DEVELOPMENT OF SCAR MARKER FOR AUTHENTICATION OF GENDER IN KODAMPULI

(*Garcinia gummi-gutta* var. *gummigutta*)

By

SHELKE SUNIL MAROTARAO

(2008-11-114)

KERALA AGRICULTURAL UNIVERSITY
CENTRAL LIBRARY
P.O.), THRISSUR

THESIS

Submitted in partial fulfilment of the requirement for the degree of

Master of Science in Agriculture

(PLANT BIOTECHNOLOGY)

Faculty of Agriculture

Kerala Agricultural University, Thrissur

Centre for Plant Biotechnology and Molecular Biology

COLLEGE OF HORTICULTURE

VELLANIKKARA, THRISSUR - 680 656

KERALA, INDIA

2010

DECLARATION

I, **Shelke Sunil Marotarao** (2008-11-114) hereby declare that this thesis entitled **"Development of SCAR marker for authentication of gender in Kodampuli (*Garcinia gummi-gutta var. gummigutta*)"** is a bonafide record of research work done by me during the course of research and this thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title of any other University or Society.

Vellanikkara
Date: 01-10-2010

Shelke Sunil Marotarao
2008-11-114

Dr. P.C. Rajendran
Professor and Head
Department of Plantation crops and Spices
College of Horticulture
Kerala Agricultural University
Thrissur, Kerala

Date 01.10.2010

CERTIFICATE

Certified that this thesis, entitled **"Development of SCAR marker for authentication of gender in Kodampuli (*Garcinia gummi-gutta var. gummigutta*)"** is a record of research work done independently by **Mr. Shelke Sunil Marotarao** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to him.

Vellanikkara

Dr. P.C. Rajendran
Major Advisor,
Advisory Committee

CERTIFICATE

We, the undersigned members of the advisory committee of **Mr. Shelke Sunil Marotarao,** a candidate for the degree of **Master of Science in Agriculture,** with major field in **Plant Biotechnology,** agree that the thesis entitled **"Development of SCAR marker for authentication of gender in Kodampuli (*Garcinia gummi-gutta var. gummigutta*)"** may be submitted by him in partial fulfilment of the requirement for the degree.

Dr. P.C. Rajendran
(Major advisor)
Professor and Head
Department of Plantation crops and spices
College of Horticulture
Vellanikkara

Dr. P.A. Nazeem
(Member, Advisory committee)
Professor and Head
Centre for Plant Biotechnology and
Molecular Biology (CPBMB)
College of Horticulture
Vellanikkara

Dr. D. Girija
(Member, Advisory committee)
Professor
Centre for Plant Biotechnology and
Molecular Biology (CPBMB)
College of Horticulture
Vellanikkara

Dr. T. V. Aravindakshan
(Member, Advisory committee)
Professor
Centre for Advanced Studies in Animal
Genetics & Breeding, College of Veterinary
& Animal Sciences, Mannuthy

E.E.

Dr. C.R. Raju
Principal Scientist,
CPCRI (RS), Kayamkulam

ACKNOWLEDGEMENT

First and foremost I bow my head before my ***Aie-Papa*** *who enabled me to successfully complete the thesis work.*

With immense pleasure I avail this opportunity to express my deep sense of gratitude and indebtedness to my major advisor ***Dr. P.C. Rajendran****, Professor Department of Plantation crops and spices , Chairperson of my Advisory Committee for his valuable guidance, constructive suggestions, unfailing patience, friendly approach, timely help at various stages of my work.*

I would like to express my extreme indebtedness and obligation to ***Dr. P. A. Nazeem,*** *Professor and Head, Centre for Plant Biotechnology and Molecular Biology, College of Horticulture and member of my Advisory Committee for her meticulous help, unwavering encouragement, forbearance, affectionate advice, well timed support and critical scrutiny of the manuscript which has helped a lot for the improvement and preparation of the thesis. She had been a support during each step of the way and I owe a deep sense of gratitude to her.*

I sincerely thank to ***Dr. D. Girija,*** *Professor, Centre for Plant Biotechnology and Molecular Biology, College of Horticulture and member of my Advisory Committee for his expert advice, constant inspiration, meticulous help and constructive criticisms rendered through out the study period.*

I express my heartiest gratitude to ***Dr. T. V. Arvindakshan,*** *Professor, Centre for Advance studies for Animal Genetics & Breeding, College of Veterinary & Animal Sciences, Mannuthy and member of my Advisory Committee for his ever willing help, valuable guidance and creative suggestions and above all good understanding during the period of my work.*

I am obliged to ***Valsala madam, Keshavachandran sir and Sujata madam*** *from Centre for Plant Biotechnology and Molecular Biology whose moral support have helped to complete this venture successfully.*

I thank Mr. ***P. K. Sreekumar*** *, Farm Assistant, CPBMB, for the help in photography.*

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

I express my deep sense of gratitude to ***Shareen chechi, Anju chechi, Simi chechi, Shailaja chechi, Firoz chettan, Praveena chechi, Sunil chetan****, of CPBMB, who helped me in several ways for the completion of this venture.*

I take this opportunity to thank my all ***seniors Dinesh, Rahul, Kiran, Shivaji, Devi, Jaba, Mani, Renu, Rashmi, Sathish, Asha, Sindhu, Jusna, Thiagrajan, Sweta, Shrividya, Niharika and Tangasuja*** *for their prompt help and cooperation for the entire period of study.*

I am in dearth of words to thank ***my friends Gajanan, Prathmesh, Arya, Lakshmy, Sabath, Tamari, Sumalata, Ranjeet, Seejani, Sudha*** *and* ***Navaneet*** *who stood by me during all the hard times.*

I wish to express my thanks to ***my loving juniors Rakesh, Ashok, Pradeep, Sarveshver, Saumya, Sahana and Lubina*** *of P. G & U. G from college of Horticulture, Forestry & Veterinary Science.*

Words cannot express the help that I relished from ***Research scholars*** *especially* ***Saifudin, Twinkal, Tom*** *and others from Centre for Plant Biotechnology and Molecular Biology for the heartfelt help and support rendered through out the study period.*

I wish to express my gratitude to all the ***chechy*** *especially to Lylla and priya chechy from Centre for Plant Biotechnology and Molecular Biology for their wholehearted co-operation and help during various stages of my work.*

The award of JNU fellowship is greatly acknowledged.

My deepest gratitude goes to my family for their unflagging love and support throughout my life.

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.

Shelke Sunil Marotarao


Dedicated to
Aie-Papa
and Friends

ABBREVIATIONS

β	Beta
bp	Base pair
BLAST	Basic Local Alignment Search Tool
CPBMB	Centre for Plant Biotechnology and Molecular Biology
°C	Degree Celsius
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribo Nucleoside Triphosphate
DIC	Distributed Information Centre
ds	Double stranded
EDTA	Ethylene diamine tetra acetic acid
pH	Hydrogen ion concentration
IPTG	Isopropyl thio galactoside
Kb	Kilo base
LB	Luria Bertani
L	Litre
µg	Micro gram
µl	Micro Litre
ml	Milli litre
mM	Milli Molar
M	Molar
MAS	Marker Assisted Selecction
NCBI	National Centre for Biotechnology information
ng	Nano gram
nm	Nano meter
OD	Optical density
ORF	Open Reading Frame
%	Percentage
PCR	Polymerase Chain Reaction
rpm	revolutions per minute
RNA	Ribonucleic acid

RNase	**Ribonuclease**
TAE	**Tris Acetate EDTA**
UV	**Ultra violet**
UTR	**Untranslated Region**
v/v	**volume/volume**
X-gal	**5bromo-4-chloro-3-indoyl-β-D-galactosidase**

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF ANNEXURES

Introduction

1. INTRODUCTION

Garcinia gummigutta var. *gummigutta* (L.) Rob., popularly known as Kodampuli or Malabar Tamarind, belonging to the family Clusiaceae, is an acidic fruit bearing tree. It occupies the backyard of Kerala's homesteads more often as one among the miscellaneous tree crops. It is a native of Malaysia and Western Ghats of Kerala. Garcinia consists of about 434 species with more than 30 edible species. It is one of the slow growing and late bearing tree of tropics. *Garcinia gummigutt*a is an evergreen androdioecious tall growing tree, with round canopy commonly seen in the Western Ghats upto 180m in Nilgiris.

Kodampuli fruit rind finds unique use in Kerala cuisine being exclusively preferred in culinary preparation involving fish. Kodampuli rind is the richest natural source of Hydroxy citric acid (HCA) and its derivatives are unique, potent metabolic regulator of obesity (Varghese, 1996). Being rich in acid, the rind possesses marked antiseptic properties. It is listed as remedy against rheumatism, rickets and enlargement of spleen and also in veterinary medicine as a rinse for disease in cattle.

Being cross pollinated and polygamodioecious in nature, segregation of seedling progenies is recognized as one of the major problems in the cultivation of Garcinia. The seedling progenies segregate into males and females. Even though vegetative propagation is standardised (Nazeema, 1992) to overcome sex determination, large scale use of this method is constrained, due to the non availability of sufficient number of orthotrops, which are required for the production of plants with normal growth habit. Hence seedling continues to be the major propagule for commercial cultivation.

Some difference between male and female with respect to growth rate, colour of the emerging flushes and the total phenol content were identified in mature tree (Muthulakshmi, 1998). Fruit and seed set through different mode of pollination is reported in Kodampuli (Sherly, 1994). But these evidences are not enough to make sure that the present field plants are male or female.

Garcinia is dioecious and segregation of sex of seedling progenies into productive females and unproductive trees. This is another main problem in its cultivation. The sex of the tree can be identified only after 7-8 years of planting.

Commercial cultivation of this crop in India is handicapped due to various reasons mentioned. The need of the hour is to develop a definite methodology for identification of sex of the plant in early stage of life cycle. With this background the present study was undertaken to establish a simple and reliable gender-diagnostic system for Kodampuli by using PCR based techniques. Therefore, in this investigation, efforts were taken for development of PCR based technique which can identify sex of plant at early stage of lifecycle. The main objectives of the study were the following:

1. Screening of RAPD primers for sex specific polymorphism.
2. Conversion of RAPD markers into SCAR marker by designing longer primers.
3. Confirming its use as marker in distinguishing male and female genotypes.

KERALA AGRICULTURAL UNIVERSITY
CENTRAL
LIBRARY
K.A.U.P.O., THRISSUR

Review of Literature

2. REVIEW OF LITERATURE

Kodampuli *(Garcinia gummi-gutta* var. *gummigutta* L. Rob.) is a potential tree spice. It is indigenous to South Asia especially, Thailand and India, abundant in Western Srilanka and Malaysia and common in evergreen forest of Western Ghats towards to Trivandrum. The fleshy fruit rind of Kodampuli is the richest natural source of anti-obesity metabolite Hydroxy Citric Acid (HCA). HCA inhibit the conversion of carbohydrate to fats without affecting Kreb's cycle through an enzyme ATP citrate lyase. Since Kodampuli is androdioecious tree, it takes 8-12 years to identify the female trees. Early sex identification, lack of orthotropic shoot for grafting, prolonged seed dormancy of 7-8 months, poor seed germination and lack of awareness of its pharmaceutical significance are hindering the extensive cultivation of this backyard companion crop in Kerala as well as to other coastal regions of the country.

Botany and Flowering:

Kodampuli is an evergreen medium size tree with oval shaped crown having orthotropic and plagiotropic branches. Sherly (1994) noticed that shoot growth in Garcinia was seasonal with one main flushing period from January to May. However, the scattered flushing occurs throughout the year. The spice Kodampuli is polygamodioecious in nature. Two types of trees are observed by George *et al.* (1992). One type was functionally male bearing only staminate flower while the other was functionally female with hermaphrodite flower. Muthulakshmi (1998) observed that male trees have more number of flowers per cluster, higher petiole length, more stamens per flower, higher pollen viability and germination. Flowers in bisexual trees have higher flower weight, petals and ovary size.

In both male and bisexual trees, the season of flowering from the end of January up to March. The flower buds are born mostly on past season's growth and early on current season shoot. In both the tree types, flowers are seen at the shoot tips as well as in leaf axils. In a male tree, the flowers are borne in clusters

of 10 to 30 in number at the leaf axils and the shoot tips. In hermaphrodite trees, the solitary flowers are commonly seen in cluster of 3 to 5 at shoot tips. Sequence of flower opening was similar that to cymose inflorescence with central bud opening first followed by the one at the two sides. Both male and bisexual flowers have four sepals and petals each arranged imbricately. In male flowers, numerous two celled anthers are seen on short filament. The bisexual flowers have 6-10 celled with 6-10 lobbed stigmas.

Garcinia pollens appeared as yellowish powder. Acetocarmine test recorded 56 per cent pollen viability in male and 28 per cent in bisexual flowers. When bisexual flowers were bagged 2-3 days prior to anthesis, it was observed that the percentage of set in selfed bag was only 20 percent. One of the reasons for poor set in bagged bisexual flower was the low pollen viability of bisexual flowers. Hence the male tree is indispensable for better fruit set in Garcinia.

Muthulakshmi (1992) observed wide variation in tree character like shape of the tree and branching pattern. Early flowering, late flowering and sporadic flowering type were identified in this crop.

Reports on molecular work in Kodampuli are scanty. Premgith and Rajendran (2008) reported screening of thirty five RAPD primers among which three primers *viz.* Kit C1, Kit C8 and Kit C9 was given male specific polymorphism. Literature on various perennial bisexual and dioecious crops is reviewed for molecular markers used for sex authentication and analysis is as follows.

2.1 Sex differentiation in plants

Charlesworth (2002) broadly classified the flowering plants into sexually monomorphic and sexually polymorphic depending upon the sex and gender systems of sexually reproducing flowering plants. The sexually monomorphic group includes the hermaphrodites (both sexual characters in same flower), monoecious (both sexual characters on same plant), gynomonoecious (male sterility) and andromonoecious (female sterility) plants. The sexually polymorphic

group consists of dioecious (both sexual characters on different plants), gynodioecious (either hermaphrodite or female) and androdioecious (either hermaphrodite or male) plants. According to Renner and Ricklefs (1995), the male and female sexual system in animal kingdom is a common phenomenon but in the plant kingdom most of the flowering plants are hermaphrodites. Only 6% of angiosperms are dioecious where individuals either bear staminate or pistillate flowers. Charlesworth (2004) points out that the plant sex chromosome evolution has taken place very recently and this accounts for very few percentages of the dioecious flowering plants. Many dioecious species with hermaphrodite relatives were evidently noticed rudiments of opposite sex structures in flowers of either sex which suggests the recent evolution of unisexual flowers (Darwin, 1877).

Lewis (1942) has described a model for evolution of dioecy from hermaphrodite ancestors; in which he suggested the occurrence of two simultaneous but independent mutations, one which results in the male or female sterility and the other suppressing the recombination between these mutations, which helps in the formation of the hermaphrodites. Llyod (1975) and Charlesworth (1985) supports the hypothesis for dioecy evolution in plants put forwarded by Lewis (1942) and described that the evolution for separate sexes in plants have begun from co-sexuality (individual plants with both sex functions), and it requires at least two mutations which will be gradual.

Anisworth (2000) had put forward a more reasonable hypothesis for the evolution of dioecy; in which he describes that a single mutation have caused either gynodioecy or androdioecy and it was intermediate step between the hermaphrodite and dioecious plants.

According to Parker (1990) mechanism of sexual differentiation in plants is almost overwhelmingly diverse. Interactions between genetic loci and plant growth hormones have been proposed for sexual differentiation in some monoecious and very few dioecious plants. Irish and Nelson (1989) given that, maleness and femaleness appear to be achieved through suppression of the

differentiation programme for opposite sex and the sex determination acts through genes regulating the relative activities of male and female differentiation pathways possibly using the growth regulators as messengers.

2.2 Sex determination in plants

Irish and Nelson (1989) used the secondary characters associated with the two different types of inflorescences; including the branching patterns, glume morphology and the extent of elongation in internodes subtending the inflorescence in maize to distinguish between the sexes. By the use of molecular markers for the study of sex determination, many X and Y linked markers have been discovered in plants with or without heteromorphic sex chromosomes (Testolin *et al.*, 1995; Harvey *et al.*, 1997; Mandolino *et al.*, 1999).

Anisworth *et al.* (1995) used the northern hybridization to determine the MADS box gene expression during flower development in hops and to correlate the gene expression data with the sex of the plant. Charlesworth (2002) suggests that the sex determination based on chromosome morphology is not functional in most of the plants since they are having homomorphic sex chromosomes and the understanding of the evolution of plant sex chromosomes, sex determination should be advanced by the use of molecular markers.

2.3 Molecular markers

DNA based molecular markers have acted as versatile tools and have found their own position in various fields like taxonomy, physiology, embryology, genetic engineering, sex determination, characterization, varietal identification, clonal fidelity testing, assessment of genetic diversity, validation of genetic relationship, phylogenetic and evolutionary studies, marker assisted selection, mapping of mutations, characterization of transformants, population genetics, molecular taxonomy and evolution, forensic analysis, estimation of genomic size etc. Based on the specific requirement, different types of marker system detecting polymorphisms/variability in different regions of DNA evolving at different rates have been used. According to Joshi *et al.* (1999) genetic

polymorphism can be defined as the simultaneous occurrence of a trait in the same population of two or more discontinuous variants or genotypes DNA based markers mostly in use include Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Variable Number of Tandem Repeats or Short Sequence Repeats (SSR) or micro satellite inter simple sequence repeat (ISSR) etc. The discovery of PCR was a landmark in the effort and proved to be a unique process that brought about a new class of DNA profiling markers.

Each marker system is characterized by a unique combination of advantages and disadvantages and the choice of marker system dictated to a significant extent by application. This facilitated the development of marker based gene tags, *amp* based cloning of agronomically interested genes, variability studies, phylogenetic analysis etc.

2.4 Restriction Fragment Length Polymorphism (RFLP)

RFLP markers were used for the first time in the construction of genetic map. RFLPs being co-dominant markers can detect coupling phase of DNA molecules, as DNA fragments from all homologous chromosomes are detected. However, their utility has been hampered due to the large amount of DNA required for restriction digestion and southern blotting. They have their origin in the DNA rearrangements that occured due to evolutionary process, point mutation within the restriction enzyme recognition site sequences, insertion or deletions within the fragments and unequal crossing over. Restriction enzymes are useful when it used with AFLP technique.

2.5 Sequence Tagged Sites (STS)

RFLP probes specifically linked to a desired trait can be converted into PCR based STS markers based on nucleotide sequence of the probe. Using this technique, tedious hybridization procedures involved in RFLP analysis can be overcome. This approach is extremely useful for studying the relationship

between various species (Parrish *et al.* 1999) when these markers are linked to some specific traits.

2.6 Amplified Fragment Length Polymorphism (AFLP)

AFLP is a technique based on the detection of genomic restriction fragments by PCR amplification. The fingerprints are produced, without any prior knowledge of sequence, using a limited set of generic primers. This technique thus shows an ingenious combination of RFLP and PCR techniques (Saiki *et al.* 1998) and is extremely useful in detection of polymorphism between closely related genotypes.

Renganayaki *et al.* (2005) reported the construction of AFLP based linkage map for both the maternal and parental plants used to develop a mapping population of Texas bluegrass. This marker provide a preliminary tool for studying sex determination and a framework for further characterization of the genomic region conferring dioecy in Texas bluegrass.

2.7 Variable Number Tandem Repeats (VNTR)

This is one of the powerful techniques for studying diversity utilizing hyper variable regions of the gene comprised of tandemly repeated simple sequences. This repeats vary in number, therefore generally called VNTRs. It identifies repeated DNA regions of differing lengths resulting from variable numbers of serial repeats of a core DNA sequence. These core sequences are referred to as mini-satellites or micro-satellites. Parasnis *et al.* (1999) develop microsatellite (GATA)*n* which reveals sex-specific differences in papaya.

2.8 Random Amplified Polymorphic DNA (RAPD)

In 1991, Welsh and McClelland developed a new PCR based genetic assay namely randomly amplified polymorphic DNA (RAPD). This procedure detects nucleotide sequence polymorphisms in DNA by using a single primer of arbitrary nucleotide sequence. In reaction, a single species of primer anneals to the genomic DNA at two different sites on complementary strands of DNA template. If these

priming sites are within an amplifiable range of each other, a discrete DNA product is formed through PCR amplification. On an average, each primer directs amplification of several discrete loci in the genome, making the assay useful for efficient screening of nucleotide sequence polymorphism between individual. They are dominant markers and hence have limitations in their use as markers for mapping, which can be overcome to some extent by selecting those markers that are linked in coupling (Williams *et al.* 1993). The application of RAPDs and their related modified markers in variability analysis and individual specific genotyping has largely been carried out, but is less popular due to problems such as poor reproducibility faint or fuzzy products and difficult in scoring bands, which lead to inappropriate inference.

2.8.1 RAPD in plant genome analysis

Jeppsson (1987) used molecular markers (RAPDs– Random Amplified Polymorphic DNA) to survey genetic variation in natural populations of *Hippophae rhamnoides* ssp. *rhamnoides*. He found 85 per cent of the molecular variation within the populations whereas the remaining 15 per cent were found among the populations.

Orozco *et al.* (1994) developed RAPD marker to detect the genetic polymorphism between 3 coffee species (*Coffee arabica, C. canephor* and *C. liberica*) and used to construct dendrograms and these was consistent with the known history and evolution of *Coffee arabica.*

Chen *et al.* (1998) studied morphological and genetic variations in somaclones of Phalaenopsis. Thirty eight selected random primers were used to generate amplified segments of genomic DNA and to differentiate polymorphisms of somaclonal variations. The random amplified polymorphic DNA data indicated that normal and variant somaclones are not genetically identical. Chen *et al.* (1998) evaluated the identification of microspore-derived plants in anther culture of flax *(Linum usitatissimum)* using molecular marker. It was determined using Inter Simple Sequence Repeats (ISSR) and RAPD markers. One ISSR and two

RAPD primers were identified. Both markers were inherited from the male parent but one showed X linked and the other a Y linked type of inheritance.

Hormaza *et al.* (1998) used RAPD markers to examine patterns of relatedness among twenty known *Pistacia vera* L. cultivars and accessions. The result showed high levels of polymorphism in the species. Komaki *et al.* (1998) developed morphological and RAPD pattern variation in Sweet potato and its closely related species. The result suggested that sweet potato and *L. trifida* are very closely related to each other and could be considered as a single taxon. Kumar *et al.* (1998) used RAPD technique and developed the data that can be used distinguished between the species and cultivars of *Heliconia.* Suk *et al.* (1998) used RAPD marker to detect genetic variation and diversity among 52 Melon *(Cucumis* lines). Among a total of 123 bands from 12 random primers, 25 polymorphic bands were selected as reliable markers.

Fourteen DNA samples, 2 from cultivated *Oryza sativa* accessions and 12 from wild *Oryza* accessions were amplified for polymorphic DNA analysis with 10 random primers by Wu *et al.* (1998). The genetic similarity coefficient was evaluated by (UPGMA) cluster analysis. Ahmad (1999) used RAPD markers to distinguish between 9 different *Cicer* species representing the cultivated chickpea and 8 other related annual wild species. Forty five 10-mer primers tested of which only 8 amplified genomic DNA across the entire species. Species diagnostic amplification product specific to all the 9 species was identified. AI-Zahim *et al.* (1999) detected somaclonal variation in *garlic (Allium sativum. L)* using RAPD and cytological analysis. Plants were regenerated by somatic embryogenesis from long-term callus cultures which were derived from five garlic cultivars. Thirty five of these plants were subjected to RAPD analysis.

Banerjee *et al.* (1999) developed male sex associated RAPD markers in *Piper longum.* L. Polymorphism in the genomic DNA of plants of 25 females and 6 males were analyzed using 40 decamer random oligonucleotide primers. Two RAPD bands consistently appeared only in the plants showing male genotype,

suggesting thereby the male associated nature of these DNA markers in dioecious *P. longum.*

Harvey *et al.* (1999) studied sex determination in *Actinida* using RAPD marker. 500 primers screened from which two sex linked markers polymorphic DNA analysis for genetic relationships among 42 grape varieties was reported.

Jionguo *et al.* (1999) reported isozyme analysis with peroxidase and RAPD of space travelled tomato. After sowing and germination, 3 lines, which showed differences in peroxidase banding patterns, were used for RAPD analysis. Among 50 primers used, 18 primers generated 166 polymorphic DNA bands with size from 200 bp to 2000 bp.

Lim *et al.* (1999) did the RAPD analysis of some species in the genus Vanda (Orchidaceae). Genetic relationship between orchids of the genus Vanda and the related genus *Ascocentrum* were evaluated. Results obtained indicated that strap-leaved Vanda species including *Vanda sanderiana* and *Ascocentrum miniatum* were more closely related to each other than to the terete-leaved Vanda species.

Ohtsubo *et al.* (1999) subjected milled grains of rice produced at several Japanese agricultural experiment stations to RAPD. DNA was extracted by CTAB method and amplified by PCR using decamer primers. The 10 most important Japanese rice cultivars were distinguished.

Padmesh *et al.* (1999) used RAPD in accessing genetic variability in Nees (*Adrographics paniculata*), a hepta protective drug. The accessions collected on molecular analysis revealed moderate variation within the species. The results indicated that RAPD could be effectively used for genetic diversity analysis in wild species of protective value as it is reliable, rapid and superior.

Sivolap *et al.* (1998) did RAPD analysis of 30 inbred lines of sunflower (*Helianthus annuus*). It was conducted using the PCR and 12 arbitrary primers. Out of these, 22 primers detected polymorphism. On the basis of RAPD analysis

genetic distances were estimated and a dendrogram was constructed reflecting genetic similarity and relatedness between the lines studied

Tang *et al.* (1999) evaluated phylogenetic relationship of *Camellia nitidissima* and its allied species based on RAPD. Six species and two botanical varieties of yellow camellia were analysed. 173 decanucleotide primers were used for DNA amplification and fourteen markers were generated. The results obtained indicated that *C. nitidissima* is closely related to *C. nitidissima* var *phaeopubisperma*.

Ganeshaiah *et al.* (2000) attempted to identify sex specific DNA markers that could potentially be used to determine the sex of *Myristica fragrans* seedlings. Out of 60 random primers screened using RAPD-PCR, one primer OPE 11 elicited a female sex specific DNA amplification product.

Gutman *et al.* (2001) used RAPD markers to fingerprint 29 genotypes of *Cereus* species and examined their genetic diversity. The result showed that *C. peruvianus* has only a limited genetic base and that further improvement of this crop may require the introduction of additional germplasm into breeding programs. RAPD markers were shown to be valuable tool for clone identification in *Cereus* species and for determining genetic relationships.

Hemanth *et al.* (2001) screened 50 mango cultivars using RAPD markers with decamer primers of arbitrary sequence. Out of 80 primers, ten were selected which gave 139 clear and bright fragments. A dendrogram based on Jaccard's coefficient of similarity implied a moderate degree of genetic diversity among the cultivars used for experimentation. Dhanaraj *et al.* (2002) used RAPD markers to estimate the diversity among 90 cashew accessions from the National Cashew Gene Bank. A dendrogram was constructed using Ward's method which confirmed that the diversity of Indian cashew collections could be considered to be moderate to high. A core collection has been identified based on the study, which represents the same diversity as the entire population.

Lakhanpaul *et al.* (2002) performed the analysis of genetic diversity in Indian taro [*Colocasia esculenta* (L.) Schott] using RAPD marker. Three out of thirteen primers showed 100 percent polymorphism. High genetic diversity was revealed as the similarity coefficient values varied from 60-100 percent. Massawe *et al.* (2003) studied the genetic diversity in bambara groundnut [*Vigna subterranean* (L) Verdc] landraces assessed by RAPD markers. DNA from individuals of each landrace was also analyzed to the level of heterogeneity within landraces. The percentage of polymorphism ranged from 63.2 - 88.2 percent.

2.8.2 Molecular markers and early sex determination in dioecious crops

Harvey *et al.* (1999) developed a female specific marker in *Pistacia vera*, after screening the bulked DNA samples of male and female plants with about seven hundred decamer primers. Kafkas *et al.* (2001) used sex pooled DNA samples prepared by mixing the DNA of ten male and ten female individuals for screening about 312 primers in Pistacia genus. Of the 312 primers used; OPC 11, BC 152, BC 360, BC 156 showed sex link polymorphism.

Semerikov *et al.* (2003) tried to identify molecular markers linked to sex determination loci in dioecious plant *Salix viminalis* L. They used a 4 x 4 factorial mating design to identify sex ratios in full sibling progenies. UBC 354 showed a 560 base pair sex specific band in females; which was obtained after a bulked segregant analysis utilizing 380 arbitrary primers. Ruas *et al.* (1998) have reported a 2075 base pair male specific DNA from *Atriplex garrettii* with OPAF-14 primer. They developed this sex associated marker after screening about 158 decamer primers in 124 males, 126 females and the hermaphrodite plants.

Gangopadhyay *et al.* (2006) identified a female specific band in papaya from ISSR profile, using primer $(GACA)_4$ and can be used for the sex identification in the pre-flowering stage. Prakash and Staden (2006) developed a sex specific RAPD marker for *Encephalartos natalensis*, after screening about 140 primers in bulked DNA samples. High degree of polymorphism was observed

in amplification profiles and primer OPD 20 generated an 850 base pair female specific marker.

Agarwal *et al.* (2007) tested 72 primers in the bulked DNA samples from *Simmondsia chinensis* and found out a unique 1,400 base pair male specific DNA band in OPG 5 primer. All the male individuals gave male specific band with the primer, while females did not.

George *et al.* (2007) have developed a male and female specific marker in *Borassus flabellifer* L. after screening about 180 RAPD primers; OPBE 12, OPA 06 gave male specific markers and OPBA 13 gave a female specific marker in bulked samples.

Korpelainen *et al.* (2008) used ISSR and RAPD primers to identify sex-specific markers in Moss plant. They also used a chromosome walking to improve the primer designing strategy for the female specific marker and were able to develop a SCAR primer, highly specific for the region.

Rania *et al.* (2008) have made an attempt to identify sex-specific DNA markers for date palm cultivars using RAPD and ISSR techniques. Their analyses have resulted in developing two male and three female specific markers.

2.9 Sequence Characterized Amplified Region (SCAR) Marker

Martin *et al.* (1991) and Michelmore *et al.* (1991), introduced this technique where in the RAPD marker termini are sequenced and longer primers are designed (22-24 nucleotide bases long) for specific amplification of a particular locus. These are similar to STS markers in construction and application. The presence or absence of the band indicates variation in sequence. These are better reproducible than RAPDs. Compared to arbitrary primers, SCARs exhibit several advantages in mapping studies (co-dominant SCARs are more informative for genetic mapping than dominant RAPDs), map-based cloning as they can be used to screen pooled genomic libraries by PCR, physical mapping, locus specificity etc. SCARs also allow comparative mapping or homology studies, sex

identification studies among the plant species, thus making it an extremely adaptable concept in near future.

2.9.1 SCAR Markers in plant genome analysis

Boukar *et al.* (2004) developed DNA markers that are useful for Marker-Assisted Selection (MAS) in breeding cowpea for resistance to *S. gesnerioides*. Two AFLP markers were validated in an F_2 population developed from crossing the resistant parent with 'Kamboinse local', a different susceptible cultivar. The AFLP fragment from marker combination E-ACT/M-CAC, which is linked in coupling with Rsg1 was cloned, sequenced, and converted into a sequence characterized amplified region (SCAR) marker, which was co-dominant and useful in breeding programmes. Gygax *et al.* (2004) by the usage of bulk segregant analysis identified three RAPD markers linked to Vbj. These markers were transformed into more reliable sequence-characterized amplified region (SCAR) markers that proved to be co-dominant. These SCAR markers were used for the selection of the scab-resistant apple cultivars for the breeding of scab-resistance among apple cultivars by pyramiding several resistance genes. In addition, three SSR markers and one SCAR were identified by comparing homologous linkage groups of existing genetic maps.

Park *et al.* (2004) identified RAPD markers linked to Ur-6 using bulked segregation analysis in an F_2 population from the Middle American (MA) common bean cross Olathe (resistant) x Nebr.#1 sel. 27 (susceptible) and developed sequence characterized amplified region (SCAR) marker based on the specific primer pair designed from the sequence of the RAPD marker OBC06. These RAPD and SCAR markers linked to Ur-6, along with markers for other independent rust resistance genes, could be utilized to pyramid multiple genes into a bean cultivar for more durable rust resistance. Radisek *et al.* (2004) developed pathotype-specific sequence-characterized amplified region (SCAR) markers from amplified fragment length polymorphism (AFLP) markers, after cloning and sequencing of the markers. When primer specificity was tested on a wide range of

Verticillium isolates, the SCAR primer pairs retained amplification specificity not only for *V. albo-atrum* Slovene hop isolates, but also amplified sequences in *V. albo-atrum* and *V. dahliae* hop isolates from different hop production areas in Europe. These new SCAR markers provide a valuable tool for rapid identification of *V. albo-atrum* PG1 and PG2 hop pathotypes.

Jacobs *et al.* (2004) used sixty RAPD primers in the parental lines, to check the salt tolerant variety; nine of these produced *Thinopyrum distichum* chromosome specific fragments. Correlations between salt tolerance and presence of the *Thinopyrum* specific bands were mostly significant. The diagnostic RAPD bands were cloned and sequenced and specific SCAR primers were designed for easy selection of the salt resistant plants.

Janila *et al.* (2004) identified molecular markers linked to the 'er' gene in pea, which confers resistance to powdery mildew caused by *Erysiphe pisi* and to screen the powdery mildew-resistant cultivar. Polymorphism revealed linkage of two RAPD primers (OPO-02 and OPU-17) to the 'er' gene and a sequence characterized polymorphic region (SCAR) primer, ScOPD-10650 with 'er' in the F2 population of 83 plants. The markers ScOPD-10650 and OPU-17 being coupled with the allele causing resistance would substantially increase the efficiency of marker-assisted selection in pea breeding for powdery mildew. Sabharwal *et al.* (2004) carried out association mapping of the seed-coat colour in 39 *Brassica juncea* lines with amplified fragment length polymorphism (AFLP) markers. The bands analyzed for association with seed-coat colour using multiple regression analysis, revealed 15 markers associated with seed-coat colour, obtained with eight AFLP primer combinations. These AFLP markers were converted to sequence-characterized amplified region (SCAR) markers and were useful in developing yellow-seeded *B. juncea* lines by means of marker-assisted selection.

Causin *et al.* (2005) developed a SCAR marker to differentiate between *Phytophthora cactorum* and *P. idaei*. These new pair of SCAR primers with

improved specificity, derived from a specific random amplified polymorphic DNA (RAPD) generated fragment, gave a single amplification product of approximately 450 bp; with absence of cross reactions with *P. pseudotsugae* and *P. idaei*. Nomura *et al.* (2005) converted the original random amplified polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP) marker into sequence-characterized amplified region (SCAR) markers to facilitate large-scale marker-assisted screening of club root resistance in cabbage breeding. Of 15 RAPD markers closely linked to the three QTLs, nine SCARs were developed as dominant markers after cloning and sequencing for the identification of the resistant varieties.

Busconi *et al.* (2006) developed a set of 14 SCAR markers starting from RAPD, AFLP and SAMPL analysis of several olive germplasm accessions. Eight RAPD, two AFLP and four SAMPL fragments were converted into dominant and codominant SCARs by cloning and sequencing the selected fragments. The markers obtained were evaluated on forty different olive cultivars from different Italian production areas (mainly from Liguria). A total of 31 different haplotypes were identified and the analysis of several individual plants indicated no intra-cultivar variability. Chatterjee *et al.* (2006) made an attempt to understand the molecular systematics and genetic differences between 10 original chrysanthemum cultivars and 11 mutants. The similarity among the cultivars and mutants varied from 0.17 to 0.90 using RAPD analyses. Two cultivars were present as a separate group showing differences from all other cultivars and they could be identified easily by the development of the SCAR markers.

Huh *et al.* (2006) carried out a study to develop a method that could be used to discriminate between *A. japonica* and *A. macrocephala* based on molecular markers. To discriminate between *A. japonica* and *A. macrocephala*, RAPD analyses were used to develop SCAR markers. Eighteen species-specific RAPD bands were obtained from 52 primer sets. Two SCAR markers were developed from these RAPD clones and they were sufficient to discriminate between samples of *A. japonica* and *A. macrocephala*. Iniguez Luy *et al.* (2006)

developed sequence characterized amplified regions (SCAR) from DNA fragments generated by randomly amplified polymorphic DNA (RAPD) primers that distinguished the U-triangle Brassica species: *B. rapa* (L.), *B. nigra* (L.) Koch, *B. oleracea* (L.), *B. juncea* (L.) Czern, *B. napus* L. and *B. carinata*. The SCAR descriptors developed in the study reveals the presence or absence of a DNA fragment and sequence length polymorphisms to distinguish the three genomes of the six cultivated *Brassica* species.

LianHua *et al.* (2006) reported for the first time, the use of sequence characterized amplified region (SCAR) markers for strain differentiation among *Lentinula edodes*. SCAR markers were created by first generating and sequencing single ISSR fragments and then designing primers based on these sequences to amplify strain-specific fragments of a certain size. One SCAR primer pair amplifying a band of 450 bp, was designed to identify one strain of *L. edodes*. The SCAR primer pair was then used to amplify the single unique fragment from DNA samples taken from a total of 85 strains representing three separate species. Rugienius *et al.* (2006) aimed to study about the development of new SCAR markers, associated with Rpf1, to make a comparative study of RAPD and SCAR markers and to select strawberry varieties and seedlings carrying the Rpf1 gene. A SCAR marker was constructed based upon RAPD marker OPO-16C, linked to the susceptibility allele of the Rpf1 gene. Then they designed SCAR primers specific to this gene.

Akkurt *et al.* (2007) developed sequence-characterized amplified regions markers (SCARs) from six randomly amplified polymorphic DNA primers linked to the major QTL region for powdery mildew (*Uncinula necator*) resistance in a test population derived from the cross of grapevine cultivars "Regent" (resistant) x "Lemberger" (susceptible). The SCAR primers resulted in the amplification of specific bands of expected sizes and were useful in identifying the plants with resistance to the disease. Anuntalabhochai *et al.* (2007) used a set of 11 unique decamer primers, for identifying the curcuma variety 'Patumma'. Polymorphic bands ranging from 100 to 2500 base pairs were used to examine 20 Curcuma

varieties from which banding patterns of interest were selected for conversion to the more reproducible and robust sequence characterized amplified region (SCAR) markers. In particular, one SCAR marker amplified a region 600 bp in length which was conserved in all 'Patumma' varieties and hybrids.

Cutler *et al.* (2007) characterized a temperature dependence in longan (*Dimocarpus longan* Lour.) cultivars using the high annealing temperature random-amplified polymorphic DNA (HAT-RAPD) methodology, using a set of 18 unique decamer primers; polymorphic bands ranging from 100 to 2500 bp were examined for 14 longan varieties from which banding patterns of interest were selected for conversion to the more reproducible and robust SCAR markers. In particular, one SCAR marker produced an electrophoresis banding pattern which could distinguish between longan varieties requiring a sustained interval at low temperatures for fruit production vs. those that do not.

Lopes *et al.* (2007) screened thirty Portuguese and eight foreign olive (*Olea europaea* L.) cultivars using random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) markers, to study the genetic variability. Twenty RAPD primers amplified 301 reproducible bands of which 262 were polymorphic and 17 ISSR primers amplified 204 bands of which 180 were polymorphic. The genetic variability observed was similar in the Portuguese and foreign olive cultivars. Seven ISSR and 12 RAPD primers were able to distinguish individually all 38 olive cultivars. Twenty specific molecular markers are converted into sequence characterized amplified region (SCAR) markers.

2.9.2 SCAR marker in plant sex determination

Jung *et al.* (2001) have used RAPD method to detect the potential male-specific markers in *Schisandra nigra*. Out of 120 primers tested, only OPA 17 produced a polymorphic nucleotide segment of approximately 800 base pair in all male samples tested. This marker was cloned, sequenced and SCAR primers were designed to amplify 486 base pair male specific region. Deputy *et al.* (2002) developed molecular markers tightly linked to sex gene in papaya. The markers

were cloned and sequenced; based on the sequences obtained SCAR primers were synthesized for the specific amplification of the sex-linked region. Gunter *et al.* (2003) have identified genetic marker linked to female sex determination locus in *Salix viminalis* L., after using a thousand RAPD primers. The consistent marker in female plants was converted to a SCAR marker to make it useful in the analyses of a number of samples. Stehlik *et al.* (2004) have developed SCAR primers based on isolated and sequenced male specific fragments as identified in an AFLP analysis of dioecious plant *Rumex nivalis.*

Xu *et al.* (2004) have identified a 569 base pair RAPD marker linked to sex determining locus in *E. ulmoides*. During their later studies they were able to identify the same marker in all the pistillate but not in staminate plants. They also confirmed the sex specific marker using the southern hybridization and developed a SCAR for the same. Yakubov *et al.* (2003) utilized a combination of touch down PCR and SCAR primers for the development of a female specific DNA marker in *Pistacia vera* (L.). The RAPD primer OPO 08 amplified a 905 base pair fragment in all females and a 909 base pair fragment in all males. A second internal set of SCAR primers were designed to clearly distinguish between the male and female RAPD markers which resulted in a 297 base pair female specific product.

Bedoya *et al.* (2007) used RAPD markers to determine the sex types of Colombian cultivars of dioecious papaya genotypes. RAPD marker of 900 base pair was found in male plants by the use of OPY 7 primer, which was later used for the development of the SCAR marker. Gao *et al.* (2007) tested hundred primers on dioecious *Asparagus officinalis* to identify sex-linked marker. An RAPD primer produced two female specific markers. The marker was sequenced and specific primers were designed to generate a 928 base pair marker in female plants.

Vinod *et al.* (2007) analyzed the bulked DNA samples from *Pandanus fascicularis* L. using 89 RAPD primers, of which OPO08 produced a 1263 base pair male specific marker in the bulked male DNA sample which was absent in

the corresponding female DNA sample. The SCAR markers designed was used for the amplification of the specific allele in male plants. The southern blotting analysis also confirmed their findings. Manoj *et al.* (2005) used differential display for studying genetic similarity and differences between morphologically similar plants. Once some potent cDNA fragments were obtained, they can be sequenced for full length gene and can be used for designing specific primers to screen the specific trait. Using this approach, they identified a male and female specific marker in *Piper longum* L., to identify the sex of the plant in very young stage.

Oyama *et al.* (2009) developed a male sex-linked marker for *Bryonia dioica* and sequenced it for the individuals representing the full geographic range of the species from Scotland to North Africa. The population genetics analyses suggest that the sex-linked SCAR marker experienced different evolutionary pressures in North and South Europe.

KERALA AGRICULTURAL UNIVERSITY
CENTRAL LIBRARY
P.O.), THRISSUR

Materials and Methods

3. MATERIALS AND METHODS

The study on the development of SCAR marker for authentication of gender in Kodampuli was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara, during the period from 2008 to 2010. The research work included mainly three aspects; PCR amplification with earlier reported primers, development of SCAR marker and validation of SCAR marker. Description of the material used and methodology adopted in the study has been furnished in this chapter.

3.1 Materials

3.1.1 Plant Material

The plant material for the present study was obtained from orchard of the Department of Olericulture and Department of Pomology and Floriculture, College of Horticulture. Twenty five each of male and female mature trees were selected for study.

3.1.2 Chemicals, glasswares and plastic wares

The chemicals used for the present study were of good quality (AR grade) from various agencies including MERCK, SRL and HIMEDIA. Molecular biology enzymes and buffers were supplied by Bangalore Genei Ltd. All the plastic wares used were obtained from Axygen and Tarson India Ltd. The decamer primers were obtained from Operon Technologies Inc. (Alameda, Calif.) and SCAR primers were synthesised through Sigma Aldrich Chemical Pvt. Ltd..

3.1.3 Equipment and machinery

The present research work was carried out using the molecular biology facilities and equipment items available at CPBMB, College of Horticulture. Quantification of DNA was done by Nanodrop® ND-1000 Spectrophotometer. The PCR was done in the thermal cycler of model Eppendorf Master Cycler (Eppendorf, USA) and horizontal gel electrophoresis system (Biorad, USA) was

used for agarose gel electrophoresis. Gel Doc-ItTS™ Imaging System (UVP Inc. CA) was used for imaging and documenting the agarose gel. The details are given in Annexure I.

3.2 Methods

3.2.1 DNA isolation (CTAB method)

Genomic DNA of male and female plants were isolated from young and tender leaves by CTAB method (Rogers and Bendich, 1994, Premjeeth, 2008) with modification such as addition of 50 µl of ß- merceptoethanol during grinding of 1 g leaf sample to overcome the problem of phenol oxidation. The isolated DNA was purified from RNA contamination by RNase treatment (Sambrook *et al.* 1989). The details of the reagents and protocol used in CTAB method are provided in Annexure II.

Protocol:

Young and tender leaf tissue (1g) was weighed and ground in liquid nitrogen using mortar and pestle along with 50 µl of ß- merceptoethanol. The sample was ground into fine powder using excess of liquid nitrogen and the powder was transferred to a sterile 50 ml centrifuge tube containing 5 ml of pre-warmed extraction buffer. The homogenate was incubated for 20 min. at 65°C with intermittent mixing. Equal volume of chloroform and isoamylalcohol (24:1) was added and centrifuged at 10,000 rpm for 15 min. at 4°C. The supernatant was collected, 1/3 volume of chilled isopropanol was added and incubated at -20°C for 30 min. The precipitated DNA was pelleted by centrifugation at 10,000 rpm for 10min. at 4°C.The pellet was collected and washed first with 70 percent alcohol and later with 100 percent alcohol. It was air dried for 30 min at room temperature and dissolved in 100 µl of T E buffer.

3.2.2 DNA purification

The DNA isolated would contain RNA as contaminant and was hence purified by phenol precipitation and RNase treatment (Sambrook *et al.*, 1989).

The RNase A from Sigma, USA was used to prepare RNAse. One per cent solution was prepared by dissolving RNase A in TE buffer at 100^0C for 15 minutes. The solution was cooled to room temperature, dispensed into aliquots and stored at -20^0C.

The procedure followed for DNA purification is as follows:

RNAse solution (2 µl) was added to 100 µl DNA sample and incubated at 37^0C in dry bath (Genei, Thermocon) for 1 hour. The volume was made up to 250 µl with distilled water and equal volume of phenol:chloroform (1:1) mixture was added. This was then centrifuged at 12,000 rpm for 10 min. at 4^0C. The aqueous phase was collected in a fresh micro centrifuge tube and added equal volume of chloroform:isoamyl alcohol (24:1). Again it was centrifuged at 12,000 rpm for 10 min. at 4^0C. The above two steps were repeated and finally DNA was precipitated from the aqueous phase with 0.6 volume of chilled isopropanol. The mixture was then incubated at -20^0C for 30 min. and centrifuged at 10,000 rpm for 15 min. at 4^0C. The pellet of DNA obtained was washed with 70 per cent ethanol. The pellets were air dried and dissolved in 100 µl TE buffer.

3.2.3 Assessing the quality of DNA by electrophoresis

The quality of isolated DNA was evaluated through agarose gel electrophoresis (Sambrook *et al.*, 1989).

Materials for agarose gel electrophoresis

1) Agarose (GeNei, Low EEO)
2) 50X TAE buffer (pH 7.0)
3) 6X Loading/ Tracking dye

4) Electrophoresis unit, power pack (GeNei), casting tray, comb

5) Ethidium bromide solution (0.5µg/ml)

6) UV transilluminator (Herolab ^{R})

7) Gel documentation and analysis system (UVP, GelDoc It™ imaging system; UK)

Chemical composition of buffers and dye are given in Annexure III.

The procedure followed for agarose gel electrophoresis is as follows:

1X TAE buffer was prepared from the 50 X TAE stock solutions. Agarose (1%) was weighed and dissolved in TAE buffer by boiling. Then ethidium bromide was added at a concentration of 0.5µg ml^{-1} and mixed well. The open end of gel casting tray was sealed with a cellotape and kept on a horizontal levelled surface. The comb was placed desirably and the dissolved agarose was poured on to the tray. The gel was allowed to set for 30 min. after which the comb was removed carefully. The tray was kept in the electrophoresis unit with well side directed towards the cathode. 1X TAE buffer was added to the tank. Then DNA sample (5µl) along with the tracking dye (1µl) was loaded into the wells using a micropipette carefully. λDNA / *Eco*RI+*Hind*III double digest was used as a molecular marker. After closing the tank, the anode and cathode ends were connected to the power pack and the gel was run at a constant voltage (100 V) and current (50 A). The power was turned off when the tracking dye reached 2/3rd length of the gel.

Then the gel was taken from the electrophoresis unit and viewed under UV light in a transilluminator (Herolab ^{R}). The DNA fluoresces under UV light due to ethidium bromide dye. The quality of DNA was judged by clarity of DNA band. The image was documented and saved in gel documentation system.

3.2.4 Assessing the quality and quantity of DNA by NanoDrop method

The quantity and quality of genomic DNA was estimated using NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies Inc., USA). Before taking sample readings, the instrument was set to zero by taking 1µl

autoclaved distilled water as blank. One microlitre from each sample was quantified and was measured in ng/µl. The absorbance of nucleic acid samples were measured at a wavelength of 260 nm and 280 nm and OD_{260}/OD_{280} and OD_{260}/OD_{230} ratios were recorded to assess the purity of DNA. A ratio of 1.8 to 2.0 for OD_{260}/OD_{280} indicated good quality DNA.

3.2.5 Bulk DNA preparation

Bulking of the samples was performed by mixing equal quantities of DNA isolated from five male and five female trees.

3.2.6 RAPD assay

3.2.6.1 Screening with earlier reported primers

The basic RAPD procedure suggested by Premjith (2008) was used for earlier reported primers. PCR reactions were carried out in 20 µl reaction tubes using three earlier reported primers *viz.* Kit C1, Kit C8 and Kit C9 (Sigma Aldrich Chemical Pvt. Ltd.) (Table 1). Each reaction tube contained 50 ng template DNA, 1.5 mmol $MgCl_2$, 200 µmol of dNTPmix, 1x *Taq* buffer, 15 pmol of decanucliotide primer and 0.9 unit of *Taq* DNA polymerase make up to a final volume of 20 µl.

Composition of the reaction mixture for PCR (20.0 µl)

Genomic DNA (50 ng)	1.0 µl
1X *Taq* buffer	2.0 µl
dNTP mix (10mmol each)	1.0 µl
$MgCl_2$ (1.5 mmol)	1.0 µl
Decamer primer (15 pmol)	1.0 µl
Taq DNA polymerase (0.9 unit)	0.3 µl
Autoclaved distilled water	13.7 µl
Total volume	= 20.0 µl

A master mix of all reagents for the required number of reactions was prepared first and aliquots were dispended into PCR tubes fallowed by addition of template DNA in each tube. Amplification was performed in Eppendorf Master Cycler (Eppendorf, USA) using following conditions:

Step 1: 94°C for 30 sec	- Initial denaturation	
Step 2: 94°C for 30 sec	- Denaturation	30 cycles
Step 3: 37°C for 1 min.	- Annealing	
Step 4: 72°C for 2 min.	- Extension	
Step 5: 72°C for 10 min.	- Final extension	
Step 6: 4°C for 10 min.	- Cooling of samples	

Modification of parameters:

The RAPD protocol described by Premjith and Rajendran (2008) was modified with different annealing temperatures and change in concentration of PCR components.

a. *Change in annealing temperature:*

Kit C1, Kit C8 and Kit C9 were amplified at different annealing temperatures *viz.* 28^0C, 30^0C, 35^0C, 37^0C and 42^0C.

b. *Modification in concentration of PCR components*

The concentration of PCR components were varied with increase in primers volume and reduction in DNA concentration. Instead of 1 µl (15 pmol.) decanucliotide primer 2 µl was used and instead of 50 ng. template DNA, 20 ng. and 30 ng. were used.

Table 1 Earlier reported primers used for initial screening

Primer	Sequence (5'→ 3')	Tm (^{0}C)
Kit C1	TTCGAGCCAG	35.8
Kit C8	TGGACCGGTG	38.7
Kit C9	CTCACCGTCC	33.1

3.2.6.2 Further screening of primers for RAPD

Further primer screening was carried out to select primers which produced good amplification in isolated male and female DNA samples. Forty six decamer oligonucleotide primers of arbitrary sequence belonging to different Operon series *viz.* OPS, OPA, RY, RN, OPAH, OPD and OPK were screened. The nucleotide sequence of the primers screened is given in Annexure V.

Primers screening with bulked DNA samples

Initially the RAPD reactions were performed with bulked DNA samples and then the primers showing good amplification were selected for individual DNA samples. The bulking of the DNA were performed by mixing equal quantities of five male DNA samples into a tube and five female DNA samples into another tube.

RAPD reactions were carried out with gradient PCR, containing 50 ng of gnomic DNA, 15 pmol of each primer, 10X PCR buffer, 1.5-2 mmol of $MgCl_2$, 10mmol each dNTP and 0.9 unit of *Taq* DNA polymerase. Amplification was performed in Gradient (Eppendorf, USA) PCR. Consistent and reproducible RAPD primers were selected for screening individual plants.

3.2.6.3 Detection and Elution of Sex Specific RAPD Band

The PCR product was resolved on a 1.2 per cent agarose gel and observed for sex specific polymorphism. The polymorphic and reproducible RAPD bands were selected for cloning purpose.

3.2.7 Gel Elution for sex specific RAPD band

The sex specific polymorphic RAPD band for individual DNA sample was eluted by using AxyPrep DNA Gel Extraction Kit (Axygen, Biosciences).

Details of procedure adapted as per the manufacturer's guidelines is as follows:

1. Polymorphic RAPD band was excised from the gel using a sterile, sharp scalpel avoiding much exposure to UV on a transilluminator.
2. Gel slice was weighed in a 1.5 ml microcentrifuge tube.
3. Added 3X gel volume of gel solubilization buffer (DEA buffer).
4. The gel was resuspended in gel solubilization buffer by vortexing. The mix was heated at 75°C until the gel was completely dissolved. Intermittent vortexing was given every 2 to 3 min. to enhance gel solubilization.
5. Added 0.5X gel solubilization volume of binding buffer (DEB buffer) and mixed properly.
6. A spin column was placed in a 2 ml collection tube. The solubilized gel slice was transferred into the spin column that was assembled in the 2 ml collection tube and centrifuged at 12,000 rpm for 1 min.
7. The filtrate was discarded. 500µl of wash buffer (W1) was added to the spin column and centrifuged at 12,000 rpm for 30 sec.
8. The filtrate was discarded and 700µl of desalting buffer (W2) was added and centrifuged at 12,000 rpm for 30 sec.
9. A second wash was given by adding 700µl of desalting buffer (W2), followed by centrifugation at 12,000 rpm for 30 sec to ensure the complete removal of salt. The filtrate was discarded and spin column was again placed in collection tube.
10. Column was again centrifuged for 1 min. at 12,000 rpm to remove any residual buffer.
11. Spin column was transferred to a fresh 1.5ml microcentrifuge tube. The eluent was prewarmed at 65°C to improve the elution efficiency.
12. To elute the DNA, 7µl of eluent was added to the centre of the spin column. It was allowed to stand for 1 min. at room temperature. Then centrifuged at 12,000 rpm for 1 min.

13. Eluted DNA fragments were checked on 0.7 percent agarose gel and stored at -20°C for further cloning works.

3.2.8 Transformation

3.2.8.1 Preparation of competent cells

Competent cells required for plasmid transformation were prepared using the competent cell preparation kit of GeNei, Bangalore in SOB agar media. (Chemical compositions of media are given in Annexure IV)

Procedure

Day I

The desired strain JM 109 was streaked on SOB plate from master plate. The plates were incubated at 37°C for 16 to18 hrs.

Day II

1. Inoculated 5 to 6 moderately sized colonies from the SOB plate in 50 ml SOB broth in a 500 ml flask.
2. Incubated the broth on a rotary shaker at 37°C; 160 rpm. When the OD reached 0.3 at A600, the growth of the bacteria was arrested quickly by chilling for 2 min. on ice.
3. The entire culture was transferred into a 50ml sterile polypropylene tube and centrifuged at 3500 rpm for 15 min. at 4°C.
4. The supernatant was discarded. Keeping the bottle on ice, the bacterial cell pellet was resuspended very gently in 16.6 ml ice cold Solution A (provided in the kit).
5. Kept on ice for 20 min. and then centrifuged at 3500 rpm for 15 min. at 4°C.
6. The supernatant was discarded and the pellet was chilled on ice.
7. The pellet was resuspended in 3 ml of ice cold Solution A (provided in kit).
8. The suspension was left on ice for 10 min. and aliquots of 100µl were added in chilled 1.5 ml microcentrifuge tubes.

9. The competent cells were covered with aluminium foil and were stored at -70°C.

3.2.8.2 Screening of competent cells

The competent cells prepared were screened to check their transformation efficiency, by transforming them using a plasmid (pUC18) containing ampicillin resistance marker. The procedure followed is as given below:

1. Prepared 50 ml LB media and 50 ml LB broth.
2. The competent cells kept at -70°C were thawed on ice.
3. pUC18 was diluted to 1:10 dilution. Added 1µl of diluted pUC18 to thawed competent cells.
4. The contents were mixed gently and kept on ice for 40 min.
5. Meanwhile, the water bath was set to 42°C.
6. The tube was rapidly taken from ice and a heat shock at 42°C was given exactly for 90 sec. Without shaking, the tube was placed back on ice for 5 min.
7. Added 250µl of LB broth to vial under sterile conditions and was inverted twice to mix the contents.
8. The tube was incubated at 37°C for 1 hour with shaking.
9. The transformed cells were plated on LB agar/ampicillin (50mg/l) overlaid with IPTG (6µl) and X gal (60µl).
10. The plates were incubated overnight at 37°C.

3.2.8.3 Cloning

The eluted sex specific RAPD fragment was cloned in pGEMT vector system supplied by Promega.

A. Ligation

The appropriate amount of eluted product (insert) required for ligation was calculated by estimating the quantity of eluted DNA using NanoDrop® Spectrophotometer. For this, 0.25µl of eluted RAPD band was diluted to 5µl with sterile water. The amount of PCR product required was calculated by the following relationship

$$\text{ng of insert} = \frac{\text{ng of vector x Kb size of insert}}{\text{Kb size of vector}} \text{ X Insert: vector molar ratio}$$

Ligation procedure was followed as per the manufacturer's guidelines.

Procedure

1. The pGEMT vector was briefly centrifuged to collect contents at the bottom of the tubes.
2. The following ligation reaction was set up as described below

Table 2 Preparation of Ligation Mix

Components	Per reaction
2X Ligation buffer	5.0µl
pGEMT vector (50ng)	1.0µl
Eluted product	3.0µl
T_4 DNA Ligase (3units/µl)	1.0µl
Total volume	**10.0µl**

3. The reaction was mixed by pipetting and incubated for 1 hr at room temperature. Then it was kept at 4°C overnight.

B. Transformation of ligated product

Reagents

1. Ampicillin – 5mg/ml in water
2. IPTG – 200mg/ml in water
3. X-gal – 10mg/ml in DMSO

The procedure adapted for plasmid DNA transformation is as follows:

The ligated PCR product was added to 100µl of thawed competent cells and kept on ice for 40 min. Heat shock was given at 42°C for 90 seconds in a water bath and immediately placed back in ice for 5 min. LB medium (250µl) was added to the cells and incubated at 37°C for 1 hr on a shaker at 160 rpm. The aliquots of transformed cells was plated on LB agar/Ampicillin (5 mg/ml) / IPTG (6µl) / X-gal (60µl) plates and incubated overnight at 37°C.

C. Confirmation of presence of insert

Presence of insert in the plasmid was confirmed by performing colony PCR with T7 and SP6 Primers specific for pGMT vector (Promega). Details are as followed:

1. A loopful of colony was diluted with 20µl of sterile water.
2. Bacterial cells were lysed by heating, with the help PCR machine at 95 ^{0}C for 2 min.
3. A spin was provided to sediment the cell debris.
4. 1 µl of supernatant was taken from lysed cells as a plasmid DNA into new PCR tube.
5. A Master Mix was prepared and the reagents were in the sequential order as shown in the table 2.
6. The contents were mixed well and briefly centrifuged.
7. The following PCR programme was run immediately:

Step1: 94°C for 2 min. - Initial denaturation
Step2: 94°C for 45 sec. - Denaturation
Step3: 41°C for 1 min. - Annealing } 30 cycles
Step 4: 72°C for 2 min. - Extension
Step 5: 72°C for 10 min. - Final extension
Step 6: 4°C for 10 min. - Cooling of samples

8. The PCR products were analyzed on 1 percent agarose gel.

Table 3 Preparation of PCR Master Mix

Components	Vol per reaction (µl)
10X Taq buffer	2.5
dNTP Mix (10mM)	1.0
T7 Primer (27.5nM)	1.0
SP6 Primer(31.3nM)	1.0
Taq DNA polymerase (0.3 units)	1.0
Sterile H_2O	16.5
Total volume	**24.0**

3.2.9 Preparation of stabs

LB agar medium was melted, cooled to 42°C and ampicillin was added. This medium was poured into storage vials aseptically in a laminar air flow. After solidification, single colony was inoculated into the medium using sterile bacterial loop. The vials were incubated at 37°C overnight and further stored at 4°C.

3.2.10 Sequencing of sex specific DNA fragment

Prepared stabs were sent for sequencing to DNA sequencing facility of Bangalore GeNei. The Primers used for sequencing were T7, SP6 (fig 1).

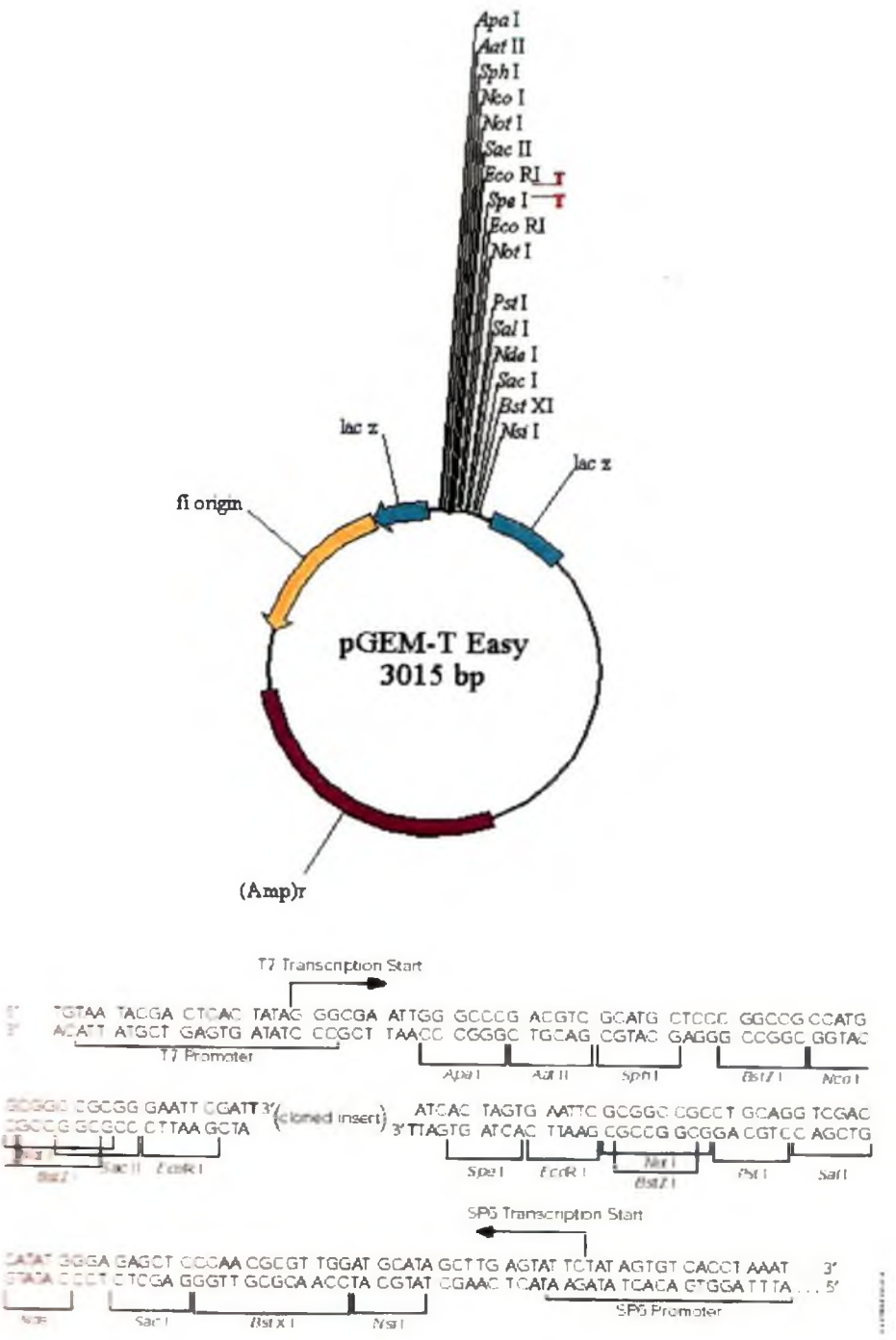


Fig. 1 pGEM-T Easy Vector (Promega) used for cloning RAPD product.

The *lacZ* region, promoter and multiple cloning sites are shown in the figure. The top strands of the sequence shown corresponds to the RNA synthesized by T7 RNA polymerase and bottom strands corresponds to the RNA synthesized by SP6 RNA polymerase.

2. The forward and reverse primers were designed based on following parameters:

    a) The end sequences selected should have GC content 40-50 per cent.
    b) Melting temperature (Tm = 4 GC + 2 AT) ranged between 55^0C to 67^0C.
    c) The distance between the primers ranged from 400 to 500 base pairs.
    d) It is preferable to have GC content at 3' end.
    e) There should not be any complementarity between forward and reverse primers.
    f) Repeats of single base should not appear within the primer sequence.
    g) Each primer should be 22 to 24 bp long.
    h) Primer sequences had no palindromic sequences or repetitive sequences.
    i) For designing primers, the sequence of the forward primer was taken as such and for the reverse primer, the reverse complementary sequence was taken.

Based on above information, two pairs of forward and reverse primers were designed.

3.2.13 PCR ampiification with SCAR primers

Two pairs of oligonucleotide primers were designed as SCAR primers. One primer contained the original 10 bases of RAPD primer plus the first 12 bases from each end of PCR product, while other primer pair contained all 23 bases from PCR product only. PCR reaction was performed in a total reaction volume of 25µl using SCAR specific primers in Eppendorf Master Cycler (Eppendorf, USA).

Table 4 PCR Master Mix for SCAR primers amplification

Components	Vol per reaction (µl)
10X Taq buffer	2.5
dNTP Mix (10mM)	1.0
Forward Primer (8 pM)	1.0
Reverse Primer (8 pM)	1.0
DNA (50 ng)	1.0
Taq DNA polymerase (0.3 units)	2.0
Sterile H_2O	16.5
Total volume	**25**

SCAR primes were used only for individual DNA samples. The reaction was set in a 50µl microfuge tube chilled over ice flakes. A momentary spin was given for the reaction and set in thermal cycler for polymerase chain reaction under suitable programme. A negative control was kept without template DNA.

The following thermal cyclers were used to amplify the template DNA with the SCAR primer:

Step 1: 94 ^{0}C for 2 min. - initial denaturation

Step 2: 94 ^{0}C for 45 sec. - denaturation

Step 3: 65 ^{0}C for 1 min. - annealing } 30 cycles

Step 4: 72 ^{0}C for 2 min. - extension

Step 5: 72 ^{0}C for 10 min. - final extension

Step 6: 4 ^{0}C for infinity to hold the sample

The PCR product was run on 1.5% agarose, documented and interpreted.

Results

4. RESULTS

The results of the investigations conducted on the 'Development of SCAR marker for authentication of gender in Kodampuli (*Garcinia gummi-gutta* var. *gummigutta*)' undertaken during the period from 2008 to 2010 at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Kerala Agricultural University, Vellanikkara are presented in this chapter under different subheadings. The research work included mainly the following aspects.

4.1 Isolation, Purification and Quantification of DNA

4.2 RAPD assay

4.3 Molecular cloning

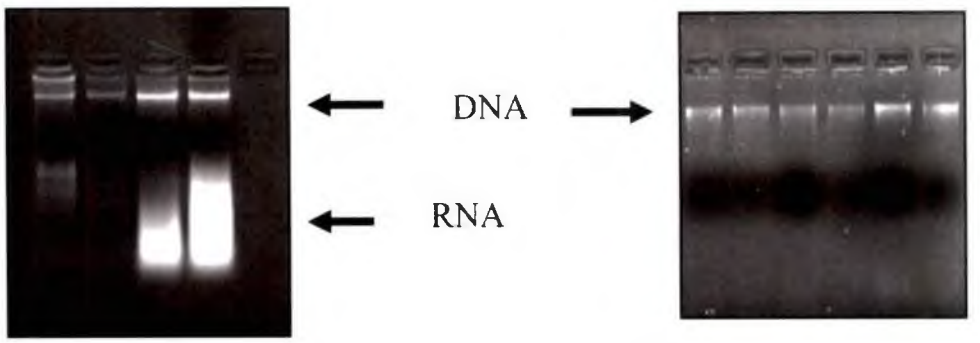
4.4 Sequence analysis

4.5 Designing of SCAR primers

4.5 Confirmation of SCAR maker

4.1 Isolation, Purification and Quantification of DNA

Genomic DNA was extracted from 25 male and 25 female trees. DNA isolation was carried out with CTAB method (Rogers and Bendich, 1994) (Premjith and Rajendran, 2008). Isolated DNA had shown a single intact band of high intensity with no sharing. But it was noticed, the RNA contamination was also high which was removed by RNase treatment (Sambrook *et al.* 1989) mentioned in 3.2.2 (Plate 1 b). The quantity of DNA isolated by CTAB method ranged from 511.4ng/µl to 976.5ng/µl. The isolated DNA, after appropriate dilutions were used as templates for RAPD and SCAR analysis.



(a) Genomic DNA with RNA contamination

(b) DNA after RNase treatment

Plate 1 Genomic DNA isolated from male and female Kodampuli plants

4.2 RAPD assay

DNA of male and female plants was subjected to RAPD analysis.

4.2.1 Screening with earlier reported primers

Primary screening of primers was done with bulked DNA samples. RAPD assay was performed with earlier reported primers *viz.* Kit C1, Kit C8 and Kit C9. The thermocycler was programmed as given in section 3.2.6.1. PCR products were resolved on 1.3 per cent agarose gel to elucidate the polymorphic band but no sex specific polymorphism was found. All three reported primers were failed to distinguish between male and female plants of Kodampuli (Plate 2).

4.2.2 Further screening of primers for RAPD

RAPD-PCR product of male and female DNA pools were attempted with further forty-six decamer primers which led to the selection some primers, the result of experiment is as follows.

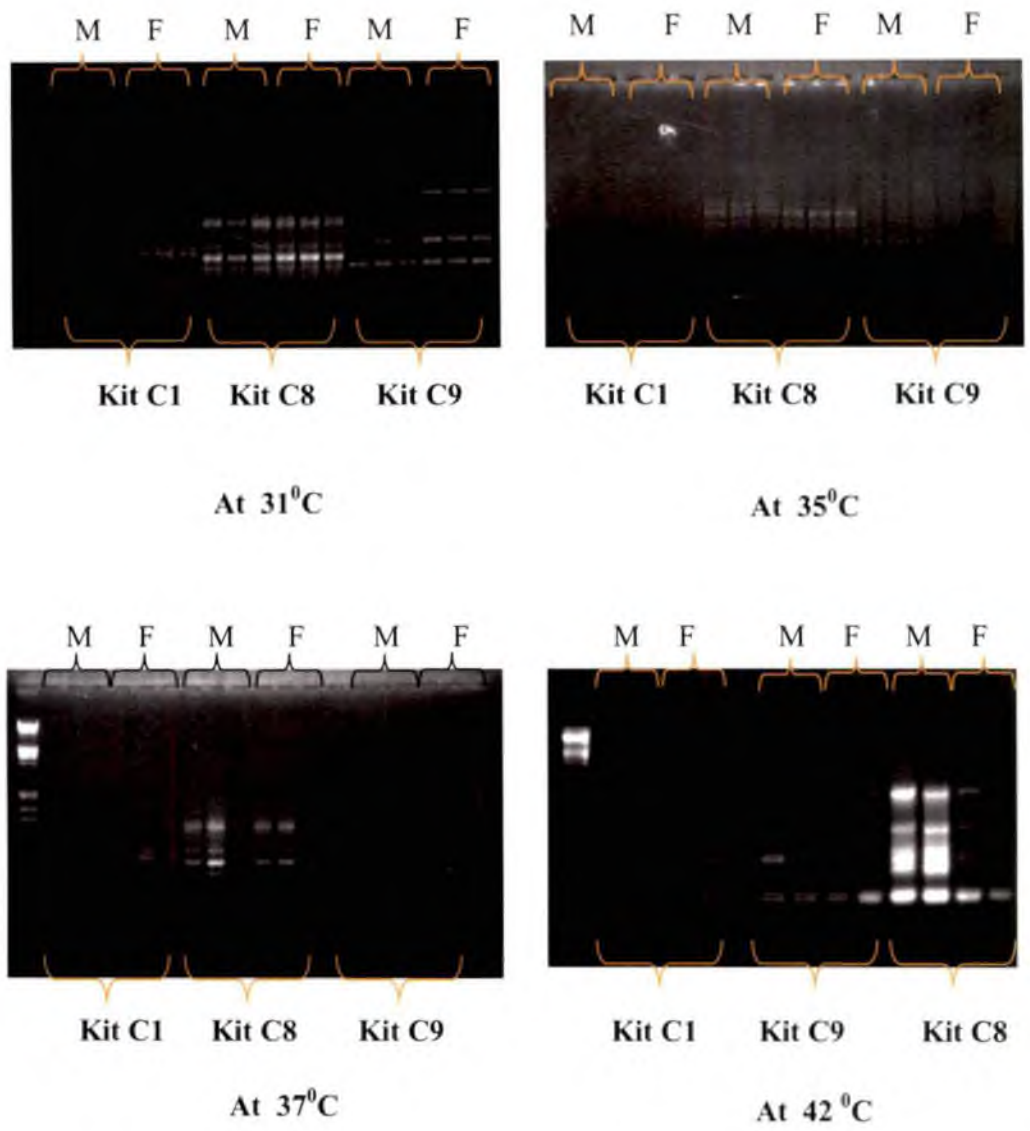
i. *Screening for RAPD*

a. Screening of OPS Series

Eight OPS series primers *viz.* OPS 1, OPS 2, OPS 3, OPS 4, OPS 5, OPS 9, OPS 10 and OPS 20 was used but no polymorphism between male and female genotypes were found.

b. Scareening of OPRY Series

Eleven random primers of this series were screened which shown good amplification among male and female DNA samples. OPRY 3, OPRY 5, OPRY 7, OPRY 8, OPRY 9, OPRY 11, OPRY 12, OPRY 14, OPRY 15, OPRY 18 and OPRY 20 were used for screening. Of which OPRY 5 showed some male specific



Screening with Kit C1, Kit C8 and Kit C9 for male and female DNA samples at different annealing temperature (M- Male and F- Female)

Plate 2 Screening with earlier reported primers

KERALA AGRICULTURAL UNIVERSITY CENTRAL LIBRARY

c. Screening of OPN Series

Two primers of this series namely OPN 6 and OPN 7 were used for screening but no polymorphism was fond.

d. Screening of RN Series

Result of screening of RN series showed some positive polymorphism towards male genotype. Total nine primers of this series were used for screening *viz.* RN 5, RN 7, RN 8, RN 9, RN 10, RN 12, RN 14, RN 16 and RN 17. Of which RN 5, RN 9 and RN 10 were showed some polymorphic bands during gradient PCR screening (Plate 3.a). But these primers were not able to reproduce the result while rescreening.

e. Screening of OPA Series

Total nine primers of this series were screened for bulk DNA samples of male and female trees. OPA 6, OPA 8, OPA 12, OPA 22, OPA 26, OPA 27 and OPA 38 were did not shown any significant polymorphism between male and female genotypes.

f. Screening of Some Random Primers

According the literature available some random primers were ordered which has reported polymorphism in different plants. It includes OPD 10, OPD 15, OPD 20, OPK1, OPF 14 and OPE 11. But these primers did not shown any polymorphism.

i. Screening of OPAH Series

Only one primer OPAH 12 of this series was used for screening of male and female genotype. This primer was showed positive polymorphism for sex throughout the process of random screening (Plate 3 and 4).

ii. Rescreening with selected primers

On rescreening of selected primers one male specific marker was observed (Plat 3). OPAH 12 was able to produce 550 bp of band for male samples in bulk so it was used with individual plant samples (Plate 4).

iii. Screening with individual plants samples

OPAH 12 was subsequently tested on individual male and female plants of Kodampuli to confirm and it reproduce the 550 bp fragment which was male specific (Plat 3, 4 and 5).

4.2.3 Detection of sex specific RAPD band

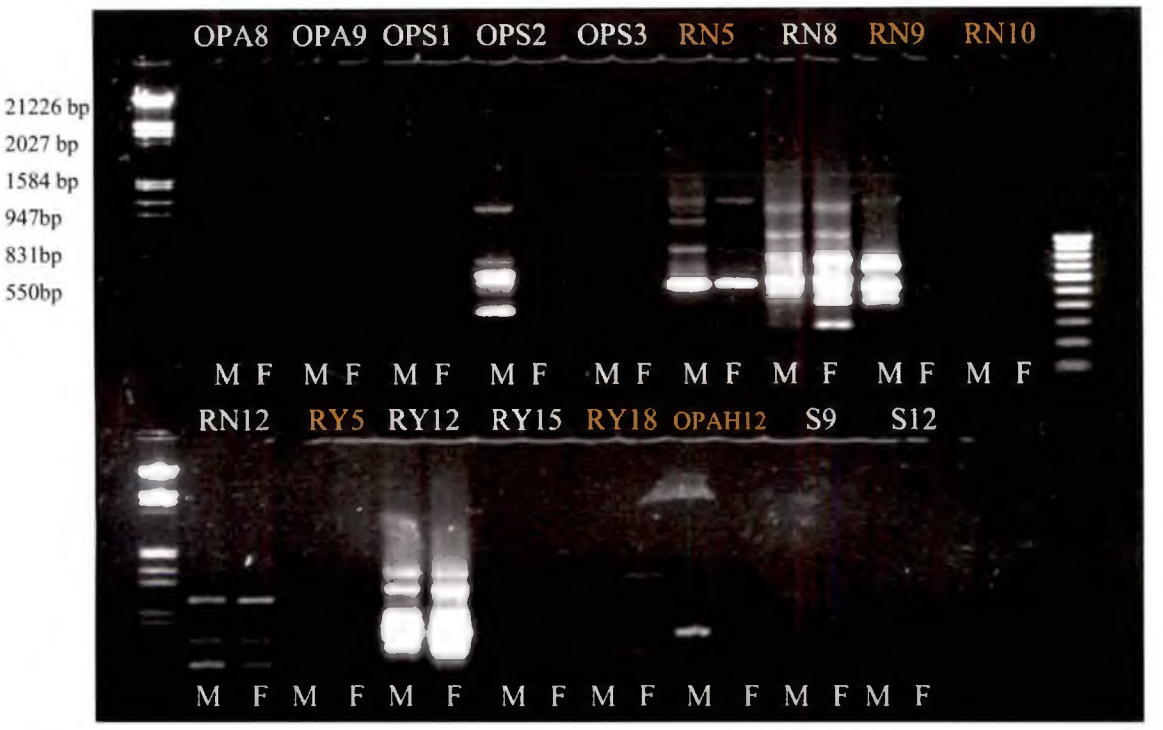
A distinct band of size 550 bp was observed when individual male and female DNA samples were amplified using OPAH 12 primer. This distinct band was polymorphic for male DNA samples only. Consistency and reproducibility of RAPD result was checked by further amplification of OPAH 12 with more number of individual male and female DNA samples. PCR product of this reaction produced same, distinct polymorphic band for male DNA samples. This help to establish RAPD marker for sex detection in Kodampuli. The sex specific RAPD band was cut under UV trasilluminator and subjected to elution with Gel Elution Kit. The quantity estimated was 0.6ng DNA μl^{-1} based on absorbance at 260 nm and 280 nm

4.3 Molecular cloning

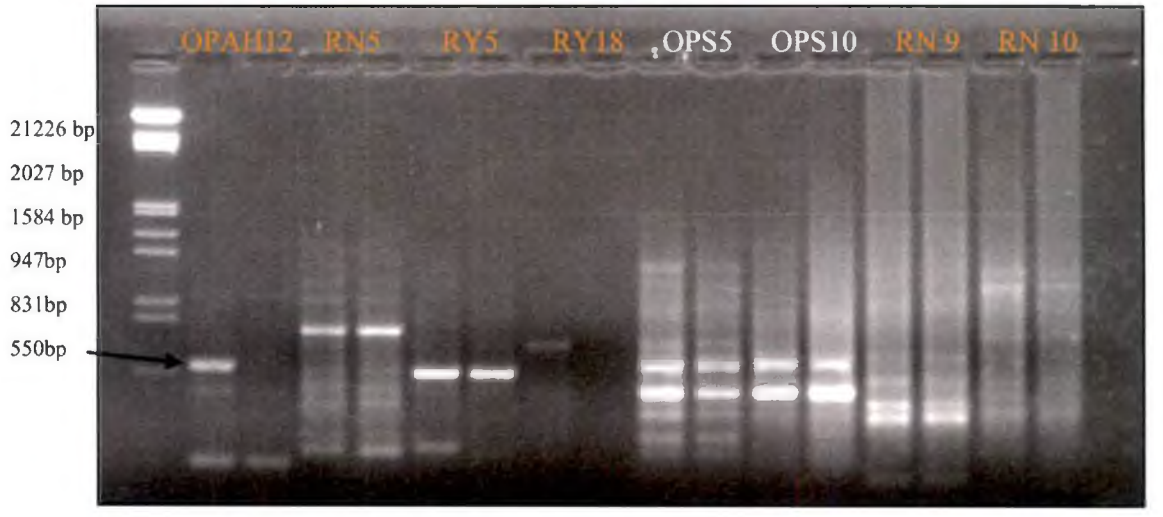
4.3.1 Transformation

4.3.1.1 Preparation and screening of competent cells

The competent cells prepared in section 3.2.8.1 were checked for competence by transforming the plasmid (pUC18) having ampicillin resistance. A large number of blue colonies were obtained which indicated a high degree of

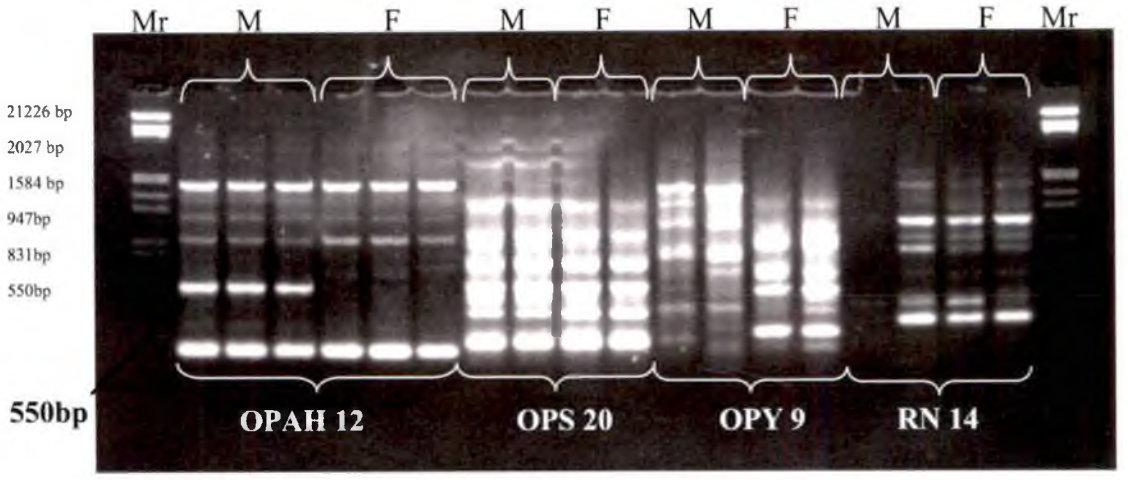


a. Random Primer Screening for Bulked male and female DNA Samples

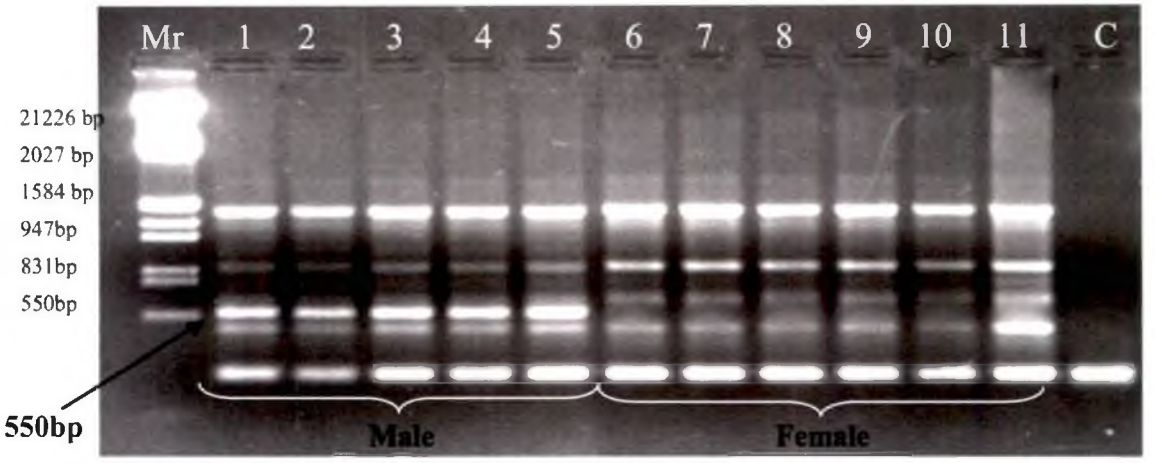


b. Screening with Selective Primers

Plate 3 Screening with RAPD primers for bulked and individual male and female DNA samples

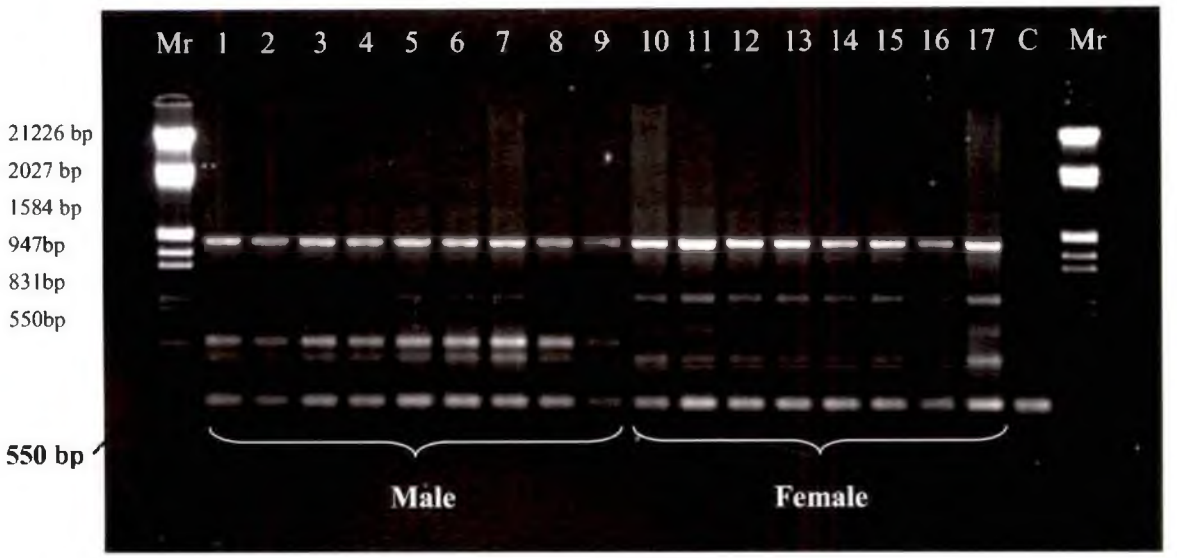


a. RAPD analysis with OPAH 12 for male and female bulked DNA sample
M- Male, F- Female, Mr- Marker.

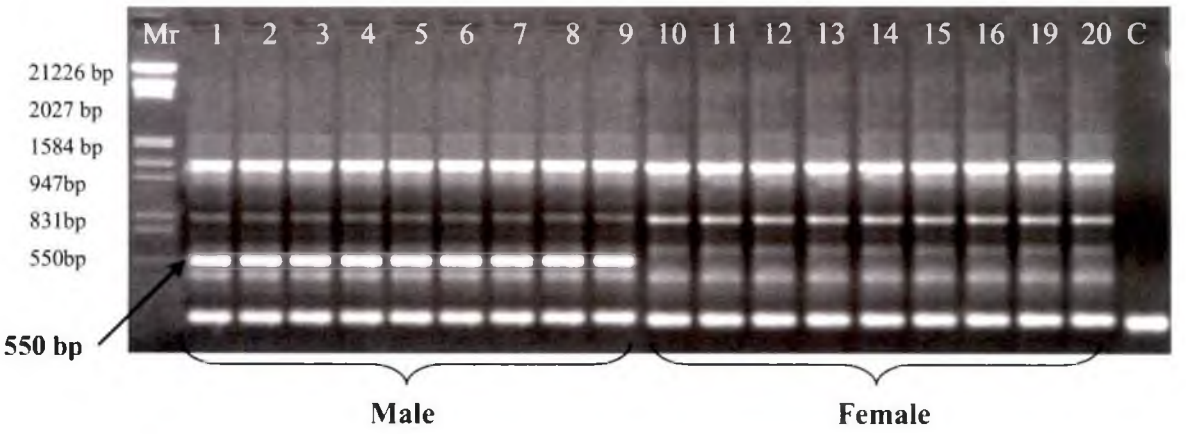


b. RAPD analysis with OPAH 12 for individual male and female DNA samples
Mr- Marker, C-negative control

Plate 4 Screening with OPAH 12 primer for bulked and individual male and female DNA samples



a. RAPD analysis with OPAH 12 primer for individual male and female
Mr- Marker, C- Negative control



b. RAPD analysis with OPAH 12 primer for individual male and female
Mr- Marker, C- negative control

Plate 5 Screening with OPAH 12 for bulked and individual male and female DNA samples

transformation efficiency. Thus the competent cells prepared were found ideal for cloning amplicons.

4.3.1.2 Ligation

The eluted RAPD product (insert) required for ligation was calculated using the equation described in section 3.2.11.3.a. Thus, 30ng of insert was used per 50ng of vector for ligation reaction using pGEMT vector, considering the fact that the eluted DNA sample had 0.6ng DNA μl^{-1}

A. Transformation of ligated product

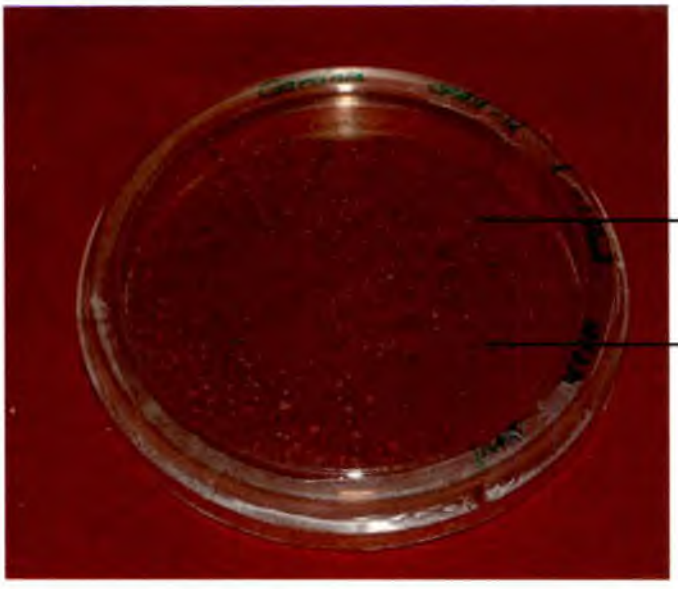
The ligated product was used to transform the prepared competent cells using the heat shock method and was incubated at 37°C. Large number of blue and white colonies was obtained after overnight incubation when the transformed cells were cultured in LB/ampicillin media, in the three plates; overlaid with X gal and IPTG (Plate 6.a).

B. Screening of the transformed colonies

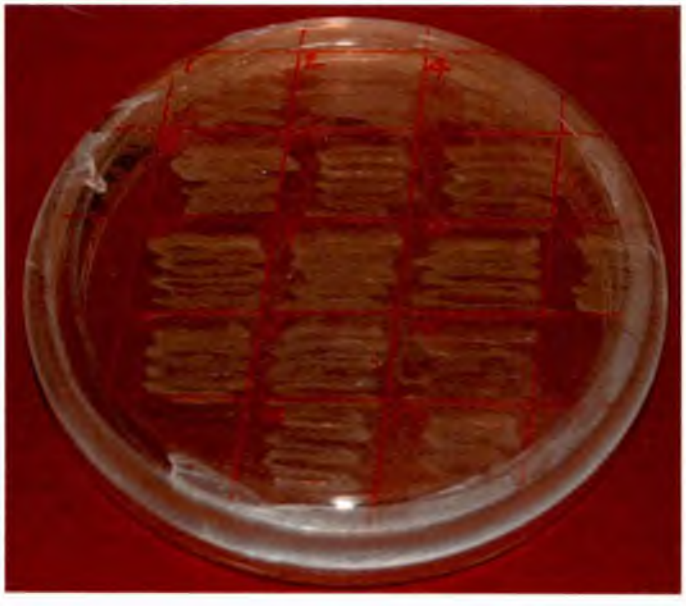
The LB ampicillin agar plates containing the transformed colonies were screened for recombinant plasmid. For maintenance of clones white colonies were picked from plate and were grown in LB ampicillin media separately. Two blue colonies were also inoculated in order to set the negative control (Palte 6.b).

C. Detection of the insert by colony PCR

Confirmation of insert presence was carried out by performing colony PCR amplification of recombinant plasmid DNA using T7 and SP6 Primers. The PCR reaction was prepared as given in 3.2.8.3.C. The PCR products when checked on 1 percent agarose gel showed amplicons of 550 bp sizes whereas no band was found in blue colony sample (Plate 7).



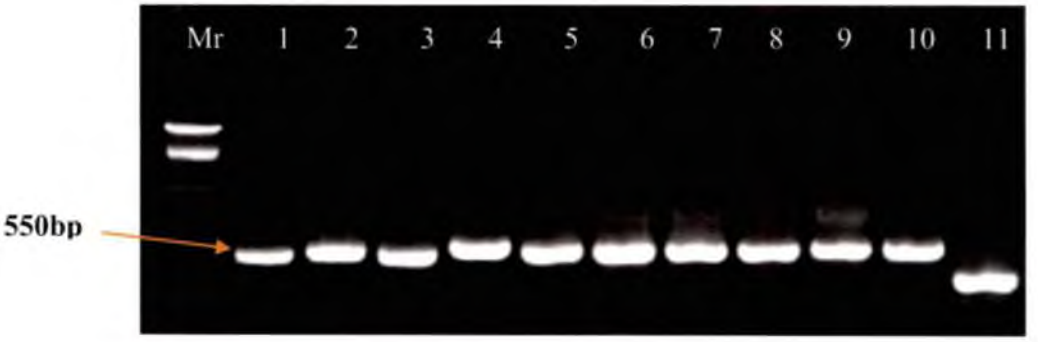
a. Blue white screening of transformed *E. coli*. Cells



b. Maintenance of Clones

1 to 15- white colonies, 16 & 17 blue colonies

Plate 6 Screening of Transformant Cells and Maintenance of Clones



Confirmation of inserts by colony PCR (white colonies 1-10, blue colony-11).

Plate 7 Confirmation of insert

CENTRAL LIBRARY

4.4 Sequence analysis

After confirmation of insert stabs were prepared and sent for automated sequencing. The outsourcing facility available at Genie, Bangalore was utilised. The sequences thus obtained were used for sequence analysis and designing SCAR primers.

4.4.1 Vector Screening

The sequences obtained after automated sequencing were subjected to vector screening using the VecScreen tool in NCBI to remove vector regions from the clones (Fig. 1a). The 977 bp obtained sequence shown vector sequence at different levels *viz,* strong, moderate and weak. The region starting from 1 to 31 bases and 589 to 977 bases showed similarity with the pGEM-T Easy Vector sequence and it was deleted from the original sequence. Which was edit out with the help of Bioedit-Biological sequence alignment editor tool (Fig. 1b). This gives about 554 bp crude sequence (Fig. 2a).

4.4.2 Search for Homology

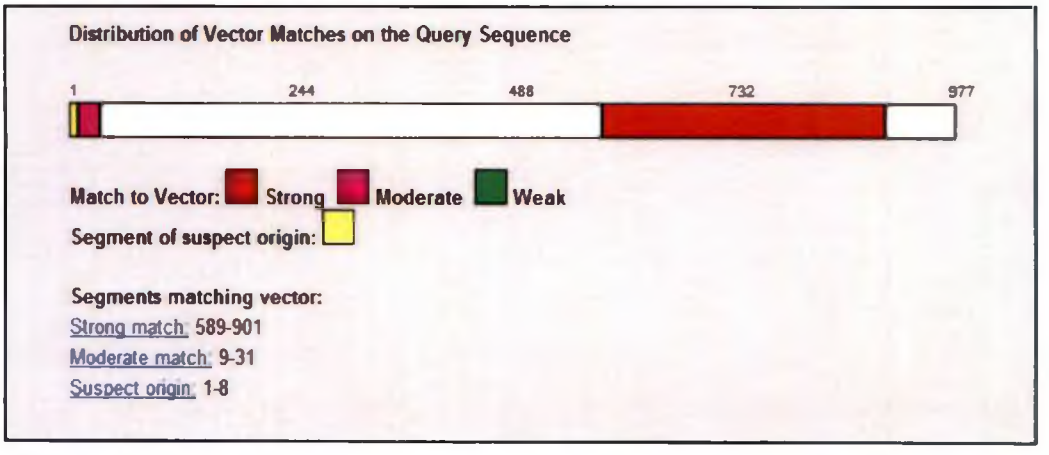
The nucleotide sequence of polymorphic band was compared with the sequences available in nucleotide database using BLAST tool (www.ncbi.nlm.nih.gov/Blast/) provided by NCBI. This was not registered any significant homology with available database (Fig. 2b).

4.4.3 Detection of Open Reading Frame (ORF)

To find the open reading frame of the insert nucleotide sequence, the programme 'ORF finder' (www.ncbi.nlm.nih.gov/gorf/gorf.html) of NCBI was used. The displayed web page showed ORF sequence in all reading frames (Fig. 3b). The longest ORF found was of 215 amino acid for Blastp.

TCCATCCTCCGGCCGCCATGGCCGCGGGATTTCAACGGCTGAGGAGG
CCAGAAAGAAGTCTTAGAGAGAGATGTAGGCTCTCTAAAATTCTTTA
ATACTCGCGACGGCTGAGACAGAGAAATACAAGAAACGCCTCCACT
GAGCCTCAAAGAGAAATGATGCCTTGAGGTGAGAGACTGATAAATA
GGCAAGGTGGAGGGAAAATATCCCTTCCACCATGGTGTTAACATATA
GTCAGTCAACAGAGTTCAAGGAGTTCGTCGAATAGGTTGGCACCAAG
GGATATTTGAAGGGTGCTTTGGCCTATAAGGAGCCAATTTTGAAGTT
TAATCTCACTTCCACAGTGGAGGGCGCCAACTCGGAAGTACCCGTAT
TAGGCGTTGATATTGGAGTGAATGAGGTTCCACCTTCTCCCCAATTCG
ACCTATCTTCTGTGGACTTAGACGGGGAGGTTCCTACTGGAACTTTTT
CAGGTCTAACTGCTCCAGTAGACACAACTGAAGTAGGGGGGACTGA
GGAGGGGGGAAATGAAGGCACCTGAGCCGAGATGCCTACGAACGAT
ATAGTTCCCTCTGATGAGCCGTTGGAAATCACTAGTGCGGCCGCCTG
CAGGTCGACCATATGGGAGAGCTCCCAACGCGTTGGATGCATAGCTT
GAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTAATCATGGTCAT
AGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACA

a. DNA sequence of male specific RAPD band

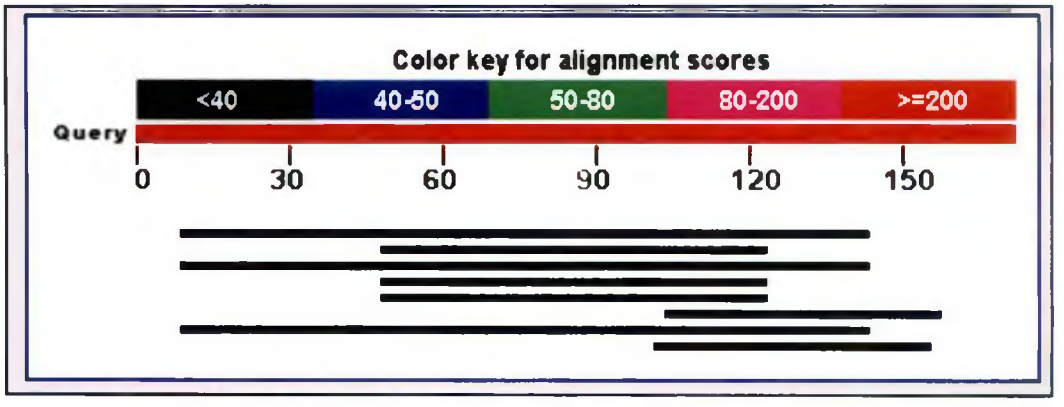


a. VecScreen output

Fig. 2 Results of sequence data analysis for the clone

TCCAACGGCTGAGGAGGCCAGAAAGAAGTCTTAGAGAGAGATGTAGGC
TCTCTAAAATTCTTTAATACTCGCGACGGCTGAGACAGAGAAATACAAG
AAACGCCTCCACTGAGCCTCAAAGAGAAATGATGCCTTGAGGTGAGAG
ACTGATAAATAGGCAAGGTGGAGGGAAAATATCCCTTCCACCATGGTG
TTAACATATAGTCAGTCAACAGAGTTCAAGGAGTTCGTCGAATAGGTTG
GCACCAAGGGATATTTGAAGGGTGCTTTGGCCTATAAGGAGCCAATTTT
GAAGTTTAATCTCACTTCCACAGTGGAGGGCGCCAACTCGGAAGTACCC
GTATTAGGCGTTGATATTGGAGTGAATGAGGTTCCACCTTCTCCCCAAT
TCGACCTATCTTCTGTGGACTTAGACGGGGAGGTTCCTACTGGAACTTTT
TCAGGTCTAACTGCTCCAGTAGACACAACTGAAGTAGGGGGGACTGAG
GAGGGGGGAAATGAAGGCACCTGAGCCGAGATGCCTACGAACGATATA
GTTCCCTCTGATGAGCCGTTGGA

a. Bioedit output



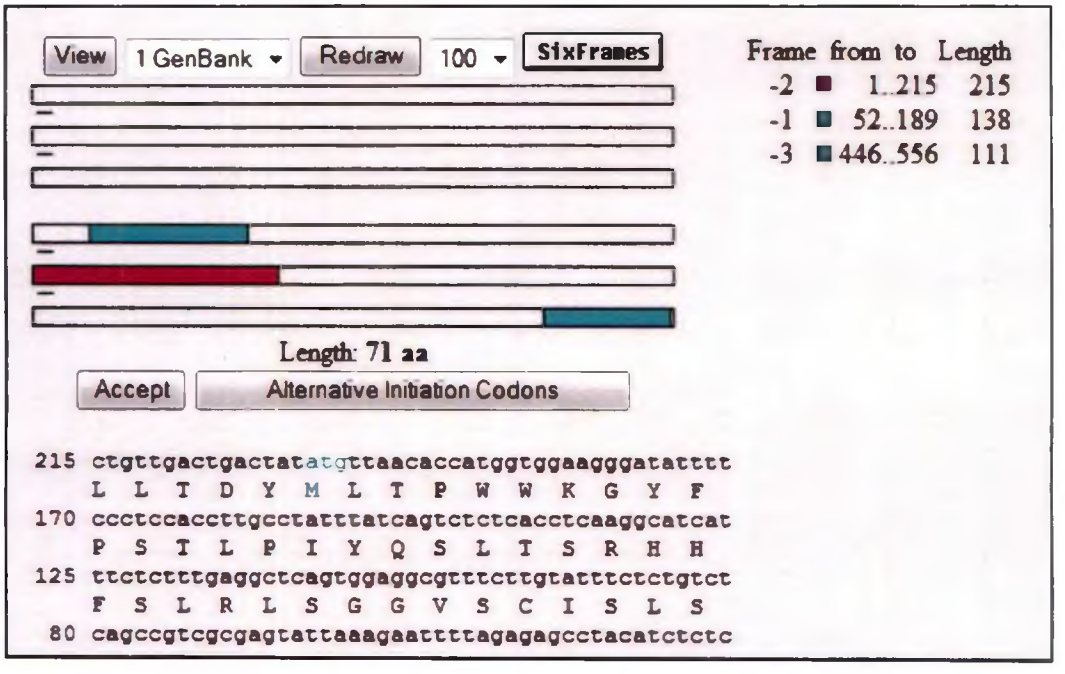
b. Blastn output

Fig. 3 Results of sequence data analysis for the clone

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Links
YP_003050891.1	Radical SAM domain protein [Methylovorus sp. SIP3-4] >gb|ACT50:	35.0	35.0	78%	3.1	G
ZP_03364103.1	hypothetical protein SentesTyph_14169 [Salmonella enterica subs	34.7	34.7	43%	3.5	
ABZ07609.1	putative Radical SAM superfamily protein [uncultured marine micro	34.7	34.7	78%	3.6	
ZP_03374678.1	hypothetical protein SentesTyp_31976 [Salmonella enterica subsp	34.7	34.7	43%	3.7	
ZP_03351508.1	hypothetical protein Salmonentericaenterica_11358 [Salmonella e	34.7	34.7	43%	3.8	
ZP_04189117.1	hypothetical protein bcere0028_51980 [Bacillus cereus AH1271] >	34.3	34.3	31%	4.5	
YP_545048.1	radical SAM family protein [Methylobacillus flagellatus KT] >ref|YP_	34.3	34.3	78%	4.8	G
XP_957245.2	hypothetical protein NCU00121 [Neurospora crassa OR74A] >gb|E	33.9	33.9	31%	5.7	UG

a. Blastn output



b. Open reading frames

Fig. 4 Results of sequence data analysis for the clone

4.5 Designing the SCAR primers

Based on the parameters described in Section 3.2.14, two pair of SCAR primers was designed for sequence of polymorphic RAPD fragment generated with OPAH 12 (Fig. 2.a)

Designed primers were named as Pt1 and Pt 2. For Pt1 forward and reverse melting temperature was 65^0C and 67^0C respectively, GC content was 63 and 56 per cent. Pt2 have melting temperature 65^0C for both forward and reverse primers, GC content was maintained at 45 and 52 per cent. Both Pt1 and Pt2 primer pair sequences had no palindromic or repetitive sequences.

4.6 Confirmation of SCAR maker

For screening SCAR primers two combinations were used. One with as given in Table 5 and other combination of Pt1 forward to Pt2 reverse and Pt1 reverse to Pt2 forward. The expected band length for Pt1 was 500bp while for Pt2 it was 400 bp.

A. Screening of SCAR primers at 55 ^{0}C annealing temperature

Initially SCAR primer amplification was performed at annealing temperature 51^0C, the lowest Tm for Pt 2. At this temperature no amplification was found for both pairs. On gradual increase of annealing temperature, some bands were observed at 55^0C (Plat 8) but it was not specific for sex. At this same temperature the combination of reverse and forward primers was performed. It results into number of spurious product formation. This combination was not used for further experiment.

B. Screening of SCAR primers at 60^0C and 65^0C

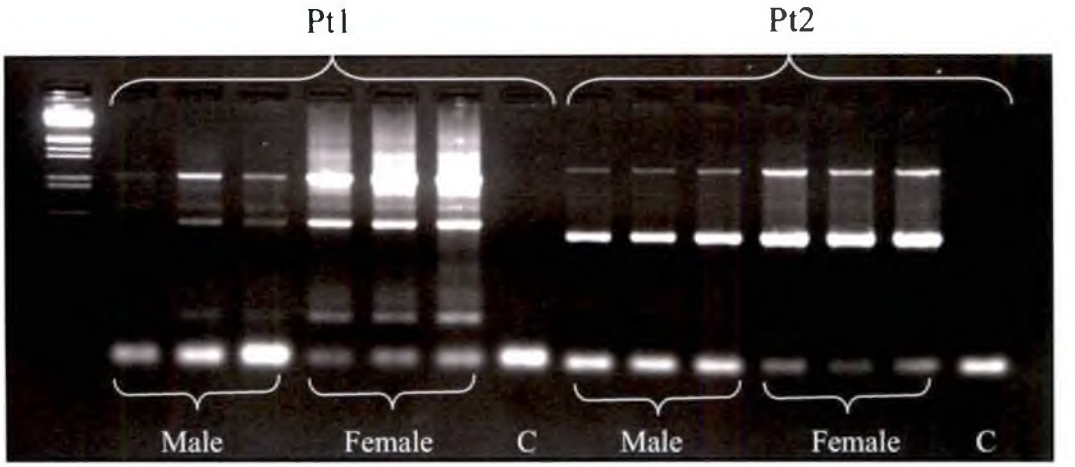
Further gradual increase in annealing temperature at 60^0C, both Pt1 and Pt2 given a distinct band of 500 bp. At 65^0C no amplification was found for Pt1 whereas Pt2 had emaciated only one distinct band with 500 bp, but present in both

DNA male and female DNA samples (Plat 9). But in both the cases SCAR primers were failed to identify male and female in Kodampuli.

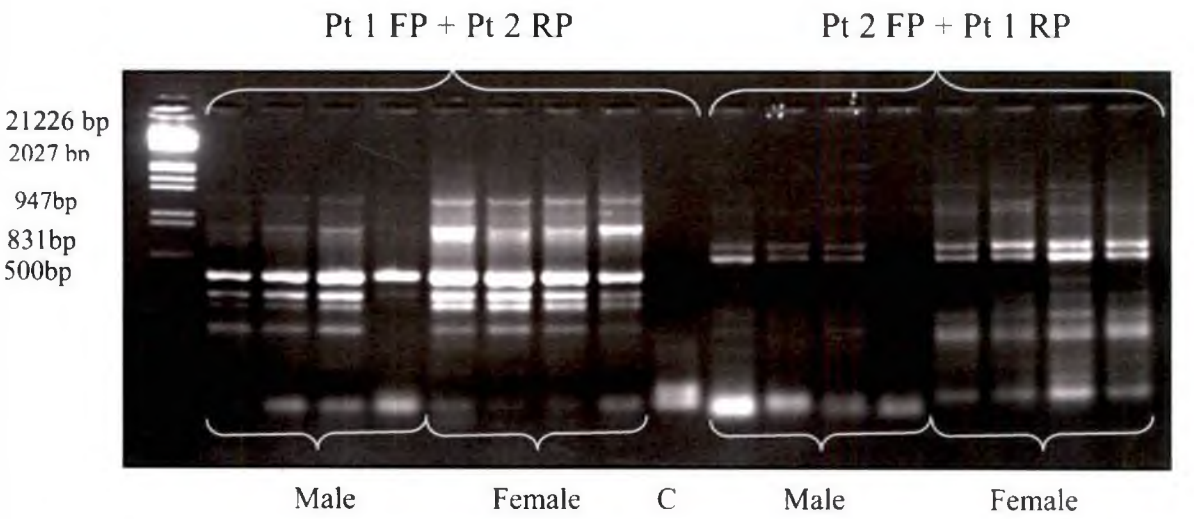
Table 5 SCAR Primers Designed for RAPD Band

No. of Base pair	SCAR primer	Sequence (5'-3')	G+C Content (%)	Tm (^{0}C)
22	Pt1FP	TCCAACGGCTGAGGAGGCCAGA	63	65
23	Pt1RP	CAAGGGAGACTACTCGGCAACCT	56	67
24	Pt2FP	GAAGTCTTAGAGAGAGATGTAGGC	45	65
23	Pt2RP	GTCTACTGGAGCAGTTAGACCTG	52	65

FP: forward primer, RP: reverse primer

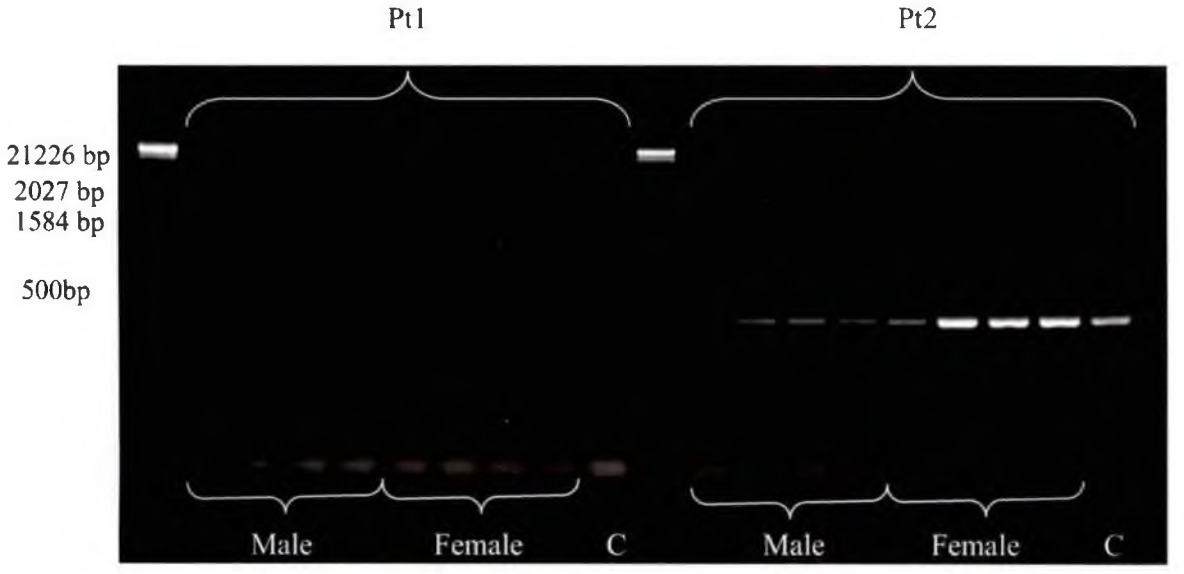


a. Screening of SCAR primers at annealing temperature 55^0C

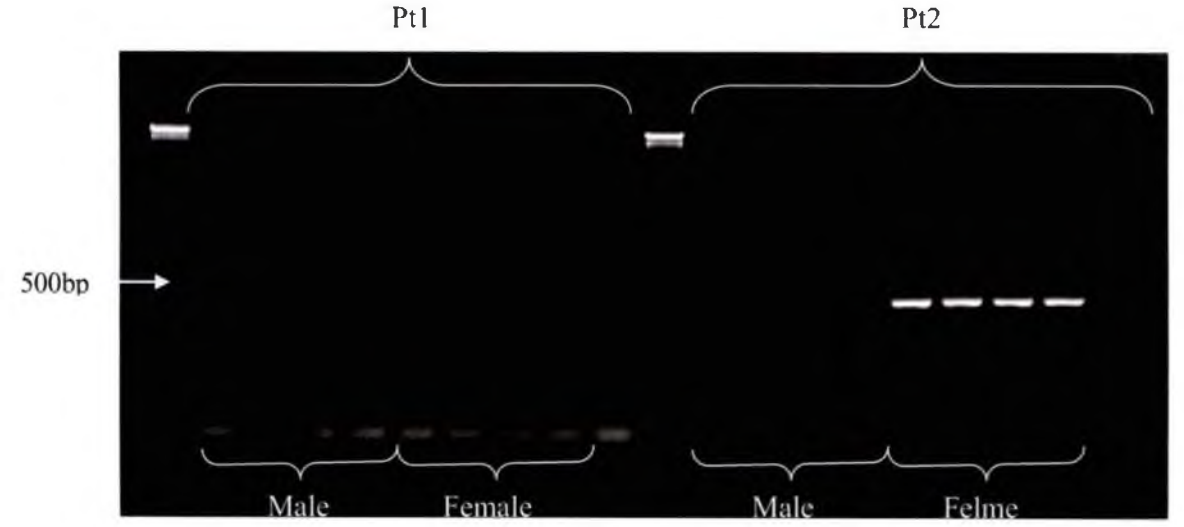


b. Screening of SCAR primers in combination at annealing temperature 55^0C

Plate 8 Amplification Profile Analysis with SCAR Primers



a. Screening of SCAR primers at annealing temperature 60^0C



b. Screening of SCAR primers annealing temperature 65^0C

Plate 9. Amplification Profile Analysis with SCAR Primer

KERALA AG CULTU
CENTRAL
LIBRARY
P.O.), THR

Discussion

5. DISCUSSION

Dioecious plants are estimated to constitute very small percent of all flowering plants and are represented in almost all families (Yamposky and Yamposky, 1992; Ainswarth, 2000). Only 6 percent of angiosperms are dioecious in nature, where individual either produce staminate or pistillat flowers (Renner and Rocklefs, 1995). Kodampuli is also belonging to the polygamodioecious group, which makes it difficult to identify sex at earlier stage. This decade has witnessed an increasing number of research efforts directed at identifying and characterizing molecular markers and gene involved in plant dioecy (Parasnis *et al.*, 1999, Kafkas *et al.*, 2002 and Khadka *et al.*, 2002). Modern DNA techniques and molecular markers are an extensive tool to study dioecious nature and variability among male and female genomes.

Progress has been made in mapping and tagging many agriculturally important genes with molecular markers, which forms the foundation for marker-assisted selection. Molecular markers have several advantages over the traditional phenotypic markers that were previously available to plant breeders. Using recombinant DNA technology, desirable cloned genes could be transferred to the varieties of different crops within limited time. Use of molecular markers gives accurate information about the particular trait of the genotypes and eliminates environmental influence in various important characters of genotypes in field conditions. Availability of sex linked markers will help in identifying plants in early stage for male or femaleness without expense of money and time. No doubt, when reliable markers are identified and gene is tagged with them they would prove to be a very powerful tool especially in perennial spice like Kodampuli.

Present investigation dealt with the development of simple PCR based gender diagnostic system in Kodampuli. The results of the various aspects of the present investigation, *viz.*, Isolation, Purification and Quantification of DNA from male and female trees, screening of RAPD primers, molecular cloning, sequence

analysis, designing of sex specific SCAR primes and confirmation of SCAR maker were presented in sequential order.

5.1 Isolation, Purification and Quantification of DNA

The genomic DNA was isolated from twenty five fully mature mature trees male and female. Kodampuli contains high amount of polysaccharides, secondary metabolites like HCA and polyphenols. The isolation of good quality of DNA from Kodampuli is complicated due to presence of phenolic compounds, highly viscous polysaccharides and DNA degrading endonucleases (Sherly, 1994).

Young, tender leaves from male and female trees were used for isolation of DNA. This is because 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), who reported that the best quality and quantity of DNA from tender leaves compared to mature and half mature leaf sample. The problem of polysaccharides is aggravated if green, over matured tissue is taken rather than etiolated leaves (Sharma *et al.*, 2000).

The homogenization, pulverisation and uniformity of grinding of plant tissue were essential during DNA extraction. Excess liquid nitrogen was used for the homogenization of leaf tissue. The use of liquid nitrogen has double functions; the maintenance of frozen tissue and prevention of nucleic acid degradation and secondary metabolites oxidation that may form some complexes and co-presipitation with DNA; and a better mechanical disruption of tissue (Hernandez and Oyarzum, 2006).

The presence of high amount of polysaccharides was a problem during DNA extraction from Kodampuli (Sharma *et al.*, 2000). The polyphenols reduce the purity of DNA by binding covalently with the extracted DNA and thus making

it unsuitable for most molecular research applications. This problem was overcome by the addition of ß- merceptoethanol and Poly Vinyl Pyrrollidone (PVP) along with the extraction buffer. ß- merceptoethanol disrupt the protein disulphide bond and thereby capable of initiating protein degradation. Poly Vinyl Pyrrollidone (PVP) removes polyphenols along with high molecular concentration of NaCl thereby inhibited co-precipitation of polysaccharides and DNA was also confirmed by De la Cruze *et al.* (1997) and Matasyoh *et al.* (2008).

The detergent used in the extraction buffer of the protocol is CTAB (Cytyl Trimethyl Ammonium Bromide), which helps in the disruption of the cell membrane thereby releasing nucleic acid into the extraction buffer and prevents co-precipitation of polysaccharides with nucleic acid by action as a selective precipitant of nucleic acids. CTAB is cationic detergent, which solubilises membrane and form a complex with DNA (Sghaier and Mohommed, 2005). The DNA isolated by Roger and Benedich (1994) protocol was free from chlorophyll by the Chloroform : Isoamyl alcohol which is added to prevent forming of mixture and to aid in the separation of organic mixture and aqueous phase.

Problem encountered in the isolation and purification of high molecular weight DNA from certain plant species include: degradation of DNA due to endonuclease, polyphenols and other secondary metabolites that directly and indirectly interfere with subsequent enzymatic reactions as reported by Weishing *et al.* (1995) and Matasyoh *et al.* (2008). EDTA present in the extraction buffer could protect the DNA from the action of DNase enzyme by chelating and blocking the action of Mg^{2+} ions, which are the major co-factor of DNase enzyme. EDTA was also major component of TE buffer in which the DNA dissolved and stored. RNase treatment was given in order to remove RNA contamination from the isolated DNA samples (Plat 1b) similar to the result of Sambrook *et al.* (1989).

5.2 RAPD Assay

The RAPD technique was developed by Williams *et al.* (1991). This technique relies on the differential enzymatic amplification of small DNA fragments using PCR with arbitrary oligonucleotide primers. In RAPD markers, polymorphism results from changes in either the sequence of the primer binding site (which prevents stable association with the primer) or from mutations like insertion, deletion, inversion etc. (which alter the size or prevent the successful amplification of a target DNA). Usually RAPD markers are dominant in nature because polymorphisms are detected as the presence or absence of bands. This procedure is rapid, requires only small amounts of DNA, which need not be of high quality, and involves no radioactivity.

5.2.1 Screening with earlier reported primers

Initial screenings of the primers were performed with bulked DNA samples. It was essential to identify those primers which produce polymorphic amplicons between the male and female plants. It was also important because Kodampuli is heterozygous, cross-pollinating crop. Premjith and Rajendran (2008) reported that three primers *viz.*, Kit C1, Kit C8 and Kit C9 were shown sex specific polymorphism.

PCR reactions for earlier reported primers were performed with protocol mentioned in 3.2.6.1. But no polymorphism was observed. Because of which optimization PCR parameters and modification of annealing temperatures were tried as similar to Horejsi *et al.* (1999) and Paran and Michelmore, (1991). At different annealing temperature all three primers were produced different banding pattern (Plat 2). When these reported primers were rechecked with use of 2 µl of primers instead of 1 µl and the use of 20-30 ng of DNA instead of 50 ng in PCR reactions, no polymorphism was found. This was the main problem regarding to RAPD marker where reproducibility will vary according to thermal cycler, annealing temperature and other parameters of reaction. These research results were in collaboration with the findings of Paran and Michelmore (1993) who

proposed that to overcome the problems associated with RAPD analysis and to improve their utility in MAS application, the RAPD markers can be converted into SCAR markers.

5.2.2 Further screening of primers for RAPD

Pooled male and female DNA samples were further screened with forty-six random decamer primers. Out of the 46 primers screened and six primers were showed good polymorphic banding pattern (Plate 3). So these six primers were used for rescreening with same bulked DNA samples. On rescreening of six primers, OPAH 12 had shown polymorphic band for male DNA samples.

At that point of time it was difficult to say that OPAH 12 could confirm RAPD marker for sex identification in Kodampuli. For confirmation of this result, OPAH 12 was tested with some individual male and female DNA samples (Plate 4 and 5). OPAH 12 reproduced the same polymorphic band of nearly 550 bp for male DNA samples. Again PCR reactions were performed with eighteen male and seventeen female individual DNA samples which showed reproducibility for OPAH 12 (Plate 5) similar results were reported by Varma (2009) in Black pepper for designing linkage map. It would be helpful to establish RAPD marker for sex identification in Kodampuli.

5.2.3 Detection and Elution of Sex Specific Amplicon

The individual DNA samples were amplified with OPAH 12 and observed distinct RAPD band of 550 bp size. This polymorphic band was eluted with process described in 3.2.7, by using AxyPrep DNA Gel Extraction Kit (Axygen, Biosciences). The eluted polymorphic band was cloned into pGEMT vector. This finding was in line with Yiping, Z. and John, R.S. (2001) who performed cloning of *Beta* gene in Tomato.

5.3 Bacterial transformation

Competence of JM109 *E. coli* cells was confirmed by transforming the cells with plasmid (pUC18) having ampicillin resistance. E. coli cells alone could not grow on ampicillin containing media, as they lack the gene for ampicillin resistance. But the competent cells harbouring the plasmid could grow in the media. In the present study, large number of blue colonies was observed on LB/ampicillin plate after overnight incubation at 37°C, confirming the competence of *E. coli* (JM109) cells for transformation.

For cloning of RAPD fragment, pGEMT vector of approx. 3kb size was used. It contains T7 and SP6 RNA polymerase promoters that flank a multiple cloning region within the α peptide coding region of the enzyme β-galactosidase. Thus, due to insertional inactivation of the α peptide region, the recombinants could be directly identified by the blue-white screening of indicator plates. The vector contains multiple restriction stress within the multiple cloning regions thus facilitating easy ligation of insert and its release by digestion with restriction enzyme.

In the present study, the ligated product containing sex specific RAPD fragment was used to transform the competent cells. The white colonies could be easily distinguished and picked up from the selection media containing 5-bromo-4-chloro-3-indolyl β-D galactoside (X-gal) and isopropyl thiogalactoside (IPTG).

pGEMT vector contained polycloning sites inside a gene encoding for β-galactosidase. Thus, insertion of a new sequence would disrupt the reading frame of galactosidase encoding gene. As a result of α-complementation, the bacterial cell and vector together provided the complete protein, because one part of the gene was present in bacteria while the other in vector (Ullmann *et al.* 1967). The colonies which have not taken up the plasmid further utilized the substrate and appeared as blue colonies on chromogenic substrate, X-gal (Horwitz et al., 1964).

Due to the disruption of α-complementation, all the transformed colonies harbouring the recombinant plasmid appeared as white (Plate 6).

The plasmid DNA isolated from white and blue colonies gave bands with different molecular weights. Most of the plasmids from white colonies had higher molecular weight than the plasmid of blue colonies due to the presence of insert.

The presence of the insert was further confirmed by PCR amplification of the plasmids with T7 and SP6 primers. The PCR product of blue colony plasmid had no bands since it lacked the insert and only T7 and SP6 regions present in it could get amplified which was very small. While, the PCR product of white colonies had higher molecular weight as the insert was also amplified (Plate 8) along with T7 and SP6 regions of the vector and given band of 550 bp. This is in line with the report of Kushawa (2008).

5.4 Sequence Analysis

Since there is no automated sequencing facility at KAU and it would be highly laborious to sequence the clones manually the outsourcing facility providing by GeNei was used. The DNA sequences that were obtained used for *in silico* analysis.

Sequence information obtained for the fragment with OPAH 12 primer had a size of 977 bp (Fig. 1a). The sequence product was subjected to vector screening using the VecScreen tool in NCBI to remove vector regions from the clones (Fig.1b). The region starting from 1 to 39 bases and 589 to 977 bases showed similarity with the pGEM-T Easy Vector sequence, and it was deleted from the original sequence. The sequence of 552 bases sequence obtained after vector screening is presented in Fig. 2a used for further analysis. This is in line with the report of Rashmi (2008).

Blastn and Blastp output did not show significant similarity with database (Fig. 2b). The sequence was found to have three ORFs with the longest one being 215 bp, in length. (Fig. 3b).

5.5 Designing of SCAR Primers

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 affected by DNA quality, primer and template concentration, different thermocyclers and even different source of DNA polymerase (Ellsworth *et al.* 1993, Muralidharan and Wakeland., 1993). Subsequent conversion of RAPD to SCAR markers could develop more specific primers with significantly improvement in the reproducibility and reliability of PCR assay as reported by Paran and Michehnore (1993).

Two pairs of SCAR primers from the termini, as well as internal site of marker were designed in order to amplify full length. One primer pair called Pt1 contained the original 10 bases of the RAPD primer plus other from each end of PCR product. The expected band length for first pair was 500bp and 400bp for other. Annealing temperature, melting temperature and GC% of the synthesized primers from Sigma Aldrich Chemicals Pvt. Ltd. used for the amplification of male and female DNA samples were depicted in Table 5.

5.6 PCR amplification using SCAR primers

RAPD polymorphisms presumably result from differences in nucleotide sequence at priming sites. In converting RAPD markers to SCARs, Paran and Michel- lettuce (1993) found that six of nine RAPD polymorphisms were derived from mismatches in one or a few nucleotides in the priming sites. These mismatches were tolerated by extended SCAR primers, producing undifferentiated amplification products from both parents of lettuce. Similarly in the present investigation of SCAR primers, specific for sex were amplified a single monomorphic PCR product in both male and female plants.

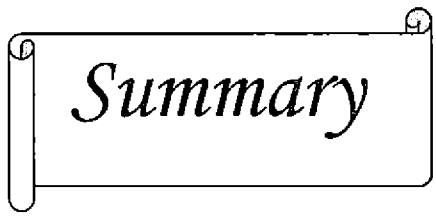
According to Horejsi *et al.* (1999) and Paran and Michelmore (1993), optimize PCR parameters like modification of annealing temperatures and Mg^{2+} concentrations were considered to be most effective. Increased annealing

temperature could eliminate amplification of the allele from one of the parents by taking advantage of differing amounts of mismatches within a primer sequence. By elevating annealing temperature from 60^0C to 67^0C, Paran and Michelmore (1993) recovered a dominant SCAR marker in lettuce (*Lactuca sativa* L.). Similar modifications were tried with the SCAR primers amplification, the annealing temperature was increase from 55^0 C to 65^0C and at each step polymorphism was checked. Initially at 55^0C, spurious product was found with number of bands (Plate 9). Optimization of PCR parameters was done with increase in annealing temperature. This result was in line with Paran and Michelmore (1993), as the annealing temperature increases only one distinct band of 550 bp was found.

However, in converting SCAR markers in citrus (*Poncirus trifoliata* L.), Deng *et al.* (1997) found that when annealing temperatures were increased from 50^0C to 66^0C to 68^0C, the polymorphic amplification products identified between two parental lines were not reliably reproduced. Lowering annealing temperature to the predicted optimal melting temperature allowed them to amplify a clear and reproducible length polymorphism. In the present study findings are in phase with Deng *et al.* (1997), as the variation in annealing temperature given single band. But the distinct band was monomorphic for sex and fails to identify male and female in Kodampuli (Plate 9).

Both RAPDs and SCARs may find applications in marker-assisted breeding applications because they are simple, fast and inexpensive, do not require the use of radioactive isotopes, and can be scaled up for analysis of high throughput applications. SCARs have several advantages over RAPD markers in MAS. Because more stringent reaction conditions are used the SCAR markers are generally more allele specific. The SCAR amplifications are more stable and reliable, and more easily reproduced in different laboratories with various thermal cyclers. Whereas the RAPDs are generally dominant markers and at the same time the codominant SCARs are more useful in high-resolution mapping and genetic studies (Paran and Michelmore, 1993). In the present study, two codominant SCAR markers were tried to develop which could be utilise in marker assisted

programs to track females of Garcinia for improving the hindering extensive cultivation of this backyard companion crop in Kerala and other coastal regions of the country. So there is need to design more efficient SCAR primers for easy detection and there is also possibility for using RAPD marker directly for sex detection directly.


Summary

6. SUMMARY

The study on "Development of SCAR marker for authentication of gender in Kodampuli (*Garcinia gummi-gutta* var. *gummigutta*)" was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara during the period 2008-2010. The main objective of the study was to establish a simple and reliable gender-diagnostic system for Kodampuli by using PCR based techniques. The genotypes used for study were adult, mature 25 male and 25 female trees of Kodampuli from KAU campus.

The salient findings of the study are summarized below:

1. As a preliminary step, adult and mature, male and female trees were selected from Department of Olericulture and Department of Pomology, College of Horticulture, Vellanikkara.

2. The CTAB method adopted could yield good quality DNA from male and female plants and the quantity of recovered DNA was between 511.4ng/µl to 976.5ng/µl . The ratio of OD_{260}/OD_{280} for both the samples was between 1.8-2.0, indicating good quality DNA.

3. Bulking of the samples was performed by mixing equal quantities of DNA isolated from five male and five female trees. This was done because Kodampuli is heterozygous and cross-pollinated in nature.

4. Primarily, earlier reported primers *viz.* Kit C1, Kit C8 and C9 were screened for bulked male and female DNA samples. Primer screening was performed with protocol given by Premjith and Rajendran (2008) as well as with suitable modifications, but no polymorphism was found.

5. Further screenings of random forty-six primers were done with bulked DNA samples. Initially primers giving good polymorphism were selected for further screening.

6. Gradient PCR screening of 10-mer primers at a time gave some positive response towards OPAH12, RN5, RY5, RY18, OPS 5, OPS10, RN 9 and RN 10. On rescreening of these eight RAPD primers OPAH 12 showed a distinct polymorphic band of 0.5 kb for male bulk sample (Plate 3a).

7. OPAH 12 was again tested for bulk and individual DNA samples. This confirmed the consistent and reproducible RAPD marker in Kodampuli.

8. The polymorphic RAPD fragment of 0.5 kb obtained OPAH 12 was eluted from agarose gel and cloned into pGEMT vector and this ligated product was transformed with competent *E.coli* cells. A large number of blue and white colonies were obtained after overnight incubation.

9. Presence of insert was checked by colony PCR. It amplified an amplicon of 550 bp in plasmid of white colony and absent in plasmid from blue colonies.

10. The cloned insert was sequenced using universal primers T7, SP6 .

11. Vector screening was performed to remove the vector sequence from Garcinia sequence using the VecScreen tool in NCBI. The cloned insert sequence 550 bases was obtained and used for *in silico* analysis.

12. The sequence obtained after vector screening was subjected to nucleotide Blast search. It did not show homology with any available nucleotide data base. In case of marker study this was not mandatory to get homology for maker linked sequence because it might be a region which present in between two genes.

13. The sequence was found to have three ORFs with the longest one being 215 bp, in length.

14. Two pairs of SCAR primers were designed for polymorphic band. Forward and reverse primers had a melting temperature of 65^0C and 67^0C for Pt1 and $65\ ^0$C for Pt 2 respectively.

15. Amplification of SCAR primer was performed at different temperature but it was not able to identify male and female plants in Kodampuli.

References

REFERENCES

Aagrwal,V., Sharma, K., Gupta, S., Kumar, R. and Prasad, M. 2007. Identificaton of sex in *Simmondsia chinensis* (jojoba) using RAPD markers. *Pl. Biotech.Rep.*1:207-210

Ahmad, F. 1999. Random Amplified Polymorphic DNA analysis reveals genetic Relationship among the annual *Cicer* species. *Theor. App. Gen.* 98: 657 -663

Al-Zahim, M.A., Ford-Loyd, B.V. and Newbury, H.J. 1999. Detection of somaclonal variation in garlic *(Allium sativum* L.) using RAPD and cytological analysis. *Pl. Cell Repo.* 18 (6): 473-477

Akkurt, M., Welter, L., Maul, E., Topfer, R. and Zyprian, E. 2007. Development of SCAR markers linked to powdery mildew (Uncinula necator) resistance in grapevine (*Vitis vinifera* L. and *Vitis sp.*). *Mol. Breed.* 19: 103-111

Anisworth, C. 2000. Boys and girls come out to play: the molecular biology of dioecious plants. *A. Bot.* 86: 211–221

Anisworth, C., Crossly, S., Buchanari,W.V., Thangavelu, M.and Parker, J. 1995. Male and female flowers of dioecious plant Sorrel show different patterns of MADS box gene expression. *Pl. Cell Phy.* 7:1583-1598

Anuntalabhochai, S., Sitthiphrom, S., Thongtaksin, W., Sanguansermsri, M. and Cutler, 2007. Hybrid detection and characterization of Curcuma spp. using sequence characterized DNA markers. *Scient. Hort.* 111: 389-393

Banerjee N.S., Manoj P., Das M.R. (1999) Male sex associate RAPD markers in *Piper longum* L. *Curr. Sci.*77:693-695

Babu, T.P.H. 2000 RAPD analysis to assess the genetic stability in tissue culture derived black pepper (*Piper nigram L.*) plant. M.Sc. (Ag) thesis. Kerala Agricultural University, Thrissur, 180p

Bedoya, C.G. and Numez, V. 2007. A SCAR marker for the sex types determination in coloumbian genotypes of *Carica papaya. Euphytica*. 153:215-220

Boukar, O., Kong, L., Singh, B.B.,Murdock, L. and Ohm, H.W. 2004. AFLP and AFLP-derived SCAR markers associated with *striga gesnerioides* resistance in cowpea. *Crop Sci*. 44(4): 1259-1264

Busconi, M., Sebastiani, L. and Fogher, C. 2006. Development of SCAR markers for germplasm characterisation in olive tree (*Olea europea* L.). *Mole.Breed.*17: 59-68

Causin, R., Scopel, C., Grendene, A. and Montecchio, L., 2005. An improved method for the detection of *Phytophthora cactorum* (L.C.) Schroeter in infected plant tissues using SCAR markers. *J. Pl. Patho*. 87: 25-35

Charlesworth, B. 2004. On chromosomes and sex determination. *Current Biology*; 14:745

Charlesworth, B. and Charlesworth, D. 2000. The degeneration of Y chromosomes. *Phil. Trans Roy. Soc. Lond.* 335:1563-1572

Charlesworth, D. 1985. Distribution of dioecy and self-incompatibility in angiosperms. *Evolution Essays in Honour of John Maynard Smith, Cambridge University Press: Cambridge*. pp: 237-268

Charlesworth, D. 2002. Plant sex determination and sex chromosomes. *J. Heredity*. 88:94-101

Chatterjee, J., Mandal, K.A., Ranade, S.A., Silva, A.T. and Datta, S.K. 2006. Molecular systematics in *Chrysanthemum* x *grandiflorum* (Ramat.) Kitamura. *Scientia Hort.* 110: 373-378

Chen, W.H., Chen, T.M., Fu, Y.M., Hsieh, R.M. and Chen, W.S. 1998. Studies on somaclonal variation in phalaenopsis. *Pl. Cell Rep.*18: 7-13

Chen, Y., Hausner, G., Kenaschuk, E., Procunier, D., Dribnenki, P. and Penner, G. 1998. Identification of microspore-derived plants in anther culture of flax *(Linum ssimum* L.) using molecular marker. *Pl. Cell Rep.* 18:44- 48

Cutler, R.W., Sitthiphrom, S., Marha, J. and Anuntalabhochai, S. 2007. Development of sequence-characterized DNA markers linked to temperature insensitivity for fruit production in longan (*Dimocarpus longan* Lour.) cultivars. *J. Agro Crop Sci.* 193: 74-78

Darwin, C.R. 1877. The different forms of flowers on plants of the same species

Deputy, C,J., Ming, R., Liu, Z., Fitch, M. M., Wang, M. and Stiles, J. I. 2002. Molecular markers for sex determination in papaya (*Carica papaya* L.). *Theor. Appl. Genet.* 106:107-111

De la Cruz, M., Romirz, F. and Hernandez, H. 1997. DNA isolation and amplification from *cacti. Plant Mol. Biol. Rep.* 15:319-325

Deng, Z., S. Huang, S. Xiao, and F.G. Gmitter. 1997. Development and characterization of SCAR markers linked to the citrus tristeza virus resistance gene from *Poncirus trifoliata. Genome* 40:697–704

Dhanaraj, A.L. Bhaskara, V.V., Swamy, R.M., Bhatt, M.G., Theertha, D. and Suresh N. S. 2002. Using RAPDs to assess the diversity in Indian cashew *(Anacardium occidentale)* germplasm. *J. Hort. Sci. Biotech.* 77:41-47

Doyle, J.J, and Doyle, J.L. 1990. Isolation of plant DNA from fresh tissue. *Focus* 12: 12–15

Ellsworth, D.L., Rittenhouse, K.D., and Honeycutt, R.L., 1993. Artifactual variation in random amplified polymorphic DNA banding pattern. *BioTech.*14:214-217

Fukuoka, S., Hosaka, K., and Kamijima, O. 1992. Use of random amplified polymorphic DNAs (RAPDs) for identification of rice accessions. *Jpn. J. Genet.* 67: 243-252

Gao L.E., Roberts G.T., Lee K., Larimer F.W. 2005. The development of SCAR markers linked to a sex determination locus in *Asparagus officinalis*. J. Heredity 94: 185–189

Ganeshaiah, K.N., Ravishankar, K.V., Anand, L., Shibu, M.P. and Shanker, U. 2000 Identification of sex specific DNA marker in the dioecious tree, nutmeg. *Pl. Gene. Resour.* 121: 59-61

Gangopadhyay, G., Roy, K.S., Ghose, K., Poddari, R., Bandyaopadhyay, T., Basu, D. and Mukherjee, K.K., 2007. Sex detection of *Carica papaya* and *Cycas circinalis* in pre-flowering stage by ISSR and RAPD. *Curr. Sci.* 92:524-526

George, J., Karun, A., Manimekalai, R., Rajesh, M.K. and Remya, P. 2007. Identification of RAPD markers linked to sex in *Palmyrah (Barassus flabellifer* L.). *Curr. Sci.* 93:1075-1077

Gunter, I.F., Roberts, I.G., Farimer, W., and Tuskan, A.G. 2003. The development of two flanking SCAR markers linked to a sex determination locus in *Salix viminalis* L. *J. Heredity*; 94: 185-189

Gutman, F., Bar-zvi, D., Nerd, A. and Mizrali, Y. 2001. Molecular typing of Cereus *Peruvian Hf.* clones and their genetic relationship and other species evaluated by RAPD analysis. *J. Hort. Sci. Biotech.* 76: 709 -713

Gygax, M., Gianfranceschi, L., Liebhard, R., Kellerhals, M., Gessler, C. and Patocchi, A. 2004. Molecular markers linked to the apple scab resistance gene Vbj derived from Malus baccata jackii. *Theor. App. Gene.* 109: 1702-1709

Harvey, C.F., Gill, C.P., Fraser, L.G. and Mc Neilage, M.A. 1997. Sex determination in *Actinidia.1* sex linked markers and progeny sex ratio in diploid *Actinidia chinensis*. *Sex. Pl. Rep*. 10:149-154

Harvey, J.I., Dollo.L and Polito.V.S. 1999.Identification of a RAPD marker linked to sex determination in *Pistacia vera* using bulked segregant analysis. *Theor. Appl.Genet.* 89:9-13

Hemanth, N.V., Narayanaswamy, P., Theertha P.D., Mukunda, G.K. and Suresh N. S. 2001. Estimation of genetic diversity of commercial mango *(Mangifera indica* L.) cultivars using RAPD markers. *J. Hort* Scie. *Biotech.* 76: 529-533

Hernandez, C.S. and Oyaszum, J.C. 2006. Two minipreperation protocol to DNA extraction from plant with high polysacchide and secondary metabolites. *Afr. J. Biotech*. 5(20):1864-1867

Horejsi, T., J.M. Box, and J.E. Staub. 1999. Efficiency of randomly a mplified polymorphic DNA to sequence characterized amplified region marker conversion and their comparative polymerase chain reaction sensitivity in cucumber. *J. Am. Soc. Hort. Sci.* 124:128–135

Hormaza, J.I., Pinney, K, and Polito, V.S. 1998.Genetic diversity *of Pistachio (Pistachio vera,* Anacardiaceae) germplasm based on randomly amplified polymorphic DNA markers. *Econ. Bot.* 52:78-87

Horwitz, J.P., Chua, J., Curby, R.J., Tomson, A.J. and Fisher, B.E. 1964. Substrates for cytochemical demonstration of enzyme activity. J. Med. Chem. 7: 574-576

Huh, M.K. and Bang, K.H. 2006. Identification of *Atractylodes japonica* and *A. macrocephala* by RAPD analysis and SCAR markers. *Silvae Gene.* 55: 101-105

Irish, E.E., and Nelson, T. 1989. Sex determination in monoecious and dioecious plants. *Pl. Cell.* 1:737-744

Jacobs, J.A., Hanekom, L., Marais, A.S. and Marais, G.F. 2004. Development of SCAR markers for a Thinopyrum distichum chromosome that appears to be involved in salt tolerance. *S. Afr. J. Pl. Soil.* 21: 236-239

Janila, P. and Sharma, B. 2004. RAPD and SCAR markers for powdery mildew resistance gene (er) in pea. *Pl. Breed.* 123: 271-274

Jeppsson, N. 1987. Progress in Research and Development of *Seabuckthorn* in Sweden

Jiang, C.X. and Sink, K.C. 1997. RAPD and SCAR marker linked to the sex expression locus *M.* in asparagus. *Euphytica* 93: 329-333

Jionguo, G., Sitthiphrom, S., Marha, J and Anuntalabhochai, S. 1999. Isozyme peroxidase and RAPD study in Tomato. *Curr. Scie.* pp. 125

Joshi, P.K., Prabhakar, K.R. and Vidya, S.G. 1999. Molecular markers in plant genome analysis. *Curr. Sci.* 77:230-240

Jung, S.J., Ham, S.H., Lee, Y.K., Bee, H.C. and., Lee, Y.H. 2001. A male associated DNA sequence in a dioecious plant *Schisandra nigra*. *Breed. sci.* 51: 219-223

Kafkas, S., Cetiner. S. and Treves, P.R. 2001. Development of sex associated RAPD markers in wild *Pistacia* species. *J. Hort. Sci. Biotech.* 76(2): 242-246.

Komaki, K., Regmi, H.N., Katayamo, K. and Tamiya, S. 1998. Morphological and RAPD pattern variation in Sweet potato and its closely related species. *Breed. Sci.* 4: 281-286

Korpelainen, H., Bisang, I., Hedenas, L. and Kolehmainen, J. 2008. The first sex specific molecular marker discovered in the moss *Pseudocalliergon trifarium*. *J. Heredity*. 99:581-587

Kumar, P.P., Yau, C.K., Jin, G.C. and Goj, C. J. 1998. Genetic analysis of *Heliconia* species and cultivars with randomly amplified polymorphic DNA markers. *J. Amer. Soc. Hort. Sci.* 123:91-97

Kushawa, R. 2008. Developement and analysis of ESTs (Express sequence tags) in Black papper (*Piper nigrum* L.) M.Sc. (Agri.). thesis, Kerala Agricultural Unoversity, thrissur, India p. 130

Lakhanapaul, S, velayudhan, K.C and Bhat K.V. 2003. Analysis Of Genetic Diversity In Indian Taro [*Colocasia esculenta* (L.) Schott] Using RAPD markers, *Genetic Resource And Crop Evolution;* 50: 603-609

Lewis, D. 1942. The evolution of sex in flowering plants. *Bio.Rev*. 17:46-67

LianHua, Q., Tan, Q. MingJie. C. and YingJie P. 2006. Use of intersimple sequence repeats markers to develop strain-specific SCAR markers for Lentinula edodes. *FEMS-Microbiology-Letters*. 257: 112-116

Lim, S., Teng, C.P., Yewhwa, L. and Chona'in, G. 1999. RAPD analysis of some species in the genus *Vanda* (Orchidaceae). *A. Bot.* 83:193- 196

Llyod, D.G. 1975. The transmission of genes via pollen and ovules in gynodioecy angiosperms. *Theor. Appl Gen.* 9:299-316

Lopes, M.P., Brito, L.J., Gomes, S., Meirinhos, J.,Santos, L. and Pinto, G. H. 2007. RAPD and ISSR molecular markers in *Olea europaea* L.: genetic variability and molecular cultivar identification. *Gene. Resou. Crop Evolution.* 54: 117-128

Louis, J.P. 1989. Genes for regulation of sex differentiation and male fertility in *Mercurialis A. J. Heredity.* 80:104-111

Luy, F.L., Sass, M.E., Jung, G.H., Johns, M.A and Nienhuis, J. 2006. Development of robust SCAR markers that distinguish the six cultivated Brassica species and subspecies of the U-triangle. *J. Amer. Soc. Hort. Sci.* 131: 424-432

Mandolino, G., Carbonia, A., Forapanis., Faetiv and Ranalli, P. 1994. Identification of DNA markers linked to male sex in dioecious hemp. *Theo. App. Genet.* 98:86-92

Manoj, P., Banaerjee, N.S. and Ravichandran, P. 2005. Development of sex-specific molecular markers in dioecious *Piper longum* L. plants by differential display. *J. Theo App Inf Tech.* 1:459-465

Martin, G.S., Williams, G.K. and Tanksley, S.D. 1991. SCAR from RAPD. *Proc. Nat. Acad. Sci.* U.S.A. 88:2336-2340

Massawe, F.J. Roberts, J.A. Azam-ali, S.N. and Davey, M.R. 2003, Genetic diversity in bambara groundnut (*Vigna* subterranean (L) verdc.) landraces assessed by RAPD markers, *Gene. Resou. Crop Evolution.* 50: 737-741.

Masuzaki, S.I., Miyazaki, T., McCallum, J.A., Heusden, S.V., Yamashita, K.I., Tashiro, Y., Yamauchi, N. and Shigyo, M. 2007. Conversion of chromosome-specific RAPDs into SCAR-based anchor markers for onion linkage maps and its application to genetic analyses in other *Allium* species. *Sci. Hort.*

Matasyoh, P., Blanchard, A. N., Bryant, J. A., Smirnoff, N., and Weir, A. F. 2008. Isolation of DNA from the highly mucilaginous succulent plant Sedum telephium. *Plant. Mol. Biol. Reptr.* 16, 133–138

Michelmore, R.W. Paran, I. and Kesseli, R.V. 1991. Development of RAPD and SCAR markers linked to the Russian wheat aphid resistance gene Dn2 in wheat. Proc. *Nat. Acad. Sci. U.S.A.* 88:9828-9832

Murlidharan, K., Wakeland, E.K., 1993. Concentration of primer and tempelate qualitatively affects products in RAPD-PCR. *BioTech.* 14: 362-364

Muthulakshmi, P. 1998. Variability analysis in *Garcenia cimbogia* Desr. (Malabar Tamarind). M.Sc. (Hort.). thesis, Kerala Agricultural Unoversity, thrissur, India p. 130

Nazeema , K.K. 1992. Standedisation of soft wood and epicotyl grafting in Garcinia combogia Desr. M.Sc. (Hort.) thesis, Kerala agricultural university, thrissur, India p. 132

Nomura, K., Minegishi, Y., Kimizuka T.C., Fujioka, T., Moriguchi, K., Shishido, R. and Ikehashi, H. 2005. Evaluation of F_2 and F_3 plants introgressed with QTLs for clubroot resistance in cabbage developed by using SCAR markers. *Pl. Breed.* 124: 371-375

Ohtslibo, K., Fufii, T., Hashino, Y., Toyoshima, H., Okadome, H., Nakamura, S., Fuse, T. and S. Kawasaki. 1999. Identification of domestic rice cultivars by RAPD method using milled rice grains as samples. *Pl. Breed. Abstr.* 69:1696

Orozco, C.C., Chalmers, K.J., Waugh, R. and Powell, W. 1994. Detection of genetic diversity and selective gene introgression in Coffee using RAPD markers. *Theo. App. Genet.* 87: 934-940

Oyama,R.K., Volz, S.M and Renner, S.S. 2009. A sex-linked SCAR marker in *Bryonia dioica*, a dioecious species with XY sex-determination and homomorphic sex chromosomes. *Journal of Evolutionary Biology.* 22:214-224

Padmesh, P., Sabu, K.K., Seeni, S. and Pushpangadan. P.1999. The use of RAPD in assessing genetic variability in *Andrographis paniculata* Nees, a hepata protective drug. *Curr. Sci.* 76 (6): 833-835

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

Parasnis, A.S., Ramakrishna, W., Chowdari, K.V., Gupta, V.S. and Ranjekar, P. K. (1999). Microsatellite (GATA)*n* reveals sex-specific differences in papaya. *Theor. Appl. Genet.* 99:1047-1052

Park, S.O., Coyne, D.P., Steadman, J.R., Crosby, K.M. and Brick, M.A. 2004. RAPD and SCAR markers linked to the Ur-6 Andean gene controlling specific rust resistance in common bean. *Crop Sci.* 44:1799-1807

Parker, J,S. 1990. Sex chromosomes and sexual differentiation in flowering plants. *Chromosomes Today.* 10: 187-198

Parrish, T.L., Koelewijn, H.P. and Dijk, P.J. 1999. Identification of a male-specific STS marker in a functionally dioecious fig, *Ficus fulva* Reinw. ex Bl. (Moraceae). *Sex Pl. Rep.* 17:17–22

Prakash, S. and Staden, V.J. 2006. Sex determination in Encephalartos natalensis (Dyer and Verdoorn) using RAPD markers. *Springer*. 152197-200

Premjith G. and Rajendran P.C. 2008. Refinement of Microropagation protocol and RAPD assay for sex determination in Kodumpuli (*Garcinia gummigutta var. gummigutta*). *Asian J. Bio Science* 4(1): 130-134

Radisek, S., Jakse, J. and Javornik, B. 2004. Development of pathotype-specific SCAR markers for detection of Verticillium albo-atrum isolates from hop. *Pl. Dis*. 88: 1115-112

Rania, A.A., Ismail, M.O. and Soliman, S.S. 2008. Identification of sex specific DNA markers for date palm (*Phoenix dactylifera* L.) using RAPD and ISSR techniques. *Res. J. Agri. Bio. Sci*. 4:278-284

Rashmi, R. 2008. Molecular characterization of chilli (*Capsicum annuum* L.) genotypes for tagging bacterial wilt resistant gene. M.Sc. (Agri.). thesis, Kerala Agricultural Unoversity, thrissur, India p. 147

Renganayaki, K., Jessup R.W., Burson, B.L., Hussey M.A. and Read, J.C. 2005. Identification of male specific AFLP markers in dioecious Texas bluegrass. *Crop Sci*. pp. 2529

Renner, S.S. and Ricklefs, R.E. 1995. Dioecy and its correlates in the flowering plants. *Am.J.Bot*. 82:596-606

Rogers, S.O and Bendich, A. J. 1994. Extraction of total cellular DNA from plants, alge and fungi. *Plant Mol. Biol. Mannual*. 91: 1-18

Ruas, F.C., Fairbanks, J.D., Evans, P.R., Stutz, H.C., Andersen, R.W and Ruas, M.P. 1998. Male specific DNA in the dioecious species *Atriplex garrettii* (chenopodiaceae). *Am. J. Bot*. 85:162-167

Rugienius, R., Siksnianas, T., Stanys, V., Gelvonauskiene, D and Bendokas, V. 2006. Use of RAPD and SCAR markers for identification of strawberry genotypes carrying red stele (*Phytophtora fragariae*) resistance gene Rpf1. *Agro Rese.* 4:335-339

Sabharwal, V., Negi, M.S., Banga, S.S. and Lakshmikumaran, M. 2004. Mapping of AFLP markers linked to seed coat colour loci in Brassica juncea (L.) Czern. *Theor. App. Genes*. 109: 160-166

Saiki, R.K., Gelfand, S., Stoffel, S.J., Scharf, R., Higuchi, Hom G.T., Mullis K.B. and Erlich, H.A. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Sci.* 239:487-491

Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. *Molecular cloning. A laboratory Manual.* Academic Press, New York, USA, 1322p

Semerikov V., Lagercrantz, U., Tsarouhas, V., Ro¨nnberg-Wa¨stljung, A. Alstro¨m-Rapaport, C. and Lascoux, M. 2003. Genetic mapping of sex-linked markers in *Salix viminalis* L. *Heredity.* 91:293–299

Sghaier, Z.A. and Mohommed, C. 2005. Genomic DNA extraction method from Pearl Millate (*Pennisetum glaucum*) leaves. *Afri. J. Biotech.* 4(8):863-866

Sharma, K.K., Lavanya, M. and Anjaiah, V. 2000. A method for isolation and purification of peanut genomic DNA suitable for analytical applications. *Plant Mol. Biol. Rep.* 18: 393a-393h

Sherly, R. 1994. Growth, flowering, fruit set and fruit development in Kodumpuli (*Garcinia combogia* Desr.). M.Sc. (Hort.) thesis, Kerala Agricultural Unoversity, thrissur, India p. 102

Sivolap, M., Solodenko, A.E. and Burlov, V.V. 1998. RAPD analysis of molecular genetic polymorphism in sunflower (*Helianthus annus*). *Pl. Breed. Abstr.* 69 (8): 1127

Suk, Y., Sunghee., Gwandal., Chongunum and Doohwan, K. 1998. RAPD analysis for genetic diversity of Melon species. *Korean J. Hort. Sci. and Tech.* 16: 21-24

Tang, S., Suhva, S. Yueqin, C., Lianghu, and Hongda, Z. 1999. Phylogenetic relationship of *Camellia nitidissima* chi and its allied species based on RAPD. Pl. Gene. Resour. Abstr. 8 (2): 147

Testolin R, Cipriani G., Costa G. 1995. Sex segregation ratio and gender expression in the genus *Actinidia*. *Sex Pl. Rep.* 8: 129–132

Ullmann, A., Jacob, F. and Monod, J. 1967. Characteriztion by *in vitro* complementation of a peptide corresponding to an operator- proximal segment of the β galactosidase structural gene of *Escherichia coli*. *J. Mol. Biol.* 24: 339-341

Verghese J. 1996. The world of spices and herbs. *Indian Spices* 34:11-12

Vinod, M.S., Prashanth, S., George, S. and Parida, A. 2007. Identification of a sex specific SCAR marker in dioecious *Pandanus fascicularis* L. *Genome*; 50: 834-839

Varma, R. 2009. Molecular marker based linkage mapping in black pepper (*Piper nigrum* L.) M.Sc. (Agri.). thesis, Kerala Agricultural Unoversity, thrissur, India p. 130

Weishing K., Nybom, H., Wolff, K. and Majer, W. 1995. DNA fingerprinting in plant and fungi. *CSR Press, Florida*. Pp. 44-59

Welsh. J. and Mc Clelland. M. 1991. Genomic finger printing using argitrarily - primed PCR and a matrix of pair wise combinations of primers. *Nucl. Acids.* 22p.

Williams, G.K., Hanafey, M.K., Rafalski, J.A. and Tingey, S.V. 1991. Genetic analysis using random amplified polymorphic markers. *Enzymol.* 218:704-740

Wu F.F., Miller N., Kaloshian I. 1998. A PCR-based marker tightly linked to the nematode resistance gene *Mi* in tomato. *Theor. Appl. Genetics* 87 : 757-763

Xu, J.E., Bing, W.W. and Ke-Ming L. 2004. RAPD and SCAR markers linked to sex. *Genetics*.11:127-132

Yakubov, B., Barazani, O. and Goldhirsh, G. 2003. Combination of SCAR primers and touchdown PCR for sex Identification in *Pistacia vera* L. *Scient Hort.* 103:473-478

Yamposky, C. and Yamposky, H. 1922. Distribution of sex forms in the phanerogamic flora. *Genetics*. 3:1-62

Yiping, Z. and John, R.S. 2001. Development of SCAR and CAPS Markers Linked to the *Beta* Gene in Tomato. *Crop Sci.* 41:1602–1608

Annexures

ANNEXURE I

Details of laboratory equipment items used for the study

Refrigerated centrifuge	: Kubota, Japan
Horizontal electrophoresis System	: Biorad, USA.
Thermal cycler	: Mastercycler personal, Eppendorf
Gel documentation system UVP (Inc. CA)	: Gel Doc-ItTS™ Imaging System
Nanodrop® ND-1000 Spectrophotometer.	: Nanodrop® Technologies Inc. USA

ANNEXURE II

CTAB method of DNA Isolation

Reagents:

(a) 4X CTAB Extraction Buffer

100 ml stock solution of each reagents were prepared separately and stored in refrigerator

Stock.1 - CTAB (10%, w/v)
Stock.11 - 1M Tris Buffer (pH 8)
Stock.111 - 0.5M EDTA (pH 8)
Stock.IV - 4M NaCl

60 ml of 4X CTAB extraction buffer was prepared by pipetting following stocks

Stock .1 - 12ml

Stock.11 - 6ml
Stock.111 - 2.4ml
Stock .IV - 21ml
Distilled water- 18.6ml
Total - 60ml

(b) 10% CTAB Solution

10% CTAB (w/v)
0.7M NaCl

(c) TE Buffer

10mM Tris (pH 8)
1mM EDTA (pH 8)

ANNEXURE III

Composition of Buffers and dyes used for gel electrophoresis

1. TAE Buffer 50X

242g Tris base

57.1ml glacial acetic acid

100ml 0.5 EDTA (pH 8.0)

2. Loading Dye (6X)

0.25% bromophenol blue

0.25% xylene cyanol

30% glycerol in water

3. Ethidium bromide

The dye was prepared as a stock solution of 10 mg/l in water and was stored at room temperature in a dark bottle.

ANNEXURE IV

Chemical composition of reagents used for Cloning and Transformation

1. Composition of SOB medium (pH 7.0)

a. 20g Tryptone
b. 5g Yeast extraxt
c. 2ml NaCl stock solution (5M)
d. 830µl KCl stock solution (3M)
e. 15 g Agar
f. 1 l Disatilled water

ANNEXURE V

List of decamer primer series OPS, OPY, RN, OPA, OPAH used for screening of male and female genotypes

Sl. No	Primer	Nucleotide Sequence (5'–3')
1	OPS 1	CTACTGCGCT
2	OPS 2	CCTCTGACTG
3	OPS 3	CAGAGGTCCC
4	OPS 4	CACCCCCTTG
5	OPS 5	TTTGGGCCTA
6	OPS 9	TCCTGGTCCC
7	OPS 12	CCTTGACGCA
8	OPS 14	TCCGCTCTGG
9	OPS 15	GGAGGGTGTT
10	OPS 20	TCTGGACGGA
11	RY 3	ACAGCCTGCT
12	RY 5	AAGGCTGACC
13	RY 7	AGAGCCGTCA
14	RY 8	AGGCAGAGCA
15	RY 9	AGCAGCGCAC
16	RY 11	AGACGATGGG
17	RY 15	AGTCGCCCTT
18	RY 18	AGCCGTGGAA
19	RY 20	CTCACG TTGG
20	RN 1	CTCACGTTGG
21	RN 2	ACCCAGGGGC
22	RN 5	ACTGAACGCC
23	RN 7	CAGCCCAGAG
24	RN 8	ACCTCAGCTC
25	RN 9	TGCCGGCTTG
26	RN 10	ACAACTGGGG

27	RN 12	CAACGACACC
28	RN 14	GGTCTGGATC
29	RN 16	AAGCCACCT
30	RN 17	GGTGCTCCGT
31	OPA 6	GTGATCGCAG
32	OPA 8	GGTCCCTGAC
33	OPA 12	TCGGCGATAG
34	OPA 22	TCGCCCGAAA
35	OPA 26	GGT CCC TGAC
36	OPA 27	AGCGTCATCC
37	OPA 38	AGTCGCCCTT
38	OPD 10	GGTCTACACC
39	OPD 15	ACCCGGTAAC
40	OPD 20	GAC CGA CCCA
41	OPK 1	TGGCGACCTG
42	OPF 14	GAG ACG CACA
43	OPE 11	ACCCCCCGAG
44	OPAH12	TCC AAC GGCT
45	OPN 6	CAT CGC CGCA
46	OPN 7	ACA GCC TGCT

DEVELOPMENT OF SCAR MARKER FOR AUTHENTICATION OF GENDER IN KODAMPULI

(*Garcinia gummi-gutta* var. *gummigutta*)

By

SHELKE SUNIL MAROTARAO

(2008-11-114)

ABSTRACT OF THE THESIS

Submitted in partial fulfilment of the
requirement for the degree of

Master of Science in Agriculture

(PLANT BIOTECHNOLOGY)

Faculty of Agriculture
Kerala Agricultural University, Thrissur

Centre for Plant Biotechnology and Molecular Biology

COLLEGE OF HORTICULTURE

VELLANIKKARA, THRISSUR - 680 656
KERALA, INDIA

2010

ABSTRACT

The diabetes and cardiovascular disease are the two serious obesity related life-style diseases, spreading at alarming rate throughout the world, especially in thickly populated third world countries in which India occupies the prime position. The fleshy fruit rind of Kodampuli (*Garcinia gummi-gutta*) is the richest natural source of anti-obesity metabolite hydroxyl citric acid (HCA). Which inhibit the conversion of carbohydrate to fats without affecting Kreb's cycle through an enzyme ATP citrate lyase.

Since Kodampuli is a polygamodioecious tree, it takes 8 to 12 years to identify the female trees. No significant reports are available for sex determination in Kodampuli on the basis of physiological, biochemical or molecular characters. Sex identification, lack of orthotropic shoots for grafting, prolonged seed dormancy, poor seed germination and lack of awareness of its pharmaceutical significance are hindering the extensive cultivation of this backyard companion crop in Kerala and other coastal regions of country.

In the present study, an attempt was made to develop simple PCR based technique which can use for gender diagnostic in this plant. DNA samples were extracted from field grown 15 to 20 years old 25 male and 25 female trees and were bulked to 5 samples each by sex type. Earlier reported RAPD primers *viz.* Kit C1, Kit C8 and Kit C9 were screened but no significant polymorphism was observed. So a total of random 46 decamer primers were tested and six primers were selected for further analysis. On rescreening of the six selected primes *viz.* RN 5, RN 9, RN 10, RY 5, RY 18 and OPAH 12 only OPAH 12 reproduce male specific band in bulked and individual samples.

Random amplified polymorphic DNA (RAPD) fragments were generated in the both bulks in order to identify markers that were polymorphic between male and female plants. A 550 base-pair (bp) male-specific DNA fragment generated with the OPAH-12 primer was identified.

The polymorphic male specific band produced by OPAH 12 primer was eluted and cloned in pGEM-T vector, and transformed into *E. coli* JM 109 cells. Cloned cells were subjected to blue-white screening and transformed one was sent for sequencing

The sequence obtained after vector screening was subjected to nucleotide blast search and ORF finder. It does not reveal any significant levels of homology and reading frame. Two pairs of SCAR primer were designed on the basis of sequence. These SCAR primers were checked for male and female samples but no polymorphic band was observed.

The future line of work can be to screen the male and female genotypes with more number of primers to obtain larger base pair polymorphic band. That can used to convert this dominant marker to co-dominant one like SCAR marker. SCAR marker would be successfully employed in breeding experiments for Marker Assisted Selection.