the Spin Column in a clean 1.5-mL recovery tube. 75 µL of preheated TE Buffer (TE) was added to the center of the column. Incubated the column for 1 minute at room temperature.
8. Centrifuged the column at 12,000 × g for 2 minutes. The recovery tube contained the purified plasmid DNA. Discarded the column. Stored plasmid DNA at 4°C (short-term) or store the DNA in aliquots at −20°C (long-term)

3.2.8 Sequencing of Dwarf specific DNA fragment

The isolated plasmid was send to eurofins genomics Pvt Ltd, Bangalore.

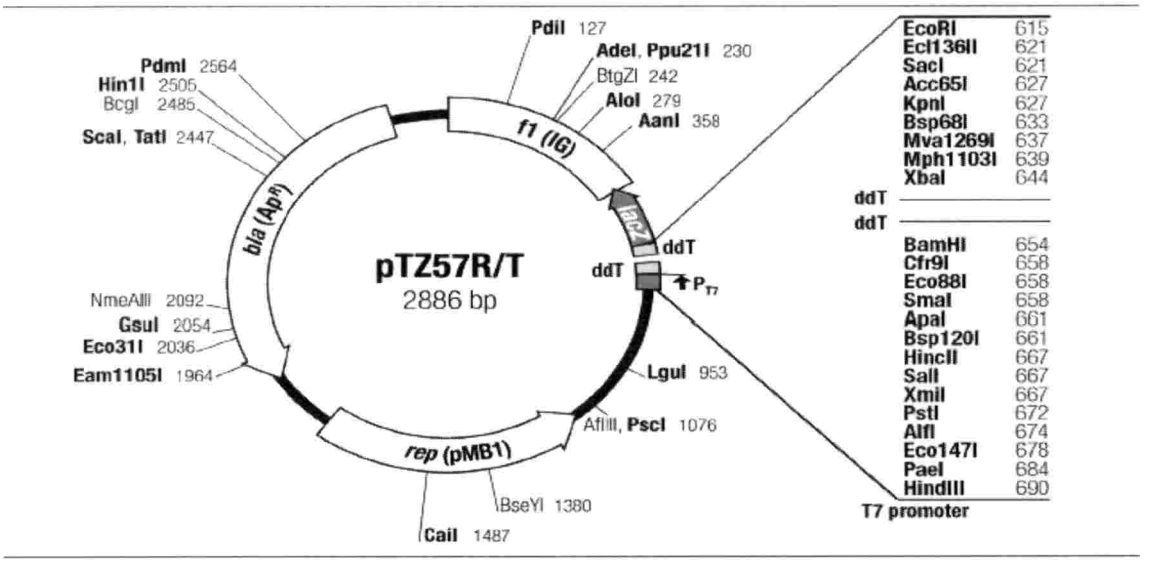

Figure 2. Map of the pTZ57R/T cloning vector. Unique restriction sites are indicated

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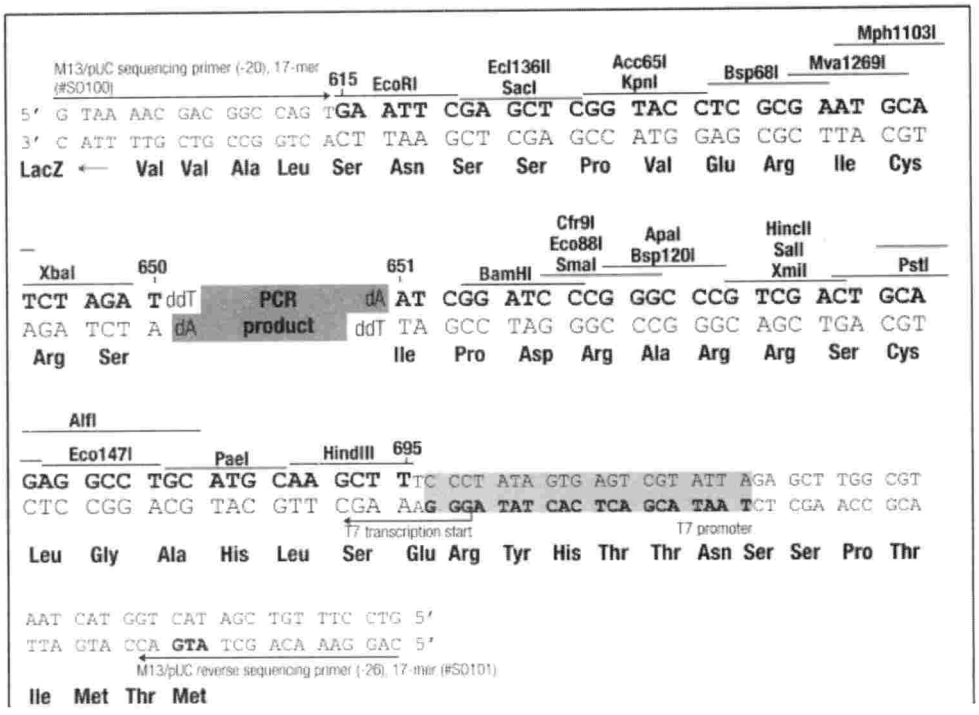

Figure 3. DNA sequence of multiple cloning site region.

3.2.9 SCAR primer designing

SCAR primer was designed from the sequence information of the desired DNA fragment by considering the following criteria. One pair of forward and reverse primer was designed.

a. GC content should be in between the range 40-50 per cent
b. Melting temperature (Tm = 4 GC + 2 AT)ranged between 45 ^{0}C and 65 ^{0}C
c. Each primer should be 20 to 24 base pair long
d. Primer sequences should not have palindromic sequences or repetitive sequences
e. There should not be any complementarity between forward and reverse primers
f. For designing primers, sequence of the forward primer as taken as such and the reverse complementary sequence was taken for reverse primer.

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3.2.10 Testing of SCAR marker

One pair of SCAR primer was designed (forward and reverse primer). Each primer has a length of 21 base pairs. Annealing temperature for the PCR reaction using designed SCAR primer was set as 60°C.PCR reaction for validating the SCAR marker in S3 inbred families was performed in a total reaction volume of 20 µl.

Table 7. The composition of reaction mixture for PCR using SCAR marker

Components	**Volume per reaction (µl)**
10X Taq buffer	2
dNTP Mix	1.6
Forward primer	0.5
Reverse primer	0.5
DNA (100ng)	0.4
Taq DNA polymerase	0.2
Sterile water	14.8
Total Volume	**20**

The amplification was done using the following thermal profile

Thermal profile for SCAR assay:

- Initial denaturation - 94^0 C for 5 min
- Final denaturation - 94^0 C for 1 min
- Primer annealing - 60^0 C for 1.2 min } 40 cycles
- Primer extension - 72^0 C for 2 min
- Final extension - 72^0 C for 10 min
- 4^0C - hold the sample

Electrophoresis of the PCR product was carried out in 1.5 per cent agarose. The gel documentation was carried out using BIO-RAD imaging system.

Results

4. RESULTS

The study was carried out during 2016-2018 in third generation (S3) selfed seedlings of coconut cultivar West Coast Tall (WCT) which were planted in 2015 at RARS Pilicode. RAPD analysis was done in S3 seedlings belonging to IAS_3-1and IBS_3-1 families in which two putative dwarf specific RAPD markers were observed in an earlier study using selected seedlings (Chethana, 2016). The present study was carried out for developing specific and reproducible dwarf specific SCAR markers from these identified RAPD markers, its validation and screening the seedlings available in these two families and 10 selected seedlings from other families of S3 generation (IIS3-1, IIIS3-1, VS3-1).

The research work included the following aspects

4.1. Isolation and quantification of DNA

4.2. RAPD assay

4.3. Molecular cloning of dwarf specific band

4.4. Sequencing and analysis of dwarf specific band

4.5. Designing SCAR primer

4.6. Validation of SCAR primer

4.7. Screening the various S3 seedlings using the primer

4.1. ISOLATION AND QUANTIFICATION OF DNA

Genomic DNA was isolated from 54 seedlings including 22 seedlings belonging to two families IAS3-1 and IBS3-1, Seedlings which showed dwarf specific bands during previous study, 10 selected seedlings from other three families belonging to Low and medium height at the time of planting. 7 seedlings of COD, 6 seedlings of CGD, one seedling of MYD which are all dwarf genotypes and 8 seedlings from the tall parental genotype, *viz.,* WCT were also used for DNA extraction (Table 8).

Table 8. Details of 3 year old coconut seedlings of third generation inbred WCT families, dwarfs and WCT selected for DNA extraction

IAS3-1 family	**IBS3-1 family**	**IIS3-1, IIIS3-1 and VS3-1 families**	**Dwarf cultivars**	**WCT**
K-M1	E-H1	S-L2	COD-1	. WCT-1
K-H1	M-L1	D-M1	COD-2	WCT-2
K-H2	M-L2	S-L2	COD-3	WCT-3
V-L1	M-M1	I1-L2	COD-4	WCT-4
V-L2	M-M2	I1-M1	COD-5	WCT-5
V-M1	M-H1	T-M1	COD-6	WCT-6
V-H2	C1-L2	T-L2	COD-7	WCT-7
J1-M1	C1-M1	T-M2	CGD-1	WCT-8
J1-M2	C1-M2	F1-L1	CGD-2	
J1-H1	C1-H1	P-M1	CGD-3	
J1-H2	C1-H2		CGD-4	
			CGD-5	
			CGD-6	
			MYD-1	

The quality and quantity of DNA isolated are provided in Table 9. The procedure yielded good quantity of DNA and electrophoresis revealed a single high molecular weight band without any degradation. The concentration of DNA varied from 120 (ng/µl) to 3810 (ng/µl). However, several samples showed RNA contamination and hence before RAPD analysis such samples were subjected to RNAse treatment before RAPD analysis.

Table 9. Quantity and quality of DNA isolated from third generation selfed seedlings, dwarf and tall genotypes of coconut

Sl. no	Seedling name(S3 families)	Conc. (ng/µl)	OD260/ OD280	Sl. No	Seedling name (Dwarfs & WCT)	Conc. (ng/µl)	OD260/ OD280
Family IAS3-1				COD			
1.	K-M1	3360	1.97	1.	COD-1	1200	1.75
2.	K-H1	1620	1.84	2.	COD-2	1500	1.78
3.	K-H2	1230	1.83	3.	COD-3	1200	1.47
4.	V-L1	1920	1.81	4.	COD-4	3600	1.53
5.	V-L2	3810	1.47	5.	COD-5	1200	1.55
6.	V-M2	1380	1.80	6.	COD-6	1440	1.77
7.	V-H2	1920	1.86	7.	COD-7	1020	1.81
8.	J1-M1	3780	1.78	CGD			
9.	J1-M2	1230	1.79	8.	CGD-1	1500	2.12
10.	J1-H1	1800	1.96	7.	CGD-2	1200	1.86
11.	J1-H2	1380	1.78	8.	CGD-3	180	2.20
Family IBS3-1				9.	CGD-4	390	2.03
12.	E-H1	1470	1.79	10.	CGD-5	210	1.74
13.	M-L1	3030	1.75	11.	CGD-6	270	1.80
14.	M-L2	1590	1.84	MYD			
15.	M-M1	540	1.80	12.	MYD-1	2670	1.74
16.	M-M2	1380	1.88	WCT			
17.	M-H1	120	1.62	13.	WCT-1	1800	1.78
18.	C1-L2	2130	2.03	14.	WCT-2	330	1.73
19.	C1-M1	1770	1.78	15.	WCT-3	180	1.64
20.	C1-M2	960	1.77	16.	WCT-4	480	1.76
21.	C1-H1	1020	1.75	17.	WCT-5	120	1.64
22.	C1-H2	1260	1.87	18.	WCT-6	720	1.76
Family IIS3-1				19.	WCT-7	720	1.76
23	S-L2	1800	1.82	20.	WCT-8	960	1.81
24	D-M1	1140	1.85				
Family IIIS3-1							
25	F-M1	1260	1.80				
26	I1-L2	3090	1.83				
27	I1-M1	690	1.85				
28	T-M1	330	1.71				
29	T-L2	1260	1.73				
30	T-M2	470	1.81				
Family VS3-1							
31	F1-L1	900	1.91				
32	P-M1	1350	1.80				

4.2 RAPD ANALYSIS

PCR amplification was carried out using the two reported decamer primers *viz*., OPAU03 and OPAW15 which gave two dwarf specific RAPD bands which were also present in the S3 inbreds belonging to IAS_3-1and IBS_3-1 family (Chethana, 2016) and the primer OPA09 which produced a band specific to tall leading to development of SCAR marker for tall (Rajesh *et al*., 2013).

Among the two PCR reaction mixtures tried by changing the quantity of DNA taken (40 ng/ µl and 100 ng/ µl DNA) the amplification was better when 40 ng/µl DNA was taken as template (Table 2, RM-1). Two different thermal profiles were also tested by making changes in annealing temperature and number of cycles. The result was better when the annealing temperature used was 37°C and the number cycles was 40 (Table 2, TP-1) than that of thermal profile with annealing temperature 34°C and 35 cycles.PCR products were loaded in 1.5 per cent agarose gel and was observed under UV light.

Table 10. PCR conditions selected for RAPD analysis using selected primers in coconut

PCR reaction mixture (RM-1)	**Thermal profile (TP-1)**
1X buffer	Initial denaturation: 94°C – 5 min
200 uMdNTPs	Denaturation: 94°C – 1min
0.75 uM primer	Annealing: 37°C – 1.2min
0.9 U Taq polymerase	Extension: 72°C -2 min
40 ng/ µl DNA	Final extension: 72°C – 10 min
	Hold : 4°C

4.2.1 Screening with primer OPAU03

Six third generation inbreds of WCT along with COD, CGD and WCT were analysed with RAPD marker OPAU 03 (Plate 1a.)

Table 11.RAPD banding pattern in coconut seedlings by primer OPAU03

		1AS3-1					1BS3-1	Dwarfs				
Sl. No	Amplicon size	K M1	V M2	J1 M1	J1 M2	V L2	C1 L2	COD 1	COD 2	CGD 1	CGD 2	WCT 8
1	2.9 Kb	+	+	+	+	+	+	+	+	+	+	+
2	2.2 Kb	+	+	+	+	+	+	+	+	+	+	+
3	1.8 Kb	+	+	-	+	+	-	+	+	+	+	-
4	1.7 Kb	+	+	-	+	+	-	-	+	+	+	+
5	1.45 Kb	+	+	+	+	-	+	+	-	-	-	+
6	1.4 Kb	+	+	+	+	+	+	+	-	-	-	-
7	1.2 Kb	+	+	+	+	+	+	+	+	+	+	+
8	1.1 Kb	-	-	+	+	+	+	-	+	+	+	+
9	0.95 Kb	+	+	+	+	+	+	+	+	+	+	+
10	0.9 Kb	+	+	+	+	+	+	+	+	+	+	+
11	0.3 Kb	+	+	+	+	+	+	+	+	+	+	+

'+' - Presence of band '-' - Absence of band

Elevenamplicons were produced within the range 0.3 kb to 2.9 kb among which, 6 were monomorphic (2.9 kb, 2.2 kb, 1.2 kb, 0.95 kb, 0.9 kb, 0.3 kb) and 5 were polymorphic (1.8 kb, 1.7 kb, 1.45 kb, 1.4 kb, 1.1 kb) (Table 11). Between 1.5 kb and 2 kb marker bands, two amplicons are present in dwarfs and WCT, wherein one is monomorphic, while the other one is present only in dwarf and not in WCT (Plate1a). This polymorphic band was observed at the range of around 1.8kb and is present in four inbreds also. However, this was repeated to confirm the amplicon, the 1,8 kb fragment failed to get amplified (Plate 1b.).Though high molecular weight amplicons were present upto 5 kb size, the clarity was less (Plate. 1a).

To confirm the result, the DNA from different dwarfs and WCT seedlings were amplified with primer OPAU03 (Plate 1c). The result showed eight amplicons in the range 0.3 kb to 5 kb out of which 6 were monomorphic (5 kb,

2.9 kb, 2.1 kb, 1.8 kb, 0.9 kb, 0.3 kb) and two were polymorphic (3.2 kb, 1.1 kb). The dwarf specific 1.8kb fragment as in Plate 1a, failed to amplify.

Table 12. RAPD banding pattern in dwarfs and WCT seedlings by primer OPAU03

		Dwarf										Tall				
Sl. No	Amplicon size	COD1	COD4	COD7	COD5	COD6	CGD1	CGD5	CGD6	CGD3	CGD4	WCT8	WCT4	WCT7	WCT2	WCT5
1	5 Kb	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	3.2 Kb	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
3	2.9 Kb	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4	2.1 Kb	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5	1.8 Kb	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6	1.1 Kb	+	+	+	-	+	+	-	-	-	-	+	+	+	-	-
7	0.9 Kb	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8	0.3 Kb	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

'+' - Presence of band '-'- Absence of band

4.2.2 Screening with primer OPAW15

Six inbreds of WCT along with COD, CGD and WCT wereanalysed with RAPD marker OPAW15. Eight amplicons were produced out of which five were monomorphic (1.45 kb, 1 kb, 0.8 kb, 0.75 kb, 0.5 kb) and three were polymorphic (1.7 kb, 1.5 kb,0.9 kb)(Table 14). Polymorphic band at 0.9 kb was seen in all six inbreds of WCT and in WCT but absent in dwarfs (Plate 2a).

Table 13. RAPD banding pattern in coconut seedlings by primer OPAW15

		1AS3-1					1BS3-1	Dwarfs				
Sl. No	Amplicon size	KM1	VM2	J1M1	J1M2	VL2	C1L2	COD1	COD2	CGD1	CGD2	WCT8
1	1.7 kb	+	+	+	+	+	+	+	-	+	+	-
2	1.5 kb	+	+	+	+	+	+	+	-	+	+	-
3	1.45 kb	+	+	+	+	+	+	+	+	+	+	+
4	1 kb	+	+	+	+	+	+	+	+	+	+	+
5	0.9 kb	+	+	+	+	+	+	+	-	-	-	+
6	0.8 kb	+	+	+	+	+	+	+	+	+	+	+
7	0.75 kb	+	+	+	+	+	+	+	+	+	+	+
8	0.5 kb	+	+	+	+	+	+	+	+	+	+	+

'+' - Presence of band '-' - Absence of band

The primer was used to amplify the DNA from more number of different dwarf cultivars (COD, CGD, and MYD) and with WCT. Six monomorphic amplicons were produced. One polymorphic band at the range 320 bp was observed in dwarf seedlings and was not present in WCT cultivar (Plate 2b). The polymorphic band seen at 0.9 kb (Plate 1a) failed to get amplified in (Plate 2b).

4.2.3 Screening with primer OPA09

RAPD primer OPA09, which was reported to produce a tall specific band of size 260bp was used for screening COD, CGD and WCT cultivars of coconut. This produced five amplicons within the range 0.25 kb to 1.1 kb out of which three were monomorphic (1.1 kb, 0.55 kb, 0.35 kb)(Table 14). Other two bands were polymorphic (03 kb, 0.25 kb). However, there was no band specifically amplified in tall (WCT) seedlings and the 250 bp fragment was also found in one of the COD seedlings.

Table 14. RAPD banding pattern in dwarfs and WCT seedlings by primer OPA09

		Dwarfs												Tall						
Sl. No	Amplicon size	COD1	COD4	COD7	COD5	COD6	COD2	CGD1	CGD5	CGD6	CGD3	CGD4	CGD2	WCT1	WCT2	WCT3	WCT4	WCT5	WCT6	MYD
1	1.1kb	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	0.55 kb	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3	0.35	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4	0.3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
5	0.25	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-

'+' - Presence of band '-' - Absence of band

Confirmation of dwarf specific band

Presence of the polymorphic band of size 320 bp observed in dwarf varieties of coconut when amplified using RAPD primer OPAW15 (Plate 2b) was confirmed by repeating the experiment. Further screening with same primer was carried out with more number of individuals for testing the consistency and reproducibility of the specific fragment by the primer. The result shows same polymorphic band, confirming the dwarf specific amplicon in coconut.The dwarf

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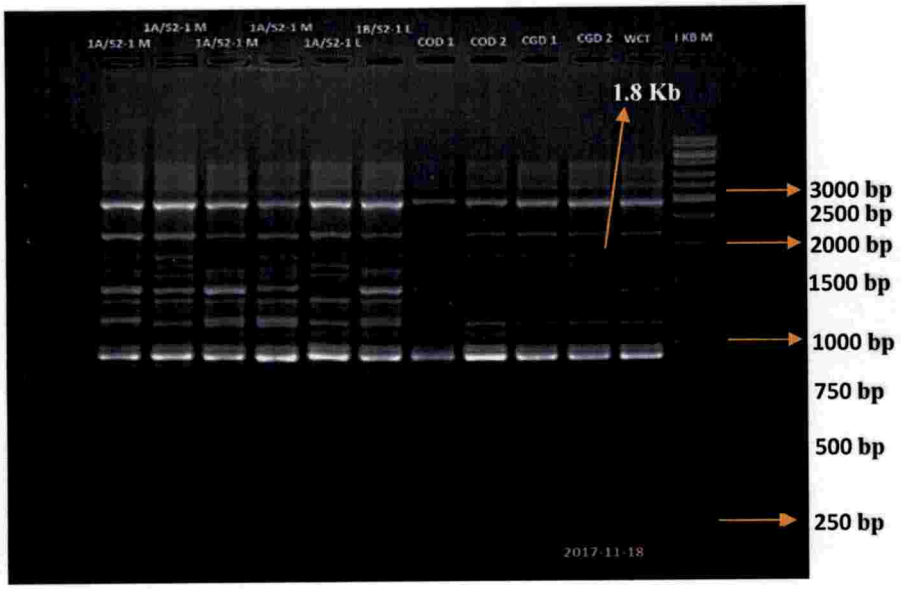
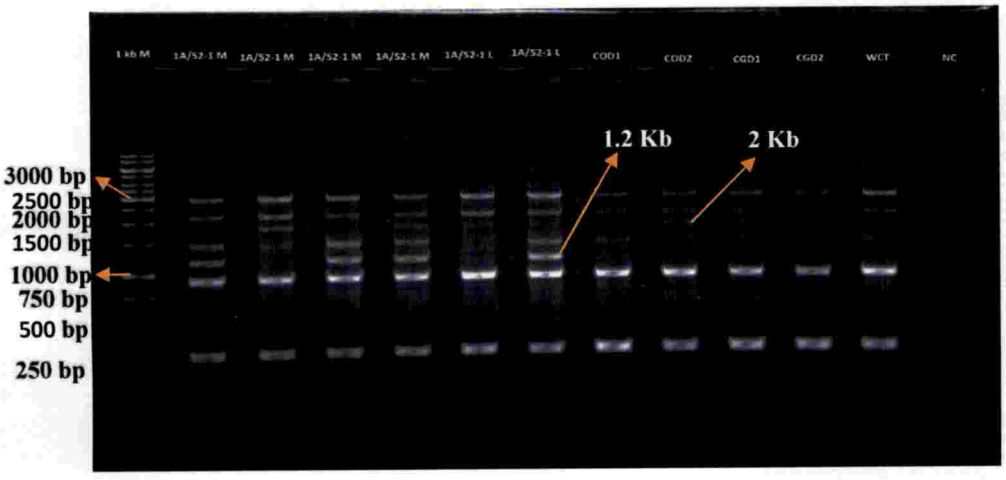
Amplification by primer OPAU 03



1A/S3-1: 1.K-M1
2. V-M2
3. J1-M1
4. j1-M2
5. V-L2
1B/S3-1 : 6.C1-L2
7. COD 1
8. COD 2
9. CGD 1
10. CGD 2
11. WCT 8
12.1 Kb DNA ladder

Plate 1a. Amplification of DNA from 3rd generation selfed seedlings, dwarf (COD, CGD) and tall (WCT) genotypes of coconut



1.1Kb DNA ladder
1A/S3-1: 2.K-M1
3. V-M2
4. J1-M1
5. J1-M2
6. V-L2
1B/S3-1 : 7.C1-L2
8. COD 1
9. COD 2
10. CGD 1
11. CGD 2
12. WCT 8
13. Negative control

Plate 1b. Amplification of DNA from 3rd generation selfed seedlings, dwarf (COD, CGD) and tall (WCT) genotypes of coconut

Amplification by primer OPAU 03

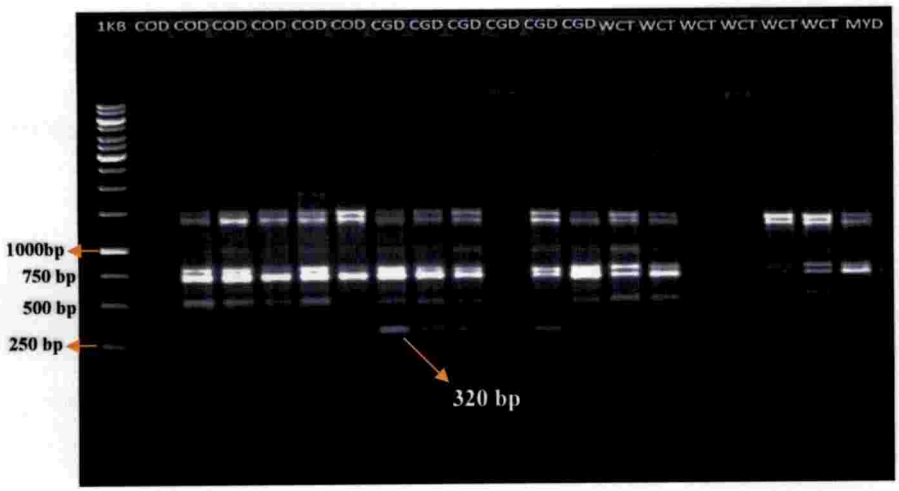
1.1Kb DNA ladder
2. COD 1
3. COD 4
4. COD 7
5. COD 5
6. COD 6
7. CGD 1
8. CGD 5
9. CGD 6
10.CGD 3
11.CGD 4
12. WCT 8
13. WCT 4
14. WCT 7
15. WCT 2
16. WCT 5
17. Negative control
18.100 bp DNA ladder

Plate 1c. Amplification of DNA from dwarf (COD, CGD) and tall (WCT) genotypes of coconut

Amplification by primer OPAW 15

1.1Kb DNA ladder
1A/S3-1: 1.K-M1
2. V-M2
3. J1-M1
4. j1-M2
5. V-L2
1B/S3-1 : 6.C1-L2
7. COD 1
8. COD 2
9. CGD 1
10. CGD 2
11. WCT 8
12.50 bp DNA
er

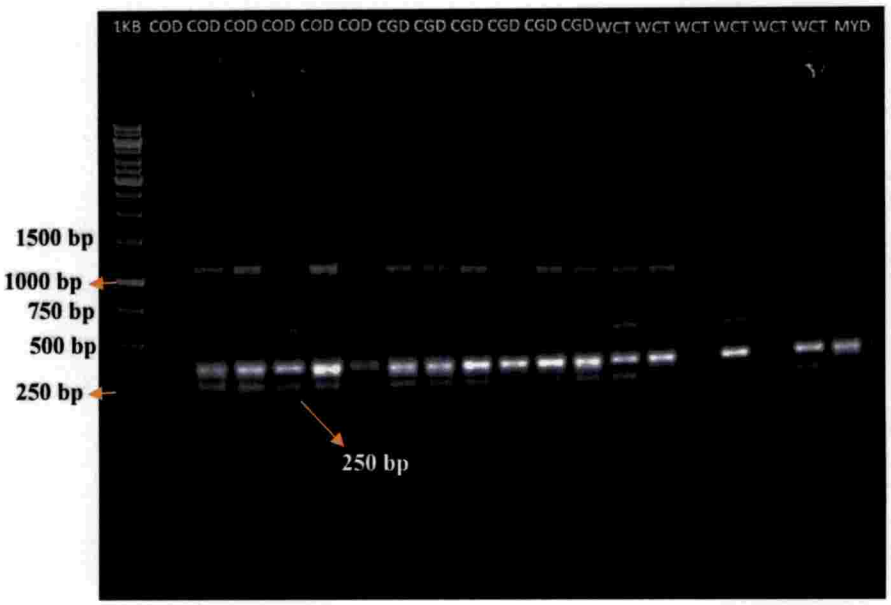
Plate 2a. Amplification of DNA from 3rd generation selfed seedlings, dwarf (COD, CGD) and tall (WCT) genotypes of coconut.



1.1 Kb DNA ladder
2. COD- (70)
3. COD- (78)
4. COD-(97)
5. COD-(79)
6. COD-(80)
7. COD-(106)
8. CGD-(72)
9. CGD-(83)
10. CGD- (84)
11. CGD-(81)
12.CGD-(82)
13. CGD- (107)
14. WCT-(74)
15. WCT- (75)
16. WCT-(76)
17. WCT- (85)
18. WCT- (88)
19. WCT- (105)
20. MYD(108)

Plate 2b. Amplification of DNA from dwarf (COD, CGD,MYD) and tall (WCT) genotypes of coconut

Amplification by primer OPA09



1.1Kb DNA ladder
2. COD 1
3. COD 4
4. COD 7
5. COD 5
6. COD 6
7. COD 2
8. CGD 1
9. CGD 5
10. CGD 6
11.CGD3
12.CGD4
13. CGD 2
14. WCT 1
15. WCT 2
16. WCT 3
17. WCT 4
18. WCT 5
19. WCT 6
20. MYD

Plate 3b. Amplification of DNA from dwarf (COD, CGD,MYD) and Tall (WCT) genotypes of coconut

specific RAPD band was cut under UV transilluminator and subjected to gel elution and purification with Gel Elution Kit. The quantity estimated was 112 ng DNA µl-1 based on absorbance at 260 nm and 280 nm.

4.3 MOLECULAR CLONING

Molecular cloning was done for incorporating the desired product into the vectorby using ThermoscientificinsTAclone PCR cloning kit. Vector pTZ57R/T was used for cloning which was included in the kit.The product (insert) size was 320 bp and 50 ng was taken for ligation reaction (as recommended in the cloning kit by thermo scientific company). Cloning procedure was done according to the directions given in the cloning kit.

4.3.1 Transformation

The competent cells(common *E. coli* laboratory strain DH5 alpha) prepared were checked for competence was transformed using the recombinant plasmid pTZ57R/Thaving ampicillin resistance.Selection of transformed cells were done by blue white screeing (Plate 4). For the maintenance of transformed colonies, four white colonies were picked from plate and were grown in four plates with LB ampicillin media separately.

4.3.2 Detection of the product (insert)

The presence of desired DNA product was checked by performing colony PCR using the primer OPAW 15.The PCR products were checked in 1.5 per cent agarose gel and 320 bp length amplicon was observed in all the colonies selected which confirmed the presence of insert after the cloning process (Plate 5)

4.3.4 Plasmid DNA isolation

Transformed colony was picked up and grown by incubating overnight at 37°C in tubes containing LB media. Plasmid DNA was isolated by using PureLink

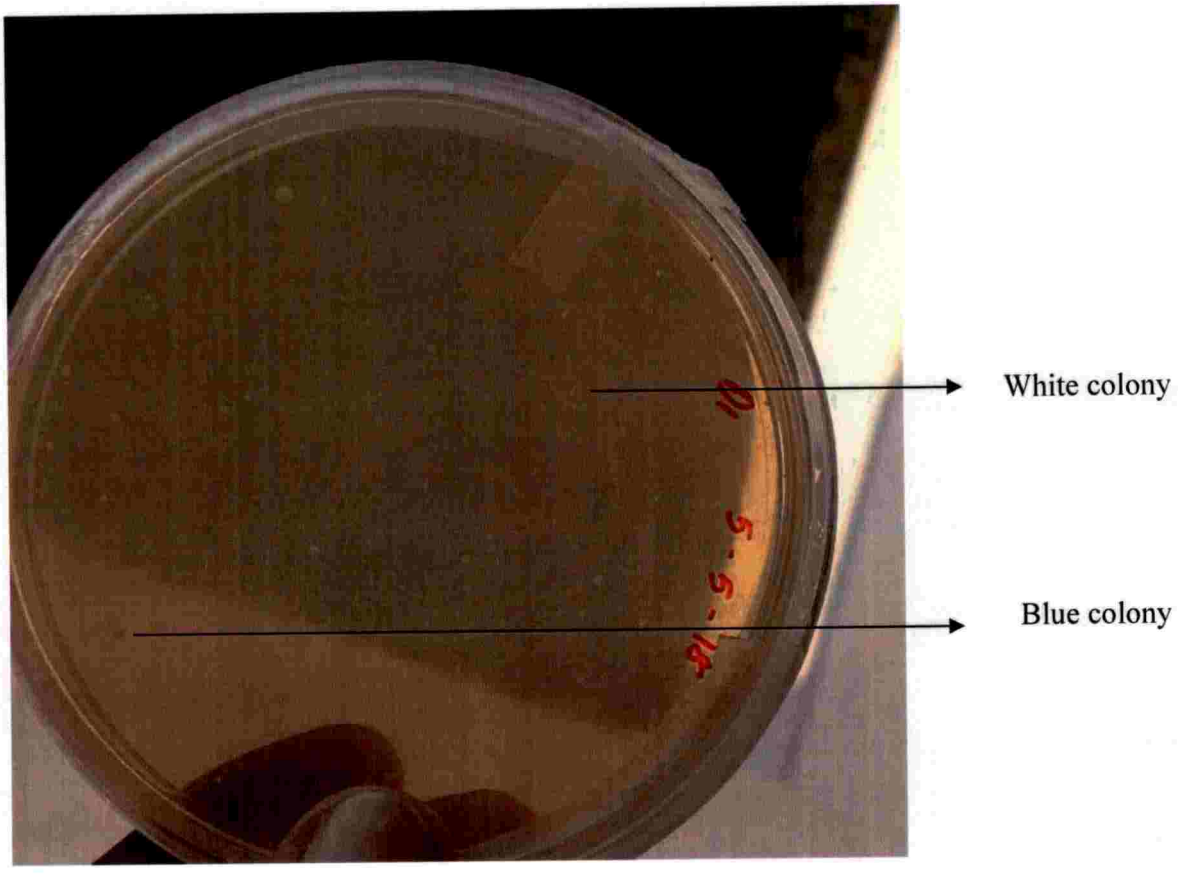


Plate 4. Blue white screening of transformed *E. coli.* Cells

Plate 5. Conformation of transformation by colony PCR (C1 - C5 - white colonies)

quick DNA Miniprep Kit, Invitrogen. The isolated DNA was tested for the presence of insert DNA and a band of size 320 obtained.

4.4 SEQUENCING AND ANALYSIS

After confirming the presence of insert, the plasmid DNA was send for sequencing. The sequencing facility of Eurofins genomics india private Ltd was utilized. The result obtained after the sequencing was analysed further for developing SCAR primer specific to dwarfing character in coconut. The total length of sequence obtained was 1455bp.

4.4.1 Vector screening

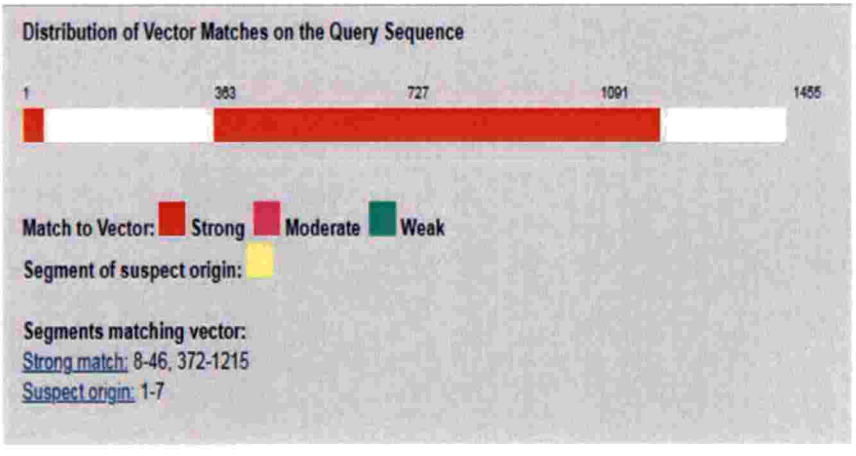
The plasmid DNA sequence obtained was subjected to vector screening using the tool vecscreen in NCBI for removing the vector portions from the sequence and to obtained the sequence of our product. The size of sequence was 1455 base pair. Vecscreen software showed the presence of strong vector sequence similarity between the regions 4-46 and 372-1215 nucleotide. This portion was removed to obtain the DNA insert of size 324 bp (Fig 4a).

4.4.2 Homology

The nucleotide sequence of polymorphic band was compared with the sequences available in nucleotide database using BLAST tool (www.ncbi.nlm.nih.gov/Blast/) provided by NCBI (Fig 5a, 5b). The data shows the DNA sequence of polymorphic band has similarity with the chromosome 2 (complete genome) of crop *Solanum lycopersicum*.

5'ccagttcccaaatccagctttacctgaggtcagaggtctctatttcagctaattcagac
ctccaacctcggctactctaaatgctattttatctaaggttgaaggtccctacttcgacttc
accagacatccgatctggatggtgctaaattgtttcatccatcaaaagtatggactttgg
cttaatgcgaccttagaactcaacgatcctgatcccgatttcattcattgtcggagatcct
aactgctttatccattatcgaagatctcgacttcggtcttatccgatcttcgacctcgaaaa
tatcctagcttgggactgg3'

4a. DNA sequence of dwarf specific band after removing vector sequence



4b. Vecscreen output

Fig 4. Sequence data analysis of the clone

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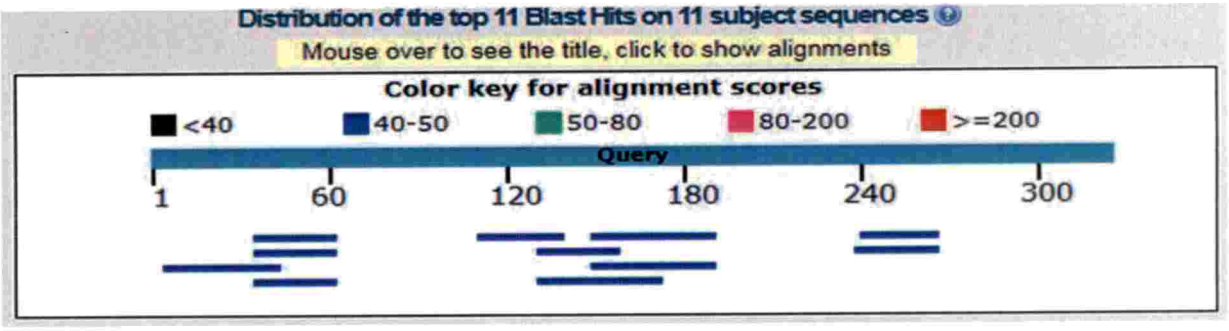

5a. Blastn output

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Solanum lycopersicum cultivar I-3 chromosome 2	42.8	42.8	13%	6.9	86%	CP023758.1
PREDICTED: Cebus capucinus imitator uncharacterized LOC108286024 (LOC108286024), ncRNA	42.8	42.8	8%	6.9	93%	XR_001817785.1
Macaca fascicularis complete genome, chromosome chr1	42.8	42.8	8%	6.9	93%	LT160000.1
Schistocephalus solidus genome assembly S_solidus_NST_G2, scaffold SSLN_scaffold0000734	42.8	42.8	8%	6.9	96%	LI900935.1
Heligmosomoides polygyrus genome assembly H_bakeri_Edinburgh, scaffold HPBE_contig0001550	42.8	42.8	8%	6.9	93%	LL203254.1
Trichobilharzia regenti genome assembly T_regenti_v1_0.4, scaffold TRE_scaffold0074836	42.8	42.8	12%	6.9	85%	LL078058.1
Thelazia callipaeda genome assembly T_callipaeda_Ticino_scaffold TCLT_contig0000115	42.8	42.8	8%	6.9	93%	LK980090.1
Solanum lycopersicum chromosome ch02, complete genome	42.8	42.8	13%	6.9	86%	HG975514.1
Schistosoma mansoni strain Puerto Rico chromosome 1, complete genome	42.8	42.8	13%	6.9	83%	HE601624.1
Pyrenophora tritici-repentis Pt-1C-BFP structural maintenance of chromosomes protein 5, mRNA	42.8	42.8	8%	6.9	93%	XM_001935971.1
Rhesus Macague BAC CH250-191M14 () complete sequence	42.8	42.8	8%	6.9	93%	AC199034.5

5b. Blastn output

Fig 5. Sequence data analysis of the clone

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4.5 DESIGNING SCAR PRIMER

One pair of SCAR primer (forward and backward primer) was designed for screening dwarf specific RAPD fragment generated with the primer OPAW 15. Both primers designed were 21 base pair length. Forward and reverse primer designed was having melting temperature 62□C and 59□C respectively and the GC content was 48 per cent.

Table 15. SCAR primers (Df and dwarf Dr) developed from sequence data of dwarf specific RAPD marker in coconut.

Sequence (5'-3')	**No of base pair**	**G+C Content (%)**	**Tm (°C)**
5'-CAAATCCAGCTTTACCTGAGG -3' forward primer	21	48	62
5'-CCAGTCCCAAGCTAGGATATT-3' Reverse primer	21	48	59

4.6 VALIDATION OF SCAR PRIMER

Validation of SCAR primer was done by using the designed primers,Df and Dr (table 16). The expected band length was 320 base pair.

4.6.1 Screening of SCAR primer at different annealing temperatures

Gradient PCR was done at different annealing temperatures viz, 50 °C, 52 °C, 55 °C, 58 °C and 60°C. No bands were found at 50 and 52°C. Band with reduced intensity was seen at annealing temperature 58°C. Clear band of size 320 base pair was observed in the samples where annealing temperature was 60 °C. So the annealing temperature was standardized as 60°C (Plate 6).

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4.6.2 Testing of SCAR primer

SCAR primer was tested in third generation selfed seedlings of coconut cultivar WCT. Testing was done in 22 seedlings belonging to two families IAS3-1 and IBS3-1 and also in 10 selected S3 inbreds belonging to the other promising families of inbreds, viz., IIS2, IIIS2 and VS2.

4.6.2.1 Testing of SCAR marker in family IAS3-1

SCAR primer was tested in 11 seedlings of family IAS3-1 along with dwarf cultivars, COD and CGD. Dwarf specific band of size 320 bp was seen in 6 seedlings viz., V-L1, V-L2, J1-M1, J1-M2, K-M1, V-M1 which comes under low and medium category (Classified based on the height of seedling at the time of planting) and in the 4 dwarf cultivars (Plate 7) and was absent in seedlings classified as with respect to height.

4.6.2.2 Testing of SCAR marker in family IBS3-1

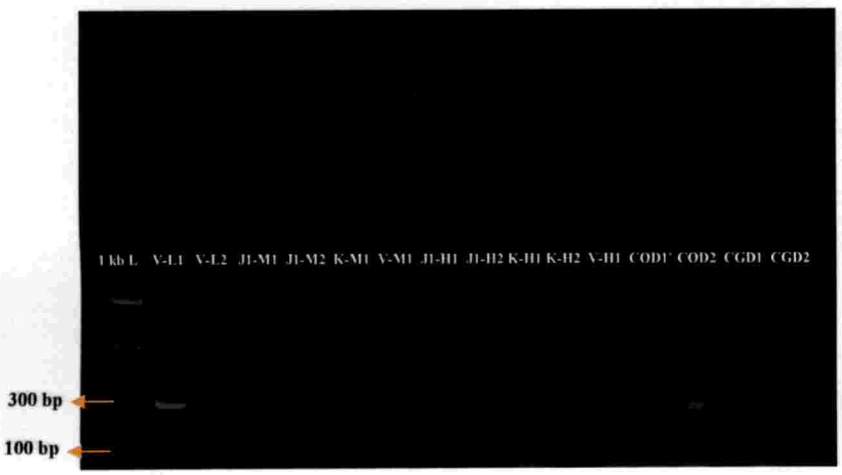
SCAR primer was tested in 11 seedlings of family IBS3-1 along with dwarf cultivars, COD and CGD. Dwarf specific band was seen in one low and two medium category seedlings *viz.,* M-L2, M-M1, C1-M2 and the dwarf cultivars (Plate 8).

4.6.2.3 Testing of SCAR marker in selected seedlings of inbred families IIS3, IIIS3 and VS3

SCAR primer was tested in 10 selected seedlings belonging to the other promising families of inbreds, *viz.,* IIS2, IIIS2 and VS2. Dwarf specific band was observed in one low and two medium seedlings *viz.,* S-L2, D-M1, T-M1 and the dwarf cultivars (Plate 9).

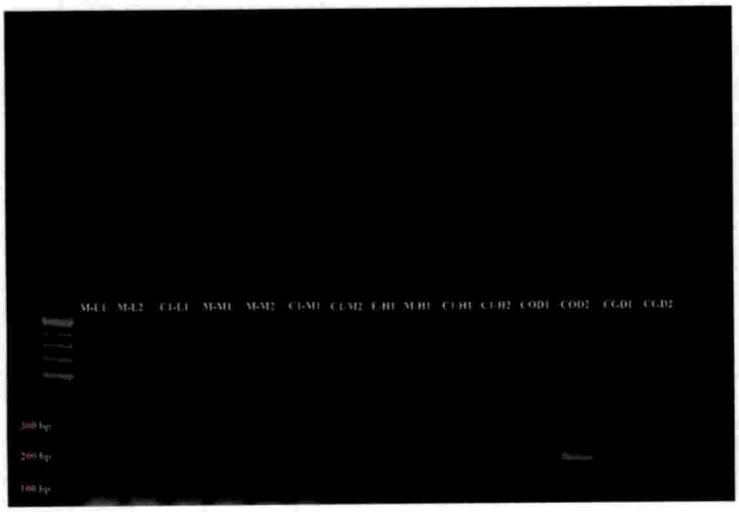
1.100 bp DNA ladder
Well no 1-7 50 °C
Well no 8-10 55 °C
Well no 11-16 58 °C
Well no 17-19 60°C

Plate 6. Testing of SCAR marker at different annealing temperature



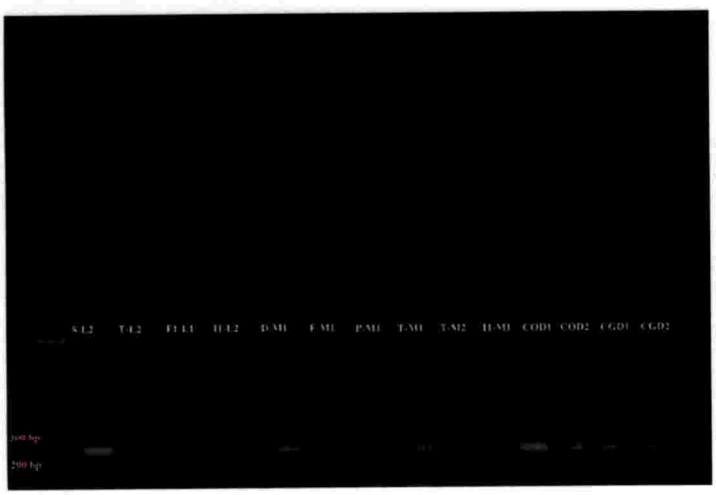
1.100 bp DNA ladder
1A/S3-1: 1.V-L1
2. V-L2
3. J1-M1
4. J1-M2
5. K-M1
6. V-M1
7. J1-H1
8. J1-H2
9. K-H1
10. K-H2
11. V-H1
11. COD1
12. COD2
13. CGD1
14. CGD2

Plate 7. Testing the S3 inbreds of WCT coconut in 1A/S3-1 family using dwarf specific SCAR marker along with COD and CGD



1.100 bp DNA ladder
1B/S3-1: 1.M-L1
2. M-L2
3. C1-L1
4. M-M1
5. M-M2
6. C1-M1
7. C1-M2
8. E-H1
9. M-H1
10. C1-H1
11. C1-H2
12. COD1
13. COD2
14. CGD1
15. CGD2

Plate 8. SCAR marker testing in 1B/S3-1 family along with COD and CGD



1.100 bp DNA ladder
1.S-L2 (II S3-1)
2. T-L2(III S3-1)
3. F1-L1(V S3-1)
4. I1-L2(III S3-1)
5. D-M1(II S3-1)
6. F-M1(IIIS 3-1)
7. P-M1(V S3-1)
8. T-M1(III S3-1)
9.T-M2(III S3-1)
10. I1-M1(III S3-1)
11. COD1
12.COD2
13. CGD1
14. CGD2

Plate 9. SCAR marker testing in IIS3-1, IIIS3-1,VS3-1 families along with COD and CGD

Discussion

5. DISCUSSION

Coconut (*Cocosnucifera* L.) palms are important subsistence and cash crop in the tropical regions of the world. In India, Kerala is having major production of coconut and the crop plays an important role in the state economy and culture by supporting millions of small and marginal farm families. The crop also serves many people in the fragile ecosystem of coastal and Island areas. Therefore, it is important to develop strategies to improve coconut crop productivity. The crop improvement in coconut palm by hybridization and selection is one of the most important objective in coconut breeding programmes. A reliable assessment of genetic relationships between coconut varieties and determination of genetic diversity present in coconut palms are pre-requisites for sustainable coconut breeding programmes (Pereraet *al.*, 2003).

Tall (*C. nucifera* L. var. *typica*) versus dwarf (*C. nucifera* L. var. *nana*) coconut ecotypes are used in most breeding programmes. This classification of coconut depends on stature and breeding habit of tall and dwarf (Menon and Pandalai, 1958). The two classes differ with respect to most of the characters such as height of the palm, colour, shape and size of the nuts as well as yield and quality of copra. Other than these types, intermediate kind of palms has also been noticed in some countries. Wide range of variation occur within the same variety in tall palms due to their cross pollinating nature while the dwarfs are comparatively homozygous in nature due to predominant self-pollination. The dwarf accessions are postulated to have evolved through inbreeding among the tall accessions (Swaminathan and Nambiar, 1961).

Breeding programmes were begun as early as 1924 to develop inbred lines in tall palms as they are highly heterozygous and to determine whether hybrid vigour occurs in crosses involving these inbreds. From 18 West Coast Tall palms of the Coconut Research Station at Kasaragod that were selfed during 1924-26, the first and second generations were planted at Pilicode during 1926 and 1961, respectively. Studies of these generations by Nair and Balakrishnan (1991)

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showed that the selfed progenies were inferior to their grandparents and sibs indicating inbreeding depression. Some of these WCT inbreds (third generation) were subjected to molecular characterisation using RAPD markers and result showed two dwarf specific RAPD bands in two of the S3 seedlings belonging to IAS_3-1and IBS_3-1 family (Chethana, 2016).

The use of biotechnology will benefit to coconut breeding, only if their application is associated with efficient conventional breeding procedures.The usefulness of the dwarf specific RAPD bands for further screening of the inbreds with dwarfing nature may be better, if they are converted to SCAR markers, as SCAR markers are reported to be powerful tools in marker-assisted selection (Kasai *et al.*, 2000). Hence the present investigation aims at developing dwarf specific SCAR markers from WCT inbreds of coconut.

The results of the research work is discussed in this chapter under the following aspects

5.1 Isolation and quantification of DNA

5.2 RAPD assay and detection of dwarf specific marker

5.3 Molecular cloning, Sequence analysis and Designing SCAR primer

5.4 Validation of SCAR primer and Screening of inbreds

5.1. ISOLATION AND QUANTIFICATION OF DNA

Genomic DNA was isolated from all the 22 seedlings belonging to two families IAS3-1 and IBS3-1 few seedlings from which showed dwarf specific bands during previous study and also from 13 dwarf cultivars (COD, CGD and MYD) AND 8 WCT cultivars. Genomic DNA was isolated from second leaf before spindle leaves of coconut as per the procedure reported (Chethana, 2016) and tested for quality and quantity. Pure, intact DNA was used for RAPD analysis.

Young tender leaves werecollected as the young tissue generally contains actively dividing cells and also contains high amount of nucleic acids as compared to older tissues. In addition to that, young tissue may have fewer metabolites which affect the performance of downstream application, this finding are in line with respect of Babu (2000).

As the RNA contamination was noticed, RNase treatment was given during the isolation procedure to avoid that. Liquid nitrogen was used during the grinding stage of DNA isolation which has functions like maintenance of frozen tissue and prevention of nucleic acid degradation and secondary metabolites oxidation and perform more disruption of the leaf tissue as reported by Hernandez and Oyarzum, 2006. Good quality DNA was obtained with a concentration of 120 (ng/µl) to 3810 (ng/µl).

5.2 RAPD ASSAY AND DETECTION OF DWARF SPECIFIC MARKER

The study was undertaken during the period from 2016-2018 in third generation (S3) selfed seedlings of coconut cultivar West Coast Tall (WCT) which were planted in 2014 at RARS Pilicode. The two families *viz.*, IAS_3-1 and IBS_3-1 in which two putative dwarf specific RAPD markers were observed by Chethana (2016) were selected for developing the SCAR marker. All the seedlings available (11 each from both families) along with the seedlings of WCT, and several dwarfs viz., COD (Chowghat Orange Dwarf), CGD (Chowghat Green Dwarf) and MYD (Malayan Yellow Dwarf) were subjected to RAPD Assay. The inbred seedlings in each family were classified into three based on height of the seedlings recorded at the time of planting itself as Low (L), Medium (M) and High (H) (Chethana, 2016).

The analysis of DNA based polymorphisms is having great importance in the construction of molecular genetic maps and identification of specific markers associated with desirable characters. RAPD developed by Williams *et al.* (1991) is still the most commonly used marker system that allows the detection of DNA sequence polymorphisms using single decamer primers of an arbitrary nucleotide sequence. The advantage of RAPD marker is that no prior knowledge about the

sequence is required for designing the primers and in a crop with limited information about DNA sequences, it provides a preliminary information about its genetic makeup.

However, for the purpose of marker development, its dominant nature and reduced repeatability poses some problems. This can be overcome by developing SCAR markers from the RAPD amplicons in which the specificity and repeatability is more as it is sequence based. Conversion of RAPD marker to SCAR marker can create more specific primers with better improvement in reliability and reproducibility of PCR assay, and to overcome the problems associated with RAPD markers and to improve their utility in marker associated selection, longer primers have been developed from RAPD fragments as reported by paran and michelmore (1993). The SCAR markers are preferred over RAPD markers as they detect only a single locus and their amplification is less sensitive to reaction conditions

Chethana (2016) reported that two primers OPAU03 and OPAW15 produced dwarf specific RAPD bands which were also present in the S3 inbreds belonging to IAS_3-1and IBS_3-1 family. RAPD analysis with the aim of identifying a specific marker in coconut was carried out by Rajesh *et al.*, (2013).Among the 200 RAPD primers initially screened with the pooled DNA from 5 tall and 5 dwarf palms, primer OPA09 was reported to produce a tall specific marker which they converted into a SCAR marker for tall character. Hence in the present investigation the genomic DNA isolated from the inbreds, dwarf and WCT seedlings were amplified using these three primers to select the amplicons for converting to SCAR marker.

Amplification of six third generation inbreds of WCT along with COD, CGD and WCT with the primer OPAU 03 produced 11 amplicons which conform to the result reported earlier (Chethana, 2016). However, since there were more number of samples the polymorphic bands were higher in the present study. A dwarf specific band of higher molecular weight was observed between 1.5kb and

2kb marker (around 1.8kb) bands which may be same as that reported earlier. However, this failed to amplify on repeating the experiment as well as when DNA from more number of dwarf and WCT seedlings were tried. This may be due to the inconsistency in amplification in RAPD experiments and such amplicons are not suitable as markers.

Amplification with OPAW15 in inbreds, dwarfs and WCT seedlings produced a 900bp amplicon in all inbreds and WCT which was also reported earlier (Chethana, 2016). But in the present study, when the experiment was repeated, the dwarf samples also produced this fragment (Plate 2a and 2b). The band of size 1200 bp which was earlier reported to be dwarf specific failed to amplify. Limited reproducibility of RAPD assay was reported earlier. Many factors can influence the reproducibility of RAPD reactions like quantity of template DNA, PCR buffer, magnesium chloride concentration, annealing temperature, Taq DNA polymerase and thermal cycler (Kundan*et al.*, 2014). Many technical disadvantages associated with RAPD were, however, raised question on its fidelity as genetic marker technique and prevented its wide spread use. The reproducibility of RAPD was adversely affected by, primer and template concentration, quality of DNA, different thermo cyclers and even different source of DNA polymerase (Ellsworth et al. 1993, Muralidharan and Wakeland., 1993).

However, in the present study a 320bp amplicon was found prominently in CGD and with lesser intensity in COD and MYD and was absent in WCT. In earlier study also this was very specifically found in CGD but with lesser intensity in COD and was absent in WCT. In repeated experiments also this amplicon was consistent. Hence this was finally selected for further cloning and sequencing to design the SCAR primer. Mitra (2010) also reported a low molecular weight fragment (420bp) in date palm (*Phoenix dactylifera L.*), which when converted to SCAR marker was found efficient and accurate in identification of sex of date palms.

Rajesh *et al.* (2013) had identified a tall specific RADP marker (260 bp) in

coconut using two samples of bulked DNA, one from five tall palms and second from five dwarf palms. After screening with 200 decamer primers, primer OPA09 revealed a unique band of around 260 bp exclusively in tall accessions. The RAPD marker OPA09 was successfully converted into a SCAR marker, which was further validated using individual samples of tall and dwarf accessions of different geographical origins. In the present study, the primer OPA09 was also included to verify the procedure followed. It produced three monomorphic and two polymorphic bands but failed to amplify any tall specific band. An amplicon of around 250 – 260 bp size was found in WCT seedlings which may be the one reported by Rajesh *et al.* (2013) but it was found in one of the COD sample also. By using more stringent conditions, the repeatability might be obtained. However, since this was a tall specific marker, this was not used for cloning and sequencing.

5.3 MOLECULAR CLONING, SEQUENCE ANALYSIS AND DESIGNING SCAR PRIMER

The 320 bp dwarf specific band observed was eluted, ligated to the vector pTZ57R/T, cloned to DH5α and sequenced for designing SCAR marker. After verifying the presence of desired DNA insert, the recombinant DNA was send for sequencing. The sequence generated were compared with the sequences available in NCBI database and the maximum homology search was done using BLASTn. The data shows that DNA sequence of polymorphic band has 86 percent similarity with the chromosome 2 of crop *Solanumlycopersicum.* But no other close similarity was seen for the DNA sequence from other plant species and no specific characters could be assigned to the fragment. Based on the sequence information 21 base pair (forward and backward) long SCAR primers were designed.

5.4 VALIDATION OF SCAR MARKER

SCAR markers, by being both accurate and cost efficient, offer the most practical method for screening numerous samples in a time and labor-saving manner. Therefore, these SCAR markers are powerful tools in marker-assisted selection (Kasai *et al.*, 2000).

One year old S3 seedlings were classified based on seedling height during transplantation into field as Low(50-100 cm), Medium(101-150), and High (151-200). Screening third generation seedlings of WCT using the newly designed SCAR primers shows amplifications in four low and eight medium category seedlings along with all the dwarf seedlings (COD and CGD).

SCAR assay in IAS3-1 family shows dwarf specific amplicons in six S3 seedlings which include two 'low' and four 'medium' and no amplicon was seen in the five 'high' category seedlings (Plate 7). This indicate that the selection of the one year old seedlings at the time of planting could be effective in identifying the inbred seedlings with dwarfing nature. When the screening was done with 11 seedlings belonging to IBS3-1 family, dwarf specific amplicon was seen in one 'low' and two 'medium' seedlings and here also it was absent in all the four seedlings which were classified as 'high' with respect to height. However, three 'low' and two' medium' also failed to amplify the marker. This emphasize the importance of selection based on morphological characters supplemented with molecular marker to get more accuracy in selection programmes.

Based on this result, only 'low' and 'medium' type seedlings were selected from the remaining three inbred families viz., IIS3, IIIS3 and VS3. Out of four 'low' and six 'medium' category seedlings, the marker was present only in one 'low' and two 'medium' types.

To conclude, the screening of S3 inbred seedlings of coconut using primer OPAW15 was successful in selectively amplifying a band of around 320 bp size in dwarf cultivars CGD, COD, and MYD and it was absent in WCT. Hence a

SCAR marker was developed based on the sequence of the specific band eluted. The validation was done using DNA from Tall, dwarf and S3 seedlings from different S3 families. The marker was found to support the classification of S3 seedlings based on their height reported by Chethana (2016) as the marker was absent in all the seedlings with height above 150 cm. However, the low and medium seedlings (height vary from 50-150 cm) showed polymorphism with the marker. This support the fact that classification based on morphological and molecular marker is more effective than classification based on morphology alone.

The dwarf specific SCAR marker developed in the present study after validation using other tall and dwarf cultivars, along with the earlier reported tall specific SCAR marker (Rajesh *et al.*) can be successfully employed in breeding experiments for marker assisted selection. The SCAR markers which distinguish tall and dwarf coconut cultivars can be used in screening seedlings at an early stage which would be of immense importance to nurseries and growers while establishing coconut plantations.

Summary

6. SUMMARY

The study on "Developing dwarf specific SCAR markers from WCT inbreds of coconut (*Cocosnucifera* L.)" was carried out in the departments of Plant Biotechnology, College of Agriculture, Padannakkad and College of Horticulture, Vellanikkara during the period 2016-2018 with an objective of developing SCAR markers from dwarf specific RAPD marker for screening superior inbreds with dwarfing nature.

DNA samples were extracted from total 54 genotypes, 22 seedlings belonging to the inbred families IAS3-1and IBS3-1, 10 seedlings from other three families *viz,* IIS3, IIIS3 and VS3, 14 dwarf seedlings (including COD, CGD and MYD) and 8 tall seedlings (WCT) using modified CTAB method. Extracted DNA was analysed in 0.8 % agarose and the quantity of DNA ranged from 120 (ng/µl) to 3810 (ng/µl).

These samples were subjected to RAPD analysis using the earlier reported primers OPAU03, OPAW15 (Chethana, 2016) and OPA09 (Rajesh *et al*, 2013). OPAU03 showed polymorphism between tall and dwarfs, but the bands lacked repeatability and was not further taken for SCAR marker development. OPA09, which was reported earlier to produce a tall specific band confirmed the result in the present study also. Primer OPAW15 successfully amplified a polymorphic band of size around 320 base pair specifically in dwarf seedlings and it was not present in the tall cultivar (WCT).

The dwarf specific band was eluted using PCR clean up Gel extraction kit (Macherey- Nagel) according to the manufacturer's protocol and the concentration after elution was estimated as 112 ng/µl. This was cloned into the vector pTZ57R/T using Thermo scientific insTAclone PCR cloning kit. Transformation was done by using *E. coli*laboratory strain *DH5 alpha.* Transformation was confirmed by doing colony PCR as the dwarf specific band of 320 bp was seen in all the five white colonies. The plasmid DNA was isolated using PureLink quick DNA Miniprep Kit, Invitrogen and send for sequencing.

Sequencing result was analysed after removing the plasmid vector portion by Vecscreen tool provided by NCBI. The sequence after vector screening was subjected to homology search using BLASTn. The DNA sequence of polymorphic band showed similarity with chromosome 2 of *Solanumlycopersicum*. Based on the sequence, one pair of SCAR primer was designed. Different annealing temperature were tested for standardizing the annealing temperature. Amplification of the dwarf specific band was clearly observed at an annealing temperature of 60°C and was selected for SCAR marker testing in S3 inbred families.

SCAR primer was tested in 11 seedlings of family IAS3-1 along with dwarf cultivars, COD and CGD. Dwarf specific band of size 312 bp was seen in 6 seedlings viz., V-L1, V-L2, J1-M1, J1-M2, K-M1, V-M1 which comes under 'low' and 'medium' category (Classified based on the height of seedling at the time of planting) and in 4 dwarf cultivars and was absent in seedlings classified as 'high' with respect to height. SCAR primer was then tested in 11 seedlings of family IBS3-1 along with dwarf cultivars, COD and CGD. Dwarf specific band was seen in one low and two medium category seedlings *viz.,* M-L2, M-M1, C1-M2 and the dwarf cultivars Here also it was absent in 'high' category seedlings. When testing with SCAR primer was done in 10 selected seedlings belonging to the other promising families of inbreds, viz., IIS3, IIIS3 and VS3, dwarf specific band was observed in one low and two medium seedlings *viz.,* S-L2, D-M1, T-M1 and the dwarf cultivars.

The result showed that the dwarf specific marker is present in S3 inbreds belonging to 'Low' and 'Medium' types but absent in all those seedlings classified as 'High' based on height.This shows that the classification based on height in the one-year-old seedlings is effective to some extent. However, some of the seedlings in 'Low' and 'Medium' category lack the marker, which indicate that a classification based on morphological and molecular marker is more effective than classification based on morphology alone.

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DEVELOPING DWARF SPECIFIC SCAR MARKERS FROM WCT INBREDS OF COCONUT (*Cocos nucifera* L.)

By

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(2016-11-127)

ABSTRACT OF THE THESIS

Submitted in partial fulfillment of the requirement for the degree of

Master of Science in Agriculture

Faculty of Agriculture

Kerala Agricultural University, Thrissur

DEPARTMENT OF PLANT BIOTECHNOLOGY

COLLEGE OF HORTICULTURE

VELLANIKKARA, THRISSUR – 680656

KERALA, INDIA

2018

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Abstract

Coconut is an important subsistence and cash crop in the humid tropical regions of the world. The coconut palm is often called "the tree of life" because of its numerous uses as food, drink, fuel, building materials and so on. Coconut cultivars are mainly classified into two: tall and dwarf. West Coast Tall (WCT) is the most common and superior tall cultivar extensively cultivated all over the west coast region, especially in Kerala. One of the main methods of crop improvement in coconut is hybridization between tall and dwarf types. However the heterozygous nature of tall types is a hurdle in selection of mother palms and seedlings. Efforts to develop homozygous inbreds of WCT were started as early as 1924.

Chethana (2016) studied the extent of inbreeding depression in second generation (S2) inbred palms and recorded morphological observations of one year old third generation (S3) inbred seedlings. Based on height, she classified the S3 seedlings into Low, Medium and High, before planting in the field. She also carried out the characterization of selected third generation (S3) inbreds using RAPD marker system along with tall (WCT) and dwarf palms (COD, CGD). The results revealed two dwarf specific RAPD bands in two of the S3 seedlings belonging to IAS3-1 and IBS3-1 family, when amplified with primers OPAU03 and OPAW15. However, as RAPD markers have low specificity and sometimes lack repeatability, converting them into Sequence Characterized Amplified Region (SCAR) markers is more advantageous. A tall specific SCAR marker was reported in coconut using primer OPA 09 (Rajesh *et al.*, 2013).

The present investigation was carried out in the departments of Plant Biotechnology, College of Agriculture, Padannakkad and College of Horticulture, Vellanikkara during the period 2016-2018 was undertaken with an objective of developing SCAR markers from dwarf specific RAPD marker for screening superior inbreds with dwarfing nature.

DNA samples were extracted from total 54 genotypes, 22 seedlings belonging to the inbred families IAS3-1and IBS3-1, 10 seedlings from other three families *viz,* IIS3, IIIS3 and VS3, 14 dwarf seedlings (including COD, CGD and MYD) and 8 tall seedlings (WCT). These were subjected to RAPD analysis using the 3 reported primers *viz*, OPAU 03, OPAW 15 and OPA 09. Though OPAU03 showed polymorphism between tall and dwarfs, the bands lacked repeatability and hence this was not included in further analysis. OPA 09, which was reported to produce a tall specific band (Rajesh *et al.*, 2013), confirmed the result in the present study also. Primer OPAW15 was successful in amplifying a polymorphic band of size around 320 base pair specifically in dwarf seedlings and it was not present in the tall cultivar (WCT).

The polymorphic dwarf specific band produced by primer OPAW 15 was eluted and cloned in pTZ57R/T vector and transformed into *E. coli* DH5 alpha cells. Cloned cells were subjected to blue white screening and transformed ones were selected, the plasmid was isolated and sequenced. The sequence after vector screening was subjected to homology search using BLASTn. The DNA sequence of polymorphic band showed similarity with chromosome 2 of *Solanum lycopersicum*. Based on the sequence, one pair of SCAR primer was designed and tested for dwarf specific band. Dwarf specific amplicon was found in all the dwarfs but absent in the tall WCT seedlings. Hence, this was used to screen the S3 inbred seedlings which were classified as Low, Medium, and High based on their height at the time of planting, to confirm the effectiveness of this classification based on morphology.

The result showed that the dwarf specific marker is present in S3 inbreds belonging to Low and Medium types but absent in all those seedlings classified as 'High' based on height. This shows that the classification based on height in the one year old seedlings is effective to some extent. However, some of the seedlings in 'Low' and 'Medium' category lack the marker, which indicate that a classification based on morphological and molecular marker is more effective than classification based on morphology alone.

The dwarf specific SCAR marker developed in the present study after validation using other tall and dwarf cultivars, along with the earlier reported tall specific SCAR marker can be successfully employed in breeding experiments for marker assisted selection. The SCAR markers which distinguish tall and dwarf coconut cultivars can be used in screening seedlings at an early stage which would be of immense importance to nurseries and growers while establishing coconut plantations.