SEX DETERMINATION IN NUTMEG (*Myristica fragrans* Houtt.) THROUGH MOLECULAR AND BIOCHEMICHAL MARKERS

By

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THESIS

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DECLARATION

I, hereby declare that this thesis entitled "Sex determination in nutmeg (*Myristica fragrans* Houtt.) through molecular and biochemical markers" is a bonafide record of research work done by me during the course of research and that it has not been previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other University or Society.

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Certified that this thesis entitled "Sex determination in nutmeg (*Myristica fragrans* Houtt.) through molecular and biochemical markers" is a bonafide record of research work done independently by Ms. Sudhamayee Maddela (2008-11-115) under my guidance and supervision and that it has not formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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Maddela Sudhamayee

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ABBREVIATIONS

A	Adenine
bp	Base pairs
β	Beta
BLAST	Basic Local Alignment Search Tool
BSA	Bovine Serum Albumin
C	Cytosine
cm	Centimeter
CPBMB	Centre for Plant Biotechnology and Molecular biology
CTAB	Cetyl Trimethyl Ammonium Bromide
^0C	Degree Celsius
DNA	Deoxyribo Nucleic Acid
DMSO	Dimethyl sulfoxide
dNTPs	Deoxyribo Nucleoside Triphosphate
EDTA	Ethylene Diamine Tetra Acetic acid
g	Gram
G	Guanine
GOT	Glutamate Oxalo acetate Transaminase
IPTG	Isopropylthio-β-D-galactoside
Kb	Kilo basepairs
KAU	Kerala Agricultural university
L	Litre
LB	Luria Broth
LBA	Luria Bertani Agar
mA	Milli Ampere

Mb	Mega base pairs
Min	Minutes
ml	Millilitre
µg	Microgram
µl	Microlitre
ng	Nanogram
NCBI	National Centre for Biotechnology Information
OD	Optical Density
ORF	Open Reading Frame
PAGE	Polyacrylamide Gel Electrophoresis
pH	Hydrogen ion concentration
PCR	Polymerase Chain Reaction
PVP	Poly vinyl pyrolidine
PMSF	Phenyl Methane Sulphonyl Fluoride
%	Percentage
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribo Nucleic acid
RNase	Ribonuclease
rpm	Rotations per minute
SCAR	Sequence Characterized Amplified Region
sec	Second (s)
T	Thymine
TAE	Tris Acetate EDTA
TE	Tris EDTA

TEMED	N,N,N',N'- Tetra methyl ethylene diamine
U	Unit
UV	Ultra violet
V	Volts
v/v	Volume by Volume
w/v	Weight by Volume
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

Introduction

1. INTRODUCTION

Nutmeg (*Myristica fragrans* Houtt.) is an important tree spice of southern India, yielding two products of commercial value, the nutmeg and the mace. Nutmeg of commerce is the dried seed and mace is the dried aril. It is a spreading evergreen tree belonging to the small, primitive family Myristicaceae. Nutmeg, originated in the Moluccas islands in the East Indian Archipelago, was introduced to India and other tropical countries during the 18th century. It is cultivated in many tropical countries like Malaysia, Indonesia, West Indies, India and Srilanka. As per 2006 statistics it is cultivated in India in an area of 11,329 ha with a total production of mace and nutmeg together 2905 tonnes per year. World production of nutmeg is around 10,000 to 12,000 tonns and that of mace is 1500-2000 tonnes per year.

Both nutmeg and mace are used as spice and in medicine. In eastern countries, they are more as a drug than as a condiment. Both the spices have stimulative, carminative, astringent and aphrodisiac properties. The volatile oil present in both contains small amounts of myristicin and elemicin, which are narcotic and poisonous when consumed in large quantities. The husk or pericarp is used in pickles, sweetmeats and jellies. Nutmeg butter, the fat extracted from the kernel is used in the manufacture of scented oils, perfumes, soaps and as a flavouring agent in cookery and confectionery.

In nutmeg, apart from the long pre bearing period, dioecy is recognized as one of the major problem of cultivation. The seedling progenies segregate into female and male in 1:1 proportion (Nichols and Pryde, 1958). The sex of tree cannot be determined until flowering has commenced which normally takes 5 to 7 years. Male trees are required only in 1:10 ratio in a plantation for effective pollination. Expecting a 1:1 segregating ratio, 50 per cent of the total trees in any nutmeg plantation raised through seedling will be males. Cutting down of the excess male trees results in irregular spacing, loss of time and resource to the grower.

Presently, the only option to overcome dioecy in nutmeg is the use of vegetatively propagated material or top working of the excess plants. Even though several vegetative methods have been reported with varying degree of success, the large scale adoption of these methods is constrained due to non availability of orthotrophs in sufficient numbers which are required for the production of plants with normal erect growth habit. So seedling continues to be the major propagating material and hence, the identification of sex of the plants in the seedling stage has great relevance. However, no definite methodology has been evolved so far for sex identification of plant growth.

Several attempts have been made to identify the sex of nutmeg plants at the seedling stage on the basis of morphological variations in male and female plants (Flach, 1966; Nayar *et al.*, 1976). But morphological markers suggested were not reliable and consistent. Biochemical methods were also tried with limited success. (Phadnis and Chowdary, 1971 and Thomas, 1997).

Dioecy occurs in certain commercial crops like papaya, asparagus, date plam and cannabis. Molecular markers have been developed to identify sex at early stage in these crops (Jiang and Sink 1997; Mandolino *et al.*, 1999; Urasaki *et al.*, 2002 and Chaves and Bedoya, 2007). Shibu *et al.* (2008) has reported about female specific RAPD DNA marker in the dioecious nutmeg. Since reproducibility of RAPD marker is sometimes difficult, usually RAPD marker will be converted to SCAR marker.

The present programme envisages a comprehensive study of the dioecy in nutmeg through molecular and biochemical analyses. The defined objective of study is to distinguish male and female nutmeg plants by molecular and biochemical marker and to develop a SCAR marker to distinguish male and female trees. This may facilitate early detection of sex in nutmeg plant.

The molecular marker study was conducted with RAPD and SCAR. The biochemical markers selected were isozyme assay of acid phosphatase and glutamate

oxaloacetate transaminase (GOT). The leaf samples from identified male and female will be analyzed with the selected markers.

Review of Literature

2. REVIEW OF LITERATURE

The study on "Sex determination in nutmeg through molecular and biochemical markers" was selected for developing molecular and biochemical markers linked to sex expression at seedling stage of nutmeg. The relevant literature on various aspects of the investigation is reviewed in this chapter.

2.1. NUTMEG PLANT

2.1.1. Habit

Nutmeg is a spreading dioecious evergreen tree, growing 5-13 m high, sometimes attaining 20 m height. All parts of the plants are aromatic. Average yield of nutmeg tree is 3000 fruits.

In nutmeg, two types of branching, erect (orthotropic) and spreading (plagiotropic) are observed. But orthotropic branches are few in number. The twigs are glabrous, slender and grayish brown (Purseglove *et al.* 1981).

The leaves of nutmeg have been described as alternate, glabrous, exstipulate with a petiole length of about 1cm. the shape of the leaves may be elliptic or oblong-lanceolate with a acute or slightly acuminate (Purseglove *et al.* 1981). The leaves contain an essential oil. Mayer (1941) obtained as yield of 1.56% oil by steam distillation of dried leaves. Khan and Krishnaswamy (1953) have reported yields of 0.41 to 0.62 per cent from fresh leaves in India. The leaf oil prepared by Mayer (1941) contained 80 per cent alphapienine and 10 per cent myristicin.

2.1.2. Inflorescence and flower

The male and female inflorescence are similar, glabrous and axillary with the flowers in umbellate cymes in which there are 1-10 flowers in the male inflorescence and 1-3 in the female (Talbot, 1976). The flowers which measures up to 1 cm in length are fragrant, waxy and fleshy. Lesila (1963) reported that the female flowers were larger

than the male and that the calyx tube appeared more oval in shape. The flower is bracteate and bracteolate. Sastri (1954) and Nair and Bahl (1956) described the perianth as receiving ten vascular traces and have postulated a pentamerous origin. The androecium consists of a solid column to which 14-22 bilocular anthers attached. The gynoecium consists of single pistle with short to non existant style and bilobed stigma. The ovule is single.

2.1.3. Fruit and Seed

The fruit is a fleshy drupe, broadly pyriform, yellow, smooth, 6-9 cm long. Ridley (1912) described the fruits when ripe as one of the most beautiful fruit in nature. There is a circumferential longitudinal ridge with persistant remains of the stigma. When ripe the suculent, aromatic yellow pericarp splits into two halves along the suture to expose the seed and red aril.

Pursuglove *et al.* (1981) described the seeds as round or oval in shape with brown testa. The kernel is broadly ovoid, grayish brown and ruminates and consists of convoluted dark brown perisperm, lighter coloured endosperm and a small embryo.

2.1.4. Growth pattern

Nutmeg trees are found to be slow growing when compared to other perennial trees. Shoot growth in nutmeg is found to be cyclic, a period of growth followed by quiescence. Nazeem *et al.* (1981) observed six flushes during a period of one year.

2.1.5. Vegetative propagation

Nutmeg being a dioecious crop, proper ratio of female and male plants is to be maintained in the plantation. Vegetative propagation is the practical way to achieve this. Many vegetative propagation methods were standardised to develop a suitable method. Vegetative propagation techniques like stem cuttings, air layering, approach grafting, epicotyl grafting, budding and top working were tried in nutmeg with varying degree of success (Sundarraju and Varadarajan, 1956; Kannan, 1973; Shanmughavelu

and Rao, 1977; Rasalam, 1978; Mathew and Joseph, 1982; Mathew, 1985 and Rethinam and Edison, 1991). *Insitu* budding method is now followed in Kerala to solve dioecy and long juvenile phase problems (Rani, 1994).

Commercial coverage through vegetative propagule is very much limited due to unavailability of orthotropic scion material. If plageotropic shoots used as scion material such plants have a spreading habit resulting in squatty plants with low yields. Because of these factors, seedling is continues to be propagating material.

2.1.6. Varieties released

Altogether three varieties have been released (Nybe *et al.*, 2007). Those were IISR- Vishwasree from IISR, Kozhikode; Konkan Sugandha and Konkan Swad from Dr. Balashaheb Sawant Konkan Krushi Vidyapeeth Regional Fruit Research Station, Vengurle.

2.2. Dioecy in nutmeg

Nutmeg is typically dioecious with male and female flowers on different trees, hermaphrodite plants and hermaphrodite flowers are noticed occasionally. Morphological variability in relation to dioecy has been reported with regards to leaf size, venation and nature of branching (Janse, 1898 and Presto, 1948).

Dienum (1931) reported that on an average 55 per cent of seeds from a female plant will be female, 40 per cent male and 5 per cent bisexual.

Nichols and Pryde (1958) observed that segregation of progenies into male and female was in 1:1 proportion.

Sastri (1962) reported that occasionally male trees after a number of years produced female flowers and eventually became female.

Flach (1966) recognized two different sexes, a female flowering sex, a male flowering sex, the later being sub divided into four different groups viz. pure male,

bisexual males, bisexual females and pure females. Hermaphrodite plants produce only smallest number of fruits and fairly high percent of double nuts.

Flach (1966) observed that female trees have a significantly higher stem diameter during the pre bearing stage while no significant difference in terms of plant height was observed.

Flach (1966) reported that *Myristica fragrans* is the first dicotyledonous plant known to possess a holokinetic chromosome and females were found to possess chromosomes that had facultative nucleolar property this may due to heterogametic nature of the females. It appears that sex in *M. fragrans* is genetically determined with a strong possibility of a mono-factorial sex-determining mechanism, such as a XX-XY system, with females being heterogametic.

Nayar *et al.* (1977) reported that calcium oxalate crystals found in the foliage epidermal cells are different in form in male and female plants. According to him it is possible to distinguish two types of plants, male having simple crystals and females having cluster of crystals in their lower epidermis.

Nazeem *et al.* (1981) studied the flowering pattern in nutmeg and reported that the male and female trees showed variation in flowering. In female, flowering was constrained to seven months where as in males flowering was observed throughout the year. Maximum flowering in both cases occurred in July followed by October.

Packiyasothy *et al.* (1991) reported that the essential oil content and the phenolic profiles of the leaves were used to identify sex in nutmeg seedlings. Significantly higher essential oil content and the absence of two phenolic spots were observed in female plants when compared with males and also observed differences in α- pinene, terpinolene, α- terpinine, myrcene and geranyl acetate content.

Thomas (1997) observed that the average leaf width larger in female plants compared to male plants. The length by width ratio of leaves was higher in male plants than in female plants. The results did not confirm the definite pattern.

Babu *et al.* (2000) standardized the DNA isolation and RAPD protocol in nutmeg for sex determination. No polymorphism was observed among male and female plants of nutmeg.

2.2.1. Dioecy in other plants

Most flowering plants are hermaphrodites. Dioecy, a condition wherein the male and female flowers are born on separate plants. Dioecy is well established in animals, but occur sporadically in plants. Dioecism is found in small number of plant species, 1500 species in 1303 genera and 170 families (Charlesworth, 1985).). In the dioecious populations, male plants with a few perfect flowers are occasionally found (Rick and Hanna 1943; Franken 1970; Lazarte and Palser 1979).

Storey (1938) and Hofmeyr (1938) proposed that sex type determination in papaya is controlled by a single gene with three alleles: M, h and m. Male individuals (Mm) and hermaphrodite individuals (Mhm) are heterozygous, where as female individuals (mm) are homozygous recessive.

Westergaard (1946) reported three different regions of the Y chromosome have been identified as having separate functions in sex determination in *silene.* One end contains a genetic factor (or factors) that suppresses the formation of the gynoecium, the opposite end contains a male fertility factor (or factors), and the middle region includes a gene or genes needed for anther initiation. So, the Y chromosome of *Silene* contains complete linkage between female-suppressor and essential male sex genes.

Westergaard, (1948) reported sex determination system in dioecious *silene.* In *Silene*, males are the heterogametic sex (XY) and females are homogametic (XX). As is the case in mammals, *Silene* has an active-Y system of sex determination, with dominant male factors and female suppressing factors. Y chromosome is decisive in determining sex in *Silene.*

Marks (1973) reported that *Asparagus officinalis* is "male dominant" and contains *male-activator-female-suppressor* genetic determinants similar to those postulated for *Silene.*

Lrish and Nelson (1989) reported that sex expression in dioecious plant may be genetic and environmental. Genetic sex determination may be controlled by genes on heteromorphic chromosomes, single locus on autosomes or multiple loci on autosomes.

Parker (1990) reported that heteromorphic sex chromosomes are rarely found in angiosperms. Chromosomal determination of sexual phenotype has been detected in only five dioecious plant families like *Rumex, Cannabis, Humulus, Silene* and *Asparagus.*

Bracale *et al.* (1991) reported that *Asparagus officinalis* is generally a dioecious plant, with sex determined by sex chromosomes in which the males (XY) are the heterogametic sex. In addition to these major sex determination genes, genetic modifiers can influence the stage of stylar degeneracy (Peirce and Currence 1962, Franken 1970).

Parker and Clark (1991) reported that in the genus *Rumex* and subgenus *Acefosa*, in which in contrast to *Silene*, the ratio X-to-autosome appears to control sex determination. Females are XX and males XYlY2 ($2n$ = 14 and $2n$ = 15 respectively); however, diploid plants with XXY and XXYlY2 genotypes are fertile females. In polyploids, a ratio of X-to-autosome is 1.0 or higher is female; X-to-autosome is 0.5 or lower are males. Intersexes (partial male/female) or hermaphrodites result in ratios between 0.5 and 1.0.

Anisworth *et al.* (1995) reported that sex of Rumex acetosa is influenced by a dosage compensation mechanism based on X chromosome to autosome ratio.

Lou *et al.* (2002) reported that one of the sexes, usually the male, is heterogametic, producing two types of gametes and bearing male or female factors. The other, the female, is homogametic and gives rise to only one type of gamete bearing the feminizing factors.

Barett and Droken (2004) reported the major evolutionary pathways for the origin of dioecy in plants (the gynodioecy and monoecy–paradioecy pathways) are often distinguished by the number of mutations involved and the magnitude of their effects. They investigated the genetic and environmental determinants of sex in *Sagittaria latifolia*. Several lines of evidence implicate two-locus control of the sex in phenotypes. In dioecious populations sex is determined by Mendelian segregation of alleles, with males heterozygous at both the male- and female-sterility loci. In monoecious populations, plants are homozygous for alleles dominant to male sterility in females and recessive to female sterility in males.

Liu *et al.* (2004) found that papaya has a primitive Y chromosome, with a male specific region of about 10% which has undergone several recombinations, depressions and DNA sequence changes. The study indicates that it may be an incipient chromosome due to the severe suppression of recombination and the extensive divergence between homologues in the region that contains the genes that determine sex type in papaya.

Ma *et al.* (2004) reported that sex type is determined not by one gene, but rather by a complex of genes that are confined to a small region on the sex chromosome within which crossing over is precluded. The sex type determination in papaya is of XX-XY type. The genotype of the male is XY, female XX, and hermaphrodite XY2, where Y2 is the modified Y chromosome. The Y chromosome has a lethal region and the Y2 chromosome preserves this lethal region.

Ming *et al.* (2007) reported that in papaya physical mapping and sample sequencing of the non-recombination region led to the conclusion that sex determination is controlled by a pair of primitive sex chromosomes with a small male-specific region (MSY) of the Y chromosome. They postulated that two sex determination genes control the sex determination pathway. One, a feminizing or stamen suppressor gene causes stamen abortion before or at flower inception while the other, a masculinizing or carpel suppressor gene, causes carpel abortion at a later flower developmental stage.

2.3. Marker assisted selection

Marker assisted selection or marker aided selection (MAS) is a process whereby a marker (morphological, biochemical or molecular markers) is used for indirect selection of a genetic determinant or determinants of a trait of interest. It is indirect selection process where a trait of interest is selected not based on the trait itself but on a marker linked to it. Molecular marker is based on DNA or RNA variation in phenotype. MAS can be useful for traits that are difficult to measure, exhibit low heritability, and/or are expressed late in development. Morphological, Biochemical and Molecular markers come under this.

2.3.1. Influence of sex on morphological characters

Differences in morphological traits have been observed in the male and female plants of different dioecious species. The possibility of using morphological characters as sex markers have been investigated by many

Lacombe (1980) observed significant difference in the intranode length, growth rate and plastochron of male and females of dioecious hemp *Cannabinus sativa*. He reported that sex of plants could be determined at the age of 15 days based on early vegetative characters.

Kotaeva *et al.* (1982) revealed that females of both species *Morus alba and Ficus arabica* were having larger leaves, denser and more spreading crown than males.

Chen *et al.* (1985) reported 100 per cent correlation between sex and length/ width (L/W) ratio of leaves of jojoba. He observed that L/W ratio of all the female was greater than average and that of all the males was smaller.

Oyama (1990) reported that dioecious palm *Chamaedorea tapijelute*, male plants showed spatial variation in growth rate but not the females. Both the sexes had different rates of production of leaves among years and males produced significantly more inflorescence than females.

Kohorn (1994) repored that in jojoba, *Simmondsia chinensis* which is a dioecious desert shrub, the females were found to have larger leaves and more open canopies than males.

Nyong *et al.* (1994) reported that in odum, (*Milicra excelsa*), a forest tree species, the females were found to possess more spreading crown and thicker stems than the males.

Machon *et al.* (1995) reported that in the dioecious perennial *Asparagus officinalis* the male plants produced more but thinner stems than females.

2.3.2. Molecular characterization

The detection of naturally occurring DNA sequence polymorphisms between individuals and using that as molecular markers for crop improvement represent one of the recent development in molecular biology. The DNA-based markers selection has been accelerated in the past few years by the advent of the PCR (Saiki *et al.* 1988; Mullis *et al.* 1986). This facilitated the development of marker-based gene tags, gender identification, cloning of agronomically important genes, variability studies, phylogenetic analysis and marker-assisted selection of desirable phenotypes.

2.3.2.1. Molecular markers in gender identification

Molecular markers have opened up new opportunities for studies in gender identification at early stage of plant. These markers have been used to identify sex in a number of agriculturally important crops, such as pistachio, cannabis, date palm, papaya, pepper and asparagus.

RAPD, RFLP and ISSR techniques were used to identify sex at early stage of crop before commencement of flowering.

2.3.2.2. Random amplified polymorphic DNA (RAPD)

The RAPD technique is based on the use of single arbitrary oligonucleotides as primers for PCR (William *et al.* 1990). These primers are synthetically produced random DNA sequences, approximately 10 nucleotides in length, with 50-60% GC content. RAPD markers have been proposed as an alternative to RFLP (Welsh and Mc clelland, 1990; William *et al.* 1990) and require less DNA, do not require southern blotting and radioactive labeling and are relatively quick to assay. It is inherited in Mendelian fashion and can be generated without any prior knowledge of the target DNA sequence (Welsh *et al.* 1991). It is inherited as dominant markers, where the presence of particular band is dominant, and its absence is recessive (Tingley and Tufo, 1993).

RAPD analysis has found applications in population studies, gender determination, biosystematics, gene tagging and finger printing. It is difficult to identify sex in many species of eukaryotic organism. Fortunately, one sex often possesses a unique chromosome termed Y or W. When DNA markers are available for these chromosomes, and then sex identification becomes straightforward. The procedure makes use of low stringency PCR to screen randomly selected primers for their ability to amplify sex-specific loci (Griffith and Tiwari, 1993).

Mulcahy *et al.* (1992) used pooled equal quantity of DNA from 18 male and female siblings separately to obtain markers for the Y- chromosome of *Silene latifolia*. They screened 60 primers out of which four given polymorphic bands in male which were not found in female DNA.

Hormaza *et al.* (1994) screened 1000 primers in *Pistichia vera* for sex determination and found only one female associated marker, which was absent males. They suggested that low frequency of sex-linked bands may indicate that the DNA segments involved in sex determination is small and probably involves a single gene or few genes.

Sakamoto *et al.* (1995) analysed male-associated DNA sequences in a dioecious plant, *Cannabis sativa* L. Two out of 15 primers yielded fragments of 500 and 730 bp which were detected in all male plants but not in any of the female plants tested. These two DNA fragments were cloned and used as probes in southern blot analysis of genomic DNA. When the male and female DNAs were allowed to hybridize with the 730-bp much more intense bands specific to male plants were detected, in addition to less intense bands that were common to both sexes.

Polley *et al.* (1997) reported that sex expression in plant controlled by a chromosome mechanism. They identified molecular markers associated with the Y or male specific chromosome in hop. They screened 900 primers, out of which only 32 primers given specific fragments in males which were absent in females. Subsequently these 32 primers were tested with unrelated male and female lines, three out of 32 primers given specific band for Y chromosome. The Y- specific product derived from one of these primers (OPJ 9) was low copy in hybridization. Primers developed from the DNA sequence of this product provided a marker for rapid sex identification.

Alstrom *et al.* (1998) identified a molecular marker linked to sex determination loci in dioecious plant *Salix viminalis*. They used 380 decamer primers to generate RAPD products. Only a single 560 bp band was linked to sex determination locus. This

marker UBC 354 $_{560}$ is associated with femaleness and is linked to allele A in the proposed two locus epistatic genetic model of sex determination for *S. viminalis*.

Claudete *et al.* (1998) identified a 2075 bp male specific band after screening 158 RAPD primers in dioecious *Atriplex garretii*. They tested DNA from ten male and female and hermaphrodites plants individually and reported that male specific fragment was present in hermaphrodites, in all males and absent in all females.

Kafkas *et al.* (2001) reported that the RAPD work in *P. terebinthus*. They used the 3 species of this cultivar and screened the 312 primers. Out of them OPC 11 and BC 152 amplified 3 bands in *P. terebinthus*, BC 156 and BC 360 amplified 3 bands in *P. eurocarpa*. The results showed that in each of putative sex related bands, the correlation with sex only is partial and some individuals have the marker phenotype of the opposite sex. It is possible that the marker is linked to sex determining locus but not very tightly.

Mulcahy *et al.* (2001) identified DNA regions specific to X and Y chromosomes in *Sphaerocarpus texans*. They used 238 decamer primers to identify sex- specific markers. Out of 238 primers, four primers had given polymorphism. Three markers specific to female and one specific to males.

Shirkot *et al.* (2001) screened the 34 random decamer primers to identify sex in kiwi fruit. Out of which 8 were identified as sex-linked markers, six were specific to female and two specific to male. RAPD data generated by using 34 primers used to develop dendrogram using UPGMA method.

Lemos *et al.* (2002) reported that RAPD analysis was used to differentiate sexual forms of *Carica papaya* belonging to solo group. BC 210 primer was able to detect hermaphrodite in all cultivars. BC_{210} 438 molecular marker was better for papaya sex determination than the other markers.

Singh *et al.* (2002) identified a female sex associated 567 bp RAPD marker in point gourd (*Trichosanthes dioca*) which is completely absent in males. They screened 100 decamer primers for this work.

Prakash and Staden (2006) reported that 69 primers were used to amplify the bulk DNA of five individuals of each male and female sample *of Encephalaris natalensis*. Out of 140 primers only one, OPD 20 generated a specific band (850 bp) in female DNA.

Agarwal *et al.* (2007) reported the RAPD technique in *Simmondisa chinensis* (jojoba). They screened 72 primers , out of them OPG-5 produced a unique 1400 bp fragment in male DNA of four cultivars and which was absent in respective female plants.

George *et al.* (2007) identified a 600 bp male specific band in palmyrah (*Borassus flabellifer* L.). 180 decamer primers were used. The band produced by the primer OPA 06 is tightly linked to male sex locus and is useful for sex determination in palmyrah.

Mokkamul *et al.* (2007) screened 37 decamer RAPD primers. Out of 37, only one RAPD primer shown polymorphism, which yielded a 750 bp male specific marker absent in respect all female plants of *Nepenthes gracilis*.

Mukherjee *et al.* (2007) studied sex of *Carica papaya* and *Cycas circinalis* using ISSR and RAPD techniques in pre-flowering stage. In papaya ISSR profile generated one female specific band using primer (GACA)4. Sequencing of a male-specific RAPD band (PCR with primer OPB 01) in *C. circinalis* revealed homology with putative retro elements of diverse plants.

Sim *et al.* (2007) screened 69 primers to search for sex- linked DNA markers for *Garcilaria changii*, and one sex-linked marker (716 bp) was identified using OPA 18.

Kumar *et al.* (2008) reported the RAPD analysis for sex determination in *Trichosanthes dioca*. They screened 41 random decamer primers with three bulk samples of male and female and parthenocarpic plants. Six primers were tested with

individual plant sample and they identified three sex associated RAPD markers. Out of three, OPC 05_{1000} is male specific, OPC 14_{400} is female specific and OPN 01_{1030}, which amplified a 1030 bp fragment in parthenocarpic fruit plants.

Rajendran *et al.* (2008) has reported the genomic DNA content was less than 60% in females compared to males in date palm. They carried out PCR to amplify alcohol dehydrogenase (AdhA) gene from genomic DNA, which yielded two fragments in female and one fragment in male.

Shibu *et al.* (2008) has reported about identification of sex specific DNA marker in the dioecious nutmeg. They isolated DNA using CTAB method (Doyle and Doyle, 1987) and bulk segregant analysis of known female and male trees was done with RAPD for detecting polymorphism between them. They screened 60 primers for selecting primers with good resolving power. Based on amplification product and similarity index RAPD analysis was conducted with 19 primers. Out of 19 primers, OPE 11 yielded a clear and characteristic amplification band (416 bp) only in female.

Yin *et al.* (2008) reported RAPD technique to determine the sex of *Calamus simplicifolius*. They used DNA samples from ten male and female plants and screened 1040 decamer primers. Out of them OPS 1_{1443} primer has given characteristic 500 bp male specific DNA fragment.

Younis *et al.* (2008) attempted to identify sex specific DNA markers in datepalm using RAPD and ISSR techniques to facilitate the identification of good male pollinators. RAPD and ISSR analyses had given three positive specific markers for females and two for males and five positive markers for males. The level of polymorphism revealed was 70% and 87% for RAPD and ISSR respectively.

Hou *et al.* (2009) identified markers that are linked to the sex expression in the dioecious *Populus tomentosa*. A total of 88 primers were screened. Twelve primers produced clear patterns with at least one band that appeared to be polymorphic between the two bulks of male and female DNA. Subsequently, five male and female individuals

were analyzed with those 12 primers, and only S60 (ACCCGGTCAC) could generate a common 1800 bp DNA fragment in all five male individuals and bulk of the same but not in any female individuals.

2.3.3. Sequence Characterized Amplified Region (SCAR)

A SCAR is a genomic DNA fragment at a single genetically cloned locus that is identified by PCR amplification using a pair of specific oligonuclotide primers (Williams *et al.* 1990). Mispriming errors occurring in PCR amplification frequently caused problems with reproducibility of RAPD profiles and specific bands. To overcome this problem, Paran and Michelmore (1993) converted RAPD fragments to simple and robust PCR markers, termed Sequenced Characterized Amplified Region (SCAR). It is similar to the RAPD method but uses longer primers, which are generally composed of between 18 and 24 bases. By increasing the specificity of the primers, the results become more reproducible and more specific (Hernandez *et al.*, 1999).

Jiang and Sink (1997) used Bulk Segregant Analysis (BSA), RAPD and SCAR methods to map molecular markers linked to the sex locus M of Asparagus. Two parents and 69 progeny were used. A total of 760 primers were used for RAPD analysis. Primer OPC- 15 produced two RAPD markers OPC 15-98 and OPC 15-30. Both of which were linked to M locus at a distance of 1.6 CM. RAPD fragment OPC 15-30 was cloned and sequenced. The sequence was used to design SCAR primers SCC 15-1 and SCC 15-2. Both of these primers amplified a single 980 bp fragment; the same size as cloned RAPD fragment.

Kafkas *et al.* (1998) reported that RAPD fragments were generated in order to identify markers specific to sex in *Atriplex garretti*. They screened 158 decamer primers. Out of 158, only OPF 14 generated a 2075 bp male specific DNA fragment. The fragment was cloned and sequenced and 24 mer SCAR primers designed. Later 124 male, 126 female and one hermaphrodite plant were tested with specific primer. The male specific fragment was present in all males one hermaphrodite and absent in all females.

Mandolino *et al.* (1999) reported that a 400 bp RAPD marker generated by a primer has been associated with the male sex in hemp (*Cannabinus sativus* L.). $OPA08_{400}$ band was present in all male plants but absent in female. The RAPD marker was sequenced and specific primers were designed. These primers generated a SCAR marker 390 bp, which was male specific and suitable for early and rapid identification of male plants.

Parasnis *et al.* (2000) reported a PCR-based Seedling Sex Diagnostic Assay (SSDA) specially designed for early sexing of papaya seedlings. They have developed a male-specific SCAR marker in papaya by cloning a male-specific RAPD (831 bp) fragment and designing longer primers.

Jung *et al.* (2001) used RAPD technique to detect potential male specific DNA marker in *Schisandra nigra*. They screened 120 primers, only one primer OPA-17 given 800 bp fragment specific to males. The nucleotide sequence was used to design a SCAR marker of 486 bp which was male specific.

Deputy *et al.* (2002) developed molecular markers in papaya, which identified sex of the plants. They synthesized the SCAR T_{12} and SCAR W_{11} primers. These amplified products in male and hermaphrodites and rarely in females. SCAR T_1 amplified a product in all papayas regardless of plant sex. A PCR based technique for determining the sex of papaya was developed by using W_{11} and T_{12} primers to detect hermaphrodite and male allele.

Khadka *et al.* (2002) identified OPB 01_{1562} and OPC 07_{303} as male specific RAPD markers in *Mercurialis annua*. These fragments were sequenced and SCAR primers were designed. Several internal segments of OPB 01_{1562} were amplified as male specific SCAR markers. The OPB 01_{1562} derived SCAR markers were absent in females as well as hexaploid male. The gender relationship of the markers was maintained in all ecotypes tested. Two internal fragments of OPB 01_{1562} were amplified in all genotypes of mercularis.

Torjek *et al.* (2002) designed SCAR markers to distinguish sex in hemp. The primers OPD 05 and UBC 354 generated specific bands in male plants. These fragments were cloned and sequenced. These markers were named as MADC 3 (OPD 05 $_{961}$) and MADC 4 (UBC 354 $_{151}$). These markers converted into SCAR markers. The SCAR markers were correlated with sex of segregating population and proved tight linkage to the male phenotype. Results of F_2 plant population analysis suggest that these markers are to be linked to the Y chromosome.

Chaves and Bedoya (2004) screened 32 decamer primers and the primer OPY 07 generated a 900 bp band in male plants of papaya, which was absent in female and hermaphrodites of papaya. The OPY 07 $_{900}$ converted to a SCAR marker and amplicon was identified as sequence discriminating sex of papaya (SDSP). The 515 bp sequence found in males and hermaphrodites were absent in females. From this sequence 20 mer SCAR primer designed it amplified a 369 bp fragment in males and hermaphrodites and was absent in female.

Wen-jie *et al.* (2004) used RAPD technique to screen markers of sex expression in *Eucommla ulmoides*. A 569 bp RAPD marker was fond in pistillate but not in staminate plants. The results were confirmed by southern blotting. The marker was sequenced and specific primers were synthesized to generate 569 bp pistillate –specific SCAR marker.

Longdou *et al.* (2006) used the RAPD technique to find markers linked sex in *Ginkgo biloba*. Out of the 158 RAPD primers only one primer S 2 generated 1080 bp marker, which was associated with male plant. The fragment was sequenced and SCS2-1 and SCS2-2 SCAR primers were synthesized. Both primers amplified a single 1080 bp fragment in male plants.

Vinod *et al.* (2007) reported that OPO-08 RAPD marker amplified a 1263 bp band in male which was absent in females of *Pandanus fascicularis*. A SCAR marker (MSSRF-01) was designed for this fragment and continued to amplify the specific allele

in all the male plants. Southern hybridization results also confirmed the RAPD work and strongly suggested that (MSSRF-01) is male specific molecular marker in *Pandanus fascicularis*.

Devaiah and Venkatasubramanian (2008) reported that the developed a SCAR marker for authentication of *Pureria tuberosa*. OPA 08_{600} bp marker specific to *P. tuberosa*. This RAPD amplicon was converted to the SCAR marker; it revealed the expected amplicon (320 bp) in male plants.

Manoj *et al.* (2008) reported the developed a SCAR marker from RAPD marker sequence in *Piper longum* fto distinguish male plants from females. The SCAR primers MPS1A and MPS1B developed from MADP1. RAPD marker sequence was effective in amplifying male specific DNA sequence.

Maki and Masayuki (2009) identified two sex linked fragments were identified by RAPD analysis in *Aucuba japonica* var *ovoidea* and were converted to markers of male specific SCAR markers. The SCAR primers were designed from RAPD amplicon OPA 10_{424}. The SCAR primers were amplified 400 bp amplicon in male plants.

Xiao *et al.* (2009) performed bulk segregant and individual analyses with RAPD and AFLP markers for sex identification in Actinida. The segregating populations were a full sibling of *Actinida deliciosa* × *Actinida eriantha* hybrid family (P_1) and two intra-specific cross families of diploid *A. chinensis* (P_2 and P_3). In the study 520 decamer primers, 64 AFLP primer combinations were analysed in bulks of 3 populations. Two RAPD primers OPA 09 and OPA 12 produced male related band in P_2. M_{450}-OPA 09, a band of 450 bp was present in all males and $1/6^{th}$ females. The band M_{700}-OPA 12 was absent in all female and present in $5/6^{th}$ male plants. In P_2, one AFLP primer EACG/M-CAA amplified a 600 bp region in male plants.

2.4. Isolation of DNA

Isolation of good quality DNA is pre requisite for all molecular analyses.

Barnwell *et al.* (1998) standardized DNA isolation in *Sedum telephium*, which is rich in polysaccharides. They modified Rogers and Bendich (1994) method by increasing the CTAB concentration from 2% to 10% with 0.7 M NaCl. The yield of pure DNA was 150-300 µg/g.

Sharma *et al.* (2000) standardized DNA isolation for mature leaves in *Arachis hypogeá* which is rich in polyphenols and polysaccharides. They modified Dellaporta *et al.* (1983) method by including DNA elution medium (2 M NaCl, 10mM Tris, 1mM EDTA) and DNA wash medium (400 mM NaCl, 10mM Tris, 1mM EDTA) and got pure DNA.

Samal *et al.* (2002) reported an efficient protocol for DNA from cashew, which is rich in polysaccharides, polyphenol and secondary metabolite. Use of extraction buffer (boric acid, CTAB, EDTA, NaCl and β-mercaptoethanol) and subsequent steps of chloroform extraction facilitated the isolation of pure DNA. Addition of 5M NaCl and ethanol increased the solubility of polysaccharides and decreased the solubility of polysacchrides and decreased the co-precipitation of polysaccharides with DNA.

Hanania *et al.* (2004) standardized DNA isolation in grape vine. They modified Bernatzly and Taksley (1986) procedure with addition of NaCl to remove polysaccharides and sodium meta bi sulphate to prevent oxidation of phenolic compounds. The quality of DNA is 150-400 µg/g.

Yang *et al.* (2007) reported DNA extraction method for sunflower leaves. They modified CTAB method with addition of 1.42M NaCl and phenol: chloroform: isoamyl alcohol extraction step to remove polysaccharides effectively.

Harini *et al.* (2008) standardized DNA isolation in *Urginea indica* and optimized conditions for RAPD-PCR analysis. Cell lysis was carried out in an

extraction buffer supplemented with CTAB (3%) and sodium chloride (5M). Two emulsification washes with phenol: chloroform: isoamyl alcohol followed by re-precipitation with salt efficiently removes high protein and polysaccharide contamination. Yield of pure DNA was 120-150 ug.

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

Sheeja *et al.* (2008) reported an efficient protocol for isolation of DNA from wild and related genera of Myristica which are rich in polysaccharides and polyphenols. CTAB (3%), 1.5% PVP and 0.3% β- mercaptoethanol utilized for isolation of DNA. Changing the centrifugation temperature from 4^0C to 25^0C facilitated easy separation of supernatant. The yield of DNA ranged between 25-175 µg/g.

2.5. Biochemical characterization

2.5.1. Isozyme analysis

In recent years the analysis of isozyme by polyacrylamide gel electrophoresis (PAGE) has been considered as a unique and powerful technique for ascertaining genetic relationship in plants.

Isozymes can be the genetic basis for polymorphism in phenotypes. Because isozymes are proteins, and directly reflect alternations in the DNA sequence through changes in aminoacid composition. This will change the electrophoretic mobility of isozyme in the PAGE. Changes in the elecrophoretic mobility of enzymes provide an extremely useful method of evaluating genetic difference among groups.

Isozymes are multiple molecular forms of enzymes that catalyse the same reaction but differing in physico chemical properties (Market and Muller, 1959). Further PAGE provides tool for species and cultivar identification where morphological and cytological data are inadequate. (Wilkinson and Beard, 1972)

Oliver and Zapater (1985) reported that among organic molecules isozymes are very useful aids in deciphering the evolutionary relationship within different groups of plants and animals. Isozyme analysis has been reported to be useful in varietal characterization and classification in a variety of crops.

Gulati (1989) reported qualitative and quantitative changes in peroxidase, acid and alkaline phosphatases of the shoot tips, nodal segment with buds, inter nodal segments, leaves and mature flowers in male and female plants of *Coccinia grandis*. Higher peroxidase and phosphatase activity was noted in the explants from male plants over those from the female plants. Variation in isoenzyme pattern has exhibited by different explants obtained from both male and female plants.

Maestri *et al.* (1990) analysed extracts from phylloclads of *Asparagus officinalis* electrophoretically for isozyme polymorphism. Fourteen enzyme systems were examined using four buffer systems: seven enzymes (acid phosphatase, catalase, glutamate-oxaloacetate transaminase, isocitrate dehydrogenase, malate dehydrogenase, peroxidase, and 6-phosphogluconate dehydrogenase) exhibited clear and consistent banding patterns. They also studied isozyme polymorphism in seven pairs of male and female doubled haploids and in their male F_1 progenies. Segregation of polymorphic loci was examined in the backcross progenies and was found to be consistent with a simple Mendelian inheritance in all cases, except for three anodical peroxidases. Based on this it has been hypothesized that no linkage could be found between isozyme markers that were segregating in the same cross, but association was demonstrated between one malate dehydrogenase locus and the sex determining genes. The availability of isozyme markers may be useful in breeding and in particular, the localization of one malate dehydrogenase locus on the sex chromosomes may be helpful in mapping the sex genes.

Bhat *et al.* (1992) demonstrated the usefulness of analyses of esterase, acid phosphotase and catalase isozymes to distinguish the different cultivars of banana.

Stejskal (1994) reported two staining methods for aspartate aminotransferase in electrophoresis resolution of its isozymes in polyacrylamide gel. The first one uses L-aspartic acid and Fast Blue BB salt (classical method), the second uses L-cysteine sulfmic acid and a redox system with phenazine methosulfate and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide. The seeds of pea, horse bean and soybean were used as a model plants for source of the enzyme. The staining method with L-cysteine sulfmic acid is very reliable and more sensitive than the Fast Blue BB method and allows detection at very low isozyme activities in the gel.

Thomas (1997) has attempted characterization of dioecy in nutmeg through morphological and biochemical analysis. She didn't get stable and specific morphological markers for distinguishing male and females. Biochemical characters studied were phenol and essential oil content, peroxidase enzyme activity and colour test with ammonium molybdate. She found high phenol content in males and high essential oil content in females. Colour test with ammonium molybdate recorded high OD values in female. Peroxidase activity is same for both male and female.

Elena *et al.* (2002) reported peroxidase and esterase isoenzymes analysis in leaves of the two sexual phenotypes of hemp. Significant differences in isoperoxidase and isoesterase patterns were found between male and female plants, both in the number and stain intensity of bands. For both esterase and peroxidase, the isoenzymatic spectrum was richer for staminate plants. Also, some differences are obvious between the two sexes concerning catalase and peroxidase activities, as well as the level of soluble protein. The quantitative analysis of flavones, polyholozides and polyphenols emphasized differences, depending not only on sex, but also on tested organ.

Saker *et al.* (2002) reported that Polyacrylamide gel electrophoresis of peroxidase (PER), polyphenol oxidase (POD), acid phosphatase showed mostly similar banding pattern of all tested date plam plants. However, remarkable and reproducible variations in esterase (EST) and glutamate oxaloacetate transaminase (GOT) isozyme banding

patterns were detected in some clones. In general, falvonoid profiles and isozyme banding patterns data can be used as an early test to screen tissue culture derived date palm clones for genetic stability.

Petrova *et al.* (2006) investigated the electrophoretic patterns of reduced proteins and ADH, ACPH, EST and GOT from leaves of *in vitro* micropropagated plantlets of Gentiana lutea L. Qualitative isoenzyme variations were found for ADH, ACPH and EST among *in vitro* plantlets whereas the variations for GOT isoenzymes were only quantitative. The isoenzyme variation was discussed in respect to the differences in the type of growth regulators included in the nutrient media.

Beksheet *et al.* (2008) has reported that acid phosphotase and glutamate oxaloacetate enzymes gave strong difference between male and female date palms. They also found that certain RAPD primers also exhibit polymorphism among male and female trees. Molecular markers using SCAR has been developed for distinguishing sex in hop (Polley *et al.*, 1997) and asparagus (Bitti *et al.*, 1995).

Materials and Methods

3. MATERIALS AND METHODS

The study on "Sex determination in Nutmeg (*Myristica fragrans* Houtt.) through molecular and biochemical markers" was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara during the period 2008-2010. The materials used and methodologies adopted are discussed in this chapter.

3.1. MATERIALS

3.1.1. Plant Materials

Five each of well differentiated male and female trees of 15 years age were selected from the Plantation and spices farm, College of Horticulture, Vellanikkara (Plate 1 and 2). The sex of the plant was confirmed by observation of inflorescence and flowers. Inflorescences in both were cyme, but flowers in males in clusters (3-8) (Plate 3A) while in female were solitary (1-2) (Plate 3B). The androecium of staminate flower consisted of solid anther comb with 10 to12 bilocular anthers (Plate 4A). The gynoecioum of pistillate flowers have single pistil of more or less flask shape with short sessile style, bilobed stigma and single ovule (Plate 4B). Occasional fruiting males have branched cymose inflorescence with cluster of 3-8 male flowers. Four occasional fruiting males were also used for testing the gender with SCAR marker. The tender, pale green, immature third leaf from apex was used as leaf sample for the RAPD and isozyme analyses.

3.1.2. Laboratory chemicals and glassware

The chemicals used in the present study were of good quality (AR grade) procured from Merck India Ltd., SRL, HIMEDIA and SISCO Research Laboratories. The Taq DNA polymerase, dNTPs, Taq buffer and molecular marker (λDNA/*Hind*III+ *Eco*RI double digest) were supplied by Bangalore Genei. The random primers were obtained from Operon Technologies, USA. The plasticwares used for the study were purchased from Tarsons India Ltd., and Axygen, USA.

Plate 1. Female nutmeg tree

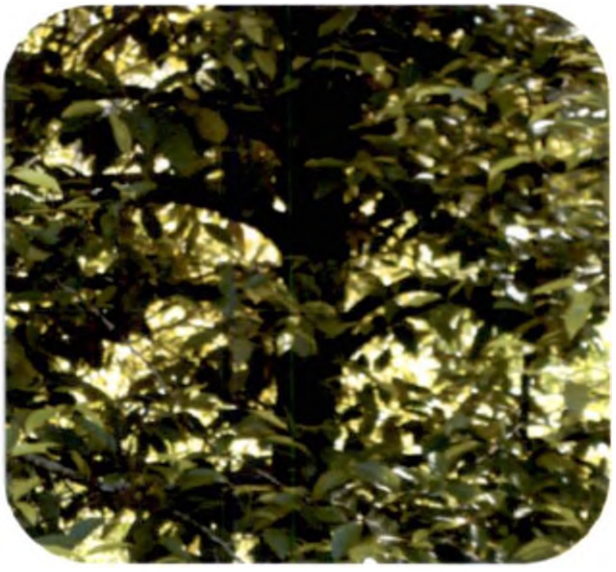


Plate 2. Male nutmeg tree

Plate 3A. Twig with male inflorescence

Plate 3B. Twig with female inflorescence

Plate 4A. Androecium of male flower

Plate 4B. Gynoecium of female flower

The equipments available at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture were used for the present study. Centrifugation was done in High speed refrigerated centrifuge (KUBOTA 6500). Estimation of quality and quantity were done by using NanoDrop^{R} ND-1000 spectrophometer. The PCR was done in Eppendrof Master Cycler (Eppendrof, USA) and agarose gel electrophoresis done in horizontal gel electrophoresis system (BIO-RAD). Gel DOC-It^{TM} Imaging system UVP (USA) was used for imaging the gel. Isozyme analysis was done by using Mini PROTEAN (BIO- RAD). The list of laboratory equipments used for the study is provided in Appendix I.

3.2 METHODS

3.2.1. MOLECULAR MARKER ANALYSIS

Molecular analysis of selected male and female plants was carried out with RAPD and SCAR marker.

3.2.1.1. Standardization of genomic DNA isolation

Isolation of good quality genomic DNA is one of the most important pre requisites for doing RAPD and SCAR analyses. The procedure reported by Rogers and Bendich (1994) for the extraction of nucleic acids was used for isolation of genomic DNA from Nutmeg with certain modifications. Modifications were made with extraction buffer and CTAB solution. Extraction buffers 2X, 3X and 4X were tried. CTAB solution concentrations of 10 per cent and 5 per cent also tested. Tender pale green and mature dark green leaves from the selected five male and female plants were collected early in the morning and used for the genomic DNA isolation

Reagents

CTAB extraction buffer (2X, 3X, 4X)

Chloroform: Isoamyl alcohol (24: 1, v/v)

CTAB solution (5%, 10%)

Isopropanol

Ethanol 70%

Distilled water

Details of composition of reagents are provided in the Appendix II.

Procedure

Leaf sample (1g) was ground to fine powder in excess liquid nitrogen using a pre-chilled mortar and pestle. A pinch of poly vinyl pyrrolidine (soluble) and 50 µl of β-mercaptoethanol were added. Later 5 ml extraction buffer was also added. The homogenate was transferred to a 50 ml of Oakridge tube. The contents were mixed well and maintained at 65^0C for 45 minutes. Equal volume of chloroform: isoamyl alcohol (24:1) mixture was added to the tube, mixed gently by inversion and centrifuged at 10000 rpm for 15 minutes at 4^0C. The contents get separated into three distinct phases. The upper aqueous phase containing DNA was pipetted out into 50 ml of Oakridge tube. Then added $1/10^{th}$ volume of 5 per cent CTAB and equal volume of chloroform: isoamyl alcohol (24:1) mixture, mixed gently by inversion and centrifuged at 10000 rpm for 15 minutes at 4^0C. The supernatant was collected into 50ml of Oakridge tube, then 0.6 volume of ice cold chilled isopropanol was added and the contents were mixed gently. The sample was then incubated at -20^0C for 30 minutes to precipitate the DNA completely. The DNA was then pelleted by centrifuging at 10000 rpm for 15 minutes at 4^0C. The isopropanol was poured out and the pellet was washed with 70 percent alcohol. The pellet was air dried to remove the alcohol and finally dissolved in 20 µl

distilled water. The isolated DNA was electrophoresed on 0.8 per cent agarose gel along with λDNA / *Eco*RI+*Hind*III double digest as molecular marker.

3.2.1.2. Electrophoresis of DNA

Reagents and Equipments

1. Agarose – 0.8 per cent (for genomic DNA)

   – 1.2 per cent (for PCR samples)

2. 50X TAE buffer (P^H 8.0)

3. Tracking dye (6X)

4. Ethidium bromide (stock 10 mg/ml; working concentration; 0.5 µg/ml)

5. Electrophoresis unit, power pack, gel casting tray, comb

6. UV transilluminator (Herolab ^{R})

7. Gel documentation and analysis system Gel DOC-It ^{TM} Imaging system UVP (USA)

Composition of reagents is provided in Appendix III.

Procedure

1X TAE buffer was prepared from the 50X 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 and mixed well. The open end of gel casting tray was sealed with a cellotape and kept on a horizontal surface. The comb was placed desirably and the dissolved agarose was poured on to the tray. The gel was allowed to set for 30 minutes 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 (5 µl) along with tracking dye (1 µl) was loaded

into the wells using a micropipette carefully. λDNA / E *co*RI+H*ind*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 (100V) 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 transilluminator for presence of DNA. The DNA fluoresces under UV light due to ethidium bromide dye. The image was documented in gel documentation system (Gel DOC-It ^{TM} Imaging system UVP (USA). The gel profile was examined for intactness, clarity of DNA band, presence of RNA and protein.

3.2.1.3. Purification of DNA

The DNA contained RNA as contaminant and was purified by phenol precipitation and RNase treatment.

Reagents

Phenol: chloroform mixture (1:1, v/v)

Chilled isopropanol

70 per cent ethanol

TE buffer

Chloroform: Isoamyl alcohol (24:1, v/v)

One per cent Rnase

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

Procedure

RNase solution (2 µl) was added to 100 µl DNA sample and incubated at 37^{0}C 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 mixture was added. This was then centrifuged at 12,000X g for 10 minutes at 4^{0}C. The aqueous phase was collected in a fresh micro centrifuge tube and added equal volume of chloroform: isoamyl alcohol (24: 1). Again they were centrifuged at 12,000Xg for 10 minutes at 4^{0}C. The above two steps were repeated and finally precipitated the DNA from the aqueous phase with 0.6 volume of chilled isopropanol. The mixture was then incubated at -20^{0}C for 30 minutes and centrifuged at 10,000 rpm for 15 minutes at 4^{0}C. The pellet of DNA was washed with 70 per cent ethanol. The pellet was air dried and dissolved in 250 µl TE buffer.

3.2.1.4. Assesing the quality and quantity of DNA

The purity of DNA was further checked by using NanoDrop^R ND-1000 spectrophometer. One µl sample was used for assessing the purity of DNA. The OD values of nuleic acid samples were measured at a wavelength of 260 nm and 280 nm. The purity of DNA was assessed by the ratio OD_{260}/OD_{280}. A ratio of 1.8 to 2.0 indicated pure DNA. The quantity of DNA in the pure sample was calculated using the formula OD_{260}= 1 is equivalent to 50 µg double stranded DNA/ µl sample.

1OD at 260 nm = 50 µg DNA/ml

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

DNA of selected male and female were isolated and checked for purity. UV absorbance ratio (OD_{260}/OD_{280}) of each sample and quantity of DNA were recorded.

3.2.1.5. RAPD (Random Amplified Polymorphic DNA) analysis

The good quality genomic DNA isolated from Nutmeg leaf samples were subjected to RAPD as per the procedure reported by William *et al.*, (1990). Random decamer primers supplied by 'Operon Technologies' USA with good resolving power were used for amplification of DNA. So decamer primers for RAPD assay were selected after an initial screening study of primers.

3.2.1.5.1. Screening of random primers for RAPD analysis

Sixty seven decamer primers in the series OPA, OPY, OPN, OPS, OPK, OPG, OPD, OPAH, OPO, OPC and OPF were screened with genomic DNA of nutmeg male and female leaves as the template. Thirteen sex specific primers as cited by review were also included. Details of the primers used for screening is given in (Table 1). Genomic DNA at the concentration of 50 ng was subjected to PCR amplification using decamer primers. The samples used were the following.

1. DNA of individual male plant

2. DNA of individual female plant

3. Negative control (without DNA)

The amplification was carried out in an Eppendrof Master Cycler (Eppendrof, USA). A master mix without the template DNA was prepared using the reaction mixture for the required number of reactions. From this master mix, 17.5 µl was pipetted out into each PCR tube. Template DNA (1 µl) was added. PCR amplification was performed in a 20 µl reaction mixture as constituted below.

Table1. List of Operon decamer primers used for screening nutmeg samples

Sl. NO	Primer	Nucleotide Sequence (5'-3')
1	OPA 38	AGTGCATTCA
2	OPA 27	ATCGGATTCA
3	OPA 36	AGCCAGGGAA
4	OPA26	GGTCCCTGAC
5	OPA28	GTGACGTAGG
6	OPA29	GGGTAACGCC
7	OPA39	CAAACGTCGG
8	OPA32	TCGGCGATAG
9	OPA12	TCGGCGATAG
10	OPA10	GTGATCGCAG
11	OPA23	AGTCAGCCAC
12	OPA22	TGCCGAGCTG
13	OPA30	GTGATCGCAG
14	OPA40	GTTGCGATCC
15	OPA24	AATCGGGCTG
16	OPA34	TCTGTGCTGG
17	OPY01	GTGGCATCTC
18	OPY02	CATCGCCGCA
19	OPY03	ACAGCCTGCT
20	OPY04	GGCTGCAATG
21	OPY05	GGCTGCGACA
22	OPY06	AAGGCTCACC
23	OPY08	AGGCAGAGCA
24	OPY09	AGCAGCGCAC
25	OPY10	CAAACGTGGG

Sl. NO	Primer	Nucleotide Sequence (5'-3')
26	OPY11	AGACGATGGG
27	OPY12	AAGCCTGCGA
28	OPY13	GGGTCTCGGT
29	OPY14	GGTCGATCTG
30	OPY15	AGTCGCCCTT
31	OPY16	GGGCCAATGT
32	OPY17	GACGTGGTGA
33	OPY18	AAGCGGTCAG
34	OPY19	TGAGGGTCCC
35	OPY20	AGCCGTGAAA
36	OPF14	GGTGCGCACT
37	OPF03	CCTGATCACC
38	OPF16	GGAGTACTGG
39	OPF09	CCAAGCTTCC
40	OPN03	GGTACTCCCC
41	OPN05	ACTGAACGCC
42	OPN04	GACCGACCCA
43	OPN06	GAGACGCACA
44	OPN12	CACAGACACC
45	OPS08	GTCCACACGG
46	OPS09	TGGGGGACTC

Sl. NO	Primer	Nucleotide Sequence (5'-3')
47	OPS10	CTGCTGGGAC
48	OPE07	AGATGCAGCC
49	OPC01	TTCGAGCCAG
50	OPC08	TGGACCGGTG
51	OPC09	CTCACCGTCC
52	OPAH 12	GTACTACCTA
53	OPAH 09	AGAACCGAGG

Table 2. List of plant gender specific decamer primers used for screening nutmeg samples

Sl. NO	Primer	Nucleotide Sequence (5'-3')	Crop	Gender
1	UBC354	CTAGAGGCCG	Basket willow	Male
2	OPK01	TGGCGACCTT	Date palm	Male
3	OPO18	CCTCCAGTGT	Pandanus	Male
4	OPA08	GTGACGTAGG	Hemp	Male
5	OPD10	GGTCCCTGAG	Asparags	Male
6	OPD15	CATCCGTGCT	Rumex	Male
7	OPG05	CTGAGACGGA	Jojoba	Male
8	OPN01	CTCACGTTGG	Atriplex	Male
9	OPC14	TGCGTGCTTG	Point gourd	Female
10	OPA06	GGTCCCTGAG	Palmyrah	Male
11	OPA17	GACCGCTTGT	*Schisandra nigra*	Male
12	OPY07	AGAGCCGTCA	Papaya	Male
13	OPE11	GAGTCTCAGG	Nutmeg	Female

Composition of the reaction mixture for PCR

a) Genomic DNA (50 ng)	- 1.0 µl
b) 10X Taq assay buffer A	- 2.0 µl
c) dNTP mix (10mM each)	- 0.5 µl
d) Taq DNA polymerase (1U)	- 0.3 µl
e) Decamer primer (10 pM)	- 1.5 µl
f) Autoclaved distilled water	- 14.7 µl
Total volume	= 20.0 µl

The PCR tubes were kept in the thermal cycler and run in the following programme:

93^0C for 4 minutes	-	Initial denaturation	
93^0C for 1 minute	-	Denaturation	40 cycles
35^0C for 1 minute	-	Primer annealing	
72^0C for 2 minutes	-	Primer extension	
72^0C for 8 minutes	-	Final extension	

4^0C for infinity to hold the sample

The amplified products were run on 1.2 per cent agarose gel using 1X TAE buffer stained with ethidium bromide along with marker (λDNA / E *co*RI+H*ind*III double digest). The profile was visualized under UV (312 nm) transilluminator and documented using gel documentation system Gel DOC-It ^{TM} Imaging system UVP (USA). The documented RAPD Profiles were carefully examined for amplification of DNA as bands. The band was considered as monomorphic if the primer amplified same molecular weight region in both

male and female and it was considered as polymorphic if present in one of the samples and absent in other. Gel profiles screened by 67 primers were carefully examined for polymorphism. Those primers, which were given polymorphism, were selected for RAPD analysis of selected male and female trees.

3.2.1.5.2. RAPD with selected primers

RAPD analysis of DNA was done with selected primers using the following samples

1. Bulk DNA of five males
2. Bulk DNA of five females
3. DNA of individual male
4. DNA of individual female
5. Negative control.

The DNA samples of 20 µl were amplified for 40 cycles by PCR reaction. PCR conditions were same as mentioned in section 3.2.1.5.1. After amplification, the products were resolved on 1.2 per cent agarose gel along with marker (λDNA / E*co*RI+H*ind*III double digest). The documented profiles were examined for polymorphic bands between male and female samples. The size of polymorphic band in kb/bp of bases was recorded in comparison with marker.

3.2.1.5.3. Reproducibility of selected primer OPK 01 amplicon

The selected primer was again rechecked with bulk samples of male and female. Three replications were followed for each bulk sample. PCR conditions were same as mentioned in section 3.2.1.5.1. After amplification, the products were resolved on 1.2 per cent agarose gel along with marker (λDNA / E*co*RI+ H*ind*III double digest). The documented profiles were examined for polymorphism between bulk male

and female samples. The size of polymorphic band in kb/bp of bases was recorded in comparison with marker.

The polymorphic nature of selected primer was again checked with individuals of bulk male and female samples. PCR conditions were same as mentioned in section 3.2.1.5.1. After amplification, the products were resolved on 1.2 per cent agarose gel along with marker (λDNA / *Eco*RI+H*ind*III double digest). The documented profiles were examined for polymorphism between individuals of bulk male ad female samples. The size of polymorphic band in kb/bp of bases was recorded in comparison with marker.

3.2.1.6. Elution of specific PCR amplicon

The specific DNA band amplified by OPK 01 primer in female plants was eluted from 1.2 per cent agarose gel by using Gel elution kit (Axygen). Elution was carried out as per the manufacture's guide lines:

1. DNA fragment of interest was excised from the gel using a sterile, sharp scalpel avoiding much exposure to UV on transilluminator.

2. Gel slice was weighed in a colorless 1.5 ml centrifuge tube.

3. 3X gel volume of gel solubilization buffer (w/v) were added.

4. The gel was resuspended in gel solubilization buffer by vortexing. It was heated at 75^0C until the gel was completely dissolved. Intermittent vortexing was given for 2-3 minutes to accelerate gel solubilization.

5. 0.5X gel solubilization buffer equal to volume of binding buffer was added and mixed properly.

6. Once the gel slice was completely dissolved, DNA fragments less than 400 bp was supplemented by adding 1X gel volume of isopropanol and mixed briefly by inversion.

7. Solubilized gel slice transferred into the spin column that was assembled in the 2 ml collection tube and centrifuged at 12,000 X g for 1 minute.

8. The filtrate was discarded. 500 µl of wash buffer was added to the spin column and centrifuged at 12,000 Xg for 30 seconds.

9. The filtrate was discarded. The spin column was again placed on collection tube.

10. Centrifugation was carried out at 12,000 Xg for 1 minute to remove any residual buffer.

11. Spin column was transferred to a fresh 1.5 ml centrifuge tube. The eluent was prewarmed at 65^0C to improve the elution efficiency.

12. To elute the DNA, 25 µl of eluent was added to the centre of the spin column. It was allowed to stand for 1 minute at room temperature. Then it was centrifuged at 12,000 Xg for 1 minute.

13. The purity of eluted DNA fragment was checked by using NanoDrop^R ND-1000 spectrophotometer and stored at -20^0C.

3.2.1.7. Cloning of the amplicon

3.2.1.7.1. Preparation of competent cells of *E. coli*

Competent cells of *Escherichia coli* strain JM 109 for plasmid transformation were prepared following the protocol of Mandel and Higa (1970). Medium used was LB medium. (Details of media prepared is given in the Appendix IV)

The steps followed for competent cell preparation was as follows:

Day 1:

1. Inoculated 18 hrs old *Escherichia coli* strain JM 109, single colony to 3 ml LB medium in sterile condition and incubated at 37^0C on a shaker set at 160 rpm.

Day 2:

1. Aseptically transferred 3 ml overnight grown culture to 50 ml LB broth and incubated for 4 hrs at 37^0C on a shaker at 160 rpm until OD_{600} reached to 0.4 – 0.5. The growth of culture was monitored at 30 minutes interval.

2. The cells were aseptically transferred to a sterile disposable ice cold 50 ml polypropylene tube.

3. The culture was cooled to 0^0C on ice for 10 minutes.

4. The cell suspension was centrifuged at 5000 rpm for 10 minutes at 4^0C.

5. The supernatant obtained was carefully discarded and the pellet obtained was gently resuspended in 10 ml ice cold filter sterilized 0.1 M $CaCl_2$.

6. The tubes were kept on ice for 10 minutes and cell suspension was centrifuged at 5000 rpm for 10 minutes at 4^0C.

7. The supernatant was decanted and resuspended the pellet in 2 ml of ice cold filter sterilized 0.1M $CaCl_2$.

Day 3:

1. Chilled glycerol (400 µl) was added to the cell suspension and mixed well using a sterile micro tip.

2. The component cells prepared were stored at -70^0C as aliquots of 100 µl in chilled 1.5 ml micro centrifuge tubes.

3.2.1.7.2. Screening of competent cells

Transformation of competent cells with a plasmid having ampicillin resistance (pUC 18) was carried out to check the competence and purity of competent cells. The procedure followed for screening of plasmid as follows:

1. The competent cells stored at -70^{0}C were thawed over ice for 10 minutes

2. Plasmid DNA (10 µl) was added to 100 µl competent cells. Negative control was placed simultaneously without adding plasmid.

3. The cells were kept in ice for 40 minutes. Heat shock was given at 42^{0}C for 2 minutes in a dry bath and plunged in ice for 5 minutes.

4. LB medium (250 µl) was added to the cells and incubated at 37^{0}C for 1 hour on a shaker set at 120 rpm

5. The transformed cells (100 µl) were plated on LBA ampicillin medium and incubated overnight at 37^{0}C in a shaker (100 rpm). The recombinant clones alone can grow on ampicillin plate.

3.2.1.7.3. Ligation of insert in pDrive vector

The eluted PCR product was ligated in pDrive vector (Fig1.) using QIAGEN PCR cloning kit (Procedure followed as per the manufacture's protocol).

1. Reaction mixture was prepared as described below.

pDrive vector (50 ng/ µl)	- 1.0 µl
PCR product	- 1.0- 4.0 µl
Ligation master mix, 2x	- 5.0 µl
Deionsed water	- variable
	= 10.0 µl

2. The reaction mixture was incubated for one hour at room temperature. Then it was kept at 4^{0}C overnight. Next day it was taken for transformation in competent cells of *E. coli*.

3.2.1.7.4. Cloning of recombinant plasmid

Cloning was done in the prepared competent cells of *Escherichia coli* strain JM 109.

Materials

1 LB media

2 IPTG- 0.5M dissolved in water

3 Ampicillin- 10%

4 X- gal – 10 mg/ml in DMSO

The procedure for the DNA transformation of *E. coli* is as given below.

1. The vial containing competent cell thawed on ice.

2. The ligated product was added to the competent cells, contents mixed gently and kept on ice for 40 minutes.

3. The tube was rapidly taken from ice; heat shock was given at 42^0C exactly for 90 seconds without shaking and placed back on ice for 5 minutes.

4. Under sterile conditions, 250 µl of LB broth was added and the tube was inverted twice to mix the cells and LB broth.

5. The tube was incubated at 37^0C for one hour with shaking.

6. Aliquots 250 µl of the transformed cells were plated and LB/ ampicillin (50 mg/l) plates layered with IPTG (6 µl) and X-gal (60 µl). (Stock: Ampicillin- 5 mg/ ml in water, IPTG- 200 mg/ ml in water, X-gal- 20 mg/ ml in DMSO) and incubated overnight at 37^0C.

7. The recombinant clones were selected based on blue-white screening.

3.2.1.7.5 Confirmation of the presence of insert using colony PCR

Loop full of recombinant and blue bacterial colonies were diluted in distilled water and used as DNA samples. First denatured the colony at 98^0C for 2 minutes. PCR was carried out as described below with the recombinant bacterial colony DNA used as template.T7 and SP6 primers were used.

Composition of the reaction mixture for Colony PCR (25.0 µl)

10 X PCR buffer A	-	2.5 µl
dNTP	-	1.0 µl
T7 primer (1:10)	-	1.0 µl
SP6 pimer (1:10)	-	1.0 µl
Taq polymerase	-	2.0 µl
DNA (colony)	-	2.0 µl
Distilled water	-	15.5 µl
		25.0 µl

The PCR tubes were kept in the thermal cycler and were run in the following programme:

94^0C for 2 minutes	-	Initial denaturation	
94^0Cfor 45 seconds	-	Denaturation	29 cycles
42^0C for 1 minute	-	Primer annealing	
72^0C for 2 minutes	-	Primer extension	
72^0C for 10 minutes	-	Final extension	

4^0C for infinity to hold the sample

The amplified products were run on 1.0 per cent agarose gel using 1X TAE buffer stained with ethidium bromide along with marker (λDNA / E *co*RI+H*ind*III double digest). In a gel amplified products of two templates DNA were run along with marker. The profile was visualized under UV (312 nm) transilluminator and documented using gel documentation system Gel DOC-It ™ Imaging system UVP (USA). The documented Profiles were carefully examined for presence of the insert.

3.2.1.8. MAINTENANCE OF CLONES

3.2.1.8.1. Preparation of pure culture of recombinant bacteria

In laminar flow, single white colony from the transformed plate was taken by using flame sterilized bacterial loop. This was streaked on LBA media containing antibiotic marker ampicilin. Ampicillin 5 per cent was used as marker. The plate was incubated overnight at 37°C for multiplication of the bacteria. Then it was stored at 4°C for further use.

3.2.1.8.2. Preparation of stabs

Pure culture of recombinant *E. coli* was raised in culture tubes. For this the LBA medium containing antibiotic ampicillin 5 per cent was melted and poured into storage vial, aseptically and allowed to solidify. Single colony of recombinant bacterial colony was carefully lifted with a sterile bacterial loop. The loop loaded with bacteria was plunged to the solid medium and incubated the culture tube overnight at 37°C. The stabs showing good growth of bacteria were further stored in refrigerator at 4°C till further use.

3.2.1.9. SEQUENCING OF DNA CLONES

The stabs of recombinant clones were sent to Bioserve, Hyderabad for sequencing. Sequencing was done with T7 and SP6 primers to obtain 5'-3' sequence information of the insert from the both forward and reverse directions. The sequence information obtained from the Bioserve was named as OPK_{1100}.

3.2.1.10. Sequence analysis with bioinformatic tools

Vector screening

The sequence OPK_{1100} was subjected to vector screening to remove vector regions from the clones by using VecScreen tool available in NCBI. The sequence thus obtained after removal of vector regions of OPK_{1100} was named as *Nut* seq_{816}

Sequence *Nut* seq_{816} was analyzed with various bioinformatic tools. The following tools were used to analyze the sequence.

1. Blastn (Nucleotide- nucleotide BLAST) and Blastp (Protein- protein BLAST)

The nucleotide sequence of Nut Seq 1 was compared with published sequence in public database using BLAST tool (Basic Local Alignment Search Tool) offered by NCBI. The BLAST programme blastn and blastp were obtained from (http://www.ncbi.nm.nih.gov/blast/; (Altschul *et al.*, 1997).

2 ORF finder

The programme ORF finder of NCBI was used (www.ncbi.nlm.gov.gorf/gorf) to find the open reading frames in the nucleotide sequence.

3 GenScan

The gene prediction tools GENSCAN (www.gene.mit.edu/GENSCAN; Burge and Karlin, 1997) was used to predict exons and peptide in the sequence.

4 Transeq

The programme Transeq (http://expasy.org/tools/transeq.html) was used to translate amino acid sequence from nucleic acid sequence.

5 Protparam

Physical and chemical properties of the given protein from the deduced aminoacid were determined by Protparam (http://expasy.org/tools/protparam.html) tool.

6 GOR

The programme GOR (http://expasy.org/tools/gor.html) for secondary structure prediction of the amino acid sequence was done using this tool.

3.2.2. DEVELOPMENT OF SCAR MARKER

3.2.2.1. SCAR primer designing

Long forward and reverse primers of 24 bp length were designed based on *Nut* seq_{816} information with primer3 programme (http://biotools.umassmed.edu/bioapps/primer3_www.cgi).

The following criteria were followed during SCAR primer designing:

1. Each primer contained the original 10 bases of RAPD primer plus the next 14 internal bases from the end.

2. The end sequences selected has GC content 40-50 percent.

4. Melting temperature (Tm = 4GC+ 2AT) ranged between 57^0C and 65^0 C.

5. The distance between the primers ranged from 200 to 400 bp.

6. GC content at 3' end was preferred.

7. There was no complementarity between forward and reverse primers.

8. There was no repetition of single base within the primer sequence.

9. The distance between forward and reverse primer was greater than 200 bp.

10. Each primer can be 24 to 27 bp long.

11. Primer sequences had no palindromic sequences or repetitive sequence.

12. 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 nucleotide sequences SP1 and SP2 were designed. Each pair had a forward and reverse primer.

3.2.2.2. PCR analysis with SCAR primer

3.2.2.2.1. Standardization of PCR conditions for SCAR analysis

Varying quantities of DNA (15 ng and 30 ng) were amplified at different annealing temperatures 61, 63, 64, 65, 66, 67 and 68^{0}C to get specific parameters for SCAR primer amplification.

3.2.2.2.2. Expression of SCAR in mature trees

Two pairs of 24 base pairs oligonucleotide primers (SP1 and SP2) synthesized based on the end sequence of RAPD clone OPK 1 $_{1100}$ were used as SCAR primers for amplifying DNA of five males, five females, four occasional fruiting males and negative control. PCR was carried out with SCAR specific primers in Eppendrof Master Cycler (Eppendrof, USA).

Composition of the reaction mixture for PCR analysis was the following:

a) Genomic DNA (15 ng) - 1.0 µl

b) 10X Taq assay buffer A - 2.5 µl

c) dNTP mix (10mM each) - 1.0 µl

d) Taq DNA polymerase (0.3U) - 2.0 µl

e) Forward primer (8 pM) - 1.5 µl

f) Reverse primer (8 pM) - 1.5 µl

g) Autoclaved distilled water - 15.5 µl

Total volume = 25.0 µl

PCR Programme

Initial denaturation - 94^{0}C for 2 minutes

Denaturation - 93^{0}C for 45 sec

Primer annealing - 68^{0}C for 1 minute } 35 cycles

Primer extension - 72^{0}C for 30 sec

Final extension - 72^{0}C for 10 minutes

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

After amplification, the amplified products were run on 1.5 per cent agarose gel along with marker (100bp). The documented gel profiles were carefully examined for presence of sex specific marker in male, female and occasional fruiting males.

3.2.2.2.3. Expression of SCAR in seedlings

Two to four year old ten seedlings were selected to check the expression of SCAR primers at seedling stage. PCR conditions were same as that discussed in 3.2.2.2. After amplification, the amplified products were run on 1.5 per cent agarose gel along with marker (100bp). The documented gel profiles were carefully examined for presence of amplicon in seedlings.

3.3. Biochemical analyses

Acid phosphotase and glutamate oxaloacetate transaminase (GOT) were studied. Polyacrylamide gel electrophoresis (PAGE) was carried out for protein banding. Biochemical analyses were carried out at the biochemistry laboratory of the CPBMB, College of Horticulture, Vellanikkara.

3.3.1. Plant material

Isozyme analysis (acid phosphatase and glutamate oxaloacetate tansaminase) was carried out using leaf samples from four mature male and five female trees.

3.3.2. Standardization of Polyacrylamide gel electrophoresis (PAGE)

PAGE protocol (Thomas, 1997) and Mini PROTEAN®3 cell (BIO-RAD) (Instruction Manual) PAGE protocol were tested for protein banding. The protocol by Mini PROTEAN®3 cell (BIO-RAD) was tested with standard protein Bovine Serum Albumin (BSA) for PAGE standardization. This standardized PAGE protocol was used for nutmeg leaf protein pattern display and isozyme analyses of acid phosphatase and glutamate oxaloacetate transaminase

3.3.2.1. PAGE protocol (Thomas, 1997)

Polyacrylamide gel was applied at 7.5 per cent and 10 per cent polymerization.

3.3.2.1.1. Polyacrylamide gel - Reagents

Solution A

Tris – 38.3 g

TEMED – 0.46 ml

1N HCl – 48 ml

Distilled water – 100 ml

p^H - 9

Solution B

	7.5% Polymerization	10% Polymerization
Acrylamide	30.0 g	40.0 g
Bis acrylamide	0.9 g	1.2 g
Made up to	100 ml	100 ml

Solution C

Ammonium persulphate	–	0.14 g
Distilled water	–	1 ml

Solution A and B were prepared and stored in amber coloured bottles at 0-4^{0}C. solution C was prepared fresh each time.

Extraction buffer

Tris buffer	–	21.1995 g
Citric acid	–	2.626 g
L- Cystein HCl	–	0.5689 g
β- mercapto ethanol	–	0.039065 g
p^H	–	7
Make up to	–	500 ml

The reagents were dissolved in 100 ml one by one and finally volume made up to 500 ml. pH was adjusted to 7.

Electrode buffer solution

Tris	–	6.0 g
Glycine	–	28.8 g
Make up to	–	1000 ml
P^H	–	8.3

Staining solution

0.2 M Acetate buffer	-	50 ml
1-naphthyl-phosphate	-	50 mg/ml
Fast Garnet GBG salt	-	50 mg/ml
10% $MgCl_2$		few drops
pH		5

3.3.2.1.2. Preparation of gel

In the gel preparation, acrylamide monomers were polymerized with N-N methylene bis acrylamide [CH2 (NHCONH=CH3)2 bis] to obtain the gel. N,N,N',N' tetra methyl ethylene di amine (TEMED) was added as polymerization initiator and freshly prepared ammonium per sulphate as catalyst. The gel was casted in Mini-PROTEAN ®3 system (BIORAD).

The size of the vertical slab gel was 16cm×14cm×0.01cm. The apparatus was set up by inserting two glass plates in border slots leaving a column of 0.5 mm thickness between them. To maintain the required hygiene, glass plates were washed and thoroughly wiped with alcohol. Stock solutions of A, B and C were taken in 1:1:2 proportion and mixed thoroughly. The solution was loaded by a micro pipette in between glass plates and was kept for 40 minutes at room temperature for solidification. Combs were placed at the top to make the wells.

3.3.2.1.3. Preparation of sample

Tender leaf samples of both male and female trees were collected in liquid nitrogen. One gram sample was ground in 5 ml extraction buffer in a pre-chilled mortar and pestle. The homogenized material was centrifuged at 15000 rpm for 15 minutes in a refrigerated centrifuge at 4^0C. After centrifugation the clear supernatant was collected and stored in a refrigerator and was used for electrophoresis.

3.3.2.1.4. Electrophoresis of the sample

After polymerization the gel was transferred to electrophoresis unit containing upper and lower tanks. The tanks were filled with pre cooled electrode buffer. Diluted the leaf sample at 1:1 ratio with sample buffer. Bromophenol blue acted as tracer dye. 10 to 20 micro litre samples were loaded to each well with micro pipette. Upper tank was connected to the cathode and lower one to the anode and electrophoresis was carried out. A constant current of 20 mA was supplied for the first half an hour and increased up to 40 mA and maintained till the end of running. A cooling system was attached to the electrophoresis unit for heat dissipation and electrophoresis was carried out at 4^0C for 3-6 hrs.

3.3.2.1.5. Staining the gel

After electrophoresis, the gel was stained with fast blue GBG stain and examined for protein pattern.

3.3.2.2. Bio RAD Protocol for protein display

Polyacrylamide gel was tested at 7 per cent and 10 per cent polymerization (Table 3)

3.3.2.2.1. Reagents- polyacrylamide gel

1. Acrylamide/Bis (30% T, 2.67% C)

Acrylamide	87.6g
N'N'-bis-methylene-acrylamide	2.4g
Distilled water	300 ml

2. 1.5 M Tris-HCl, pH 8.8

Tris base	18.15g
Deionized water	80ml
Made up to	100 ml

3. 0.5 M Tris-HCl, pH 6.8

Tris base	6.0g
Deionised water	60ml
Made up to	100 ml

4. 10% APS solution

Ammonium persulphate	100 mg
Deionised water	1 ml

5. Sample Buffer

Deionised water	5.55 ml
0.5 M Tris-HCl, pH 6.8	1.25 ml
Glycerol	3.0 ml
Bromopheol blue (0.5%)	0.2 ml
Total volume	10.0 ml

6. 10x Electrode Buffer, pH 8.3

Tris base	30.3 g
Glycine	144.0 g

Made up to 1000 ml

Stock Solutions were prepared and stored in amber colored bottles at 0-4^{0}C. Sample buffer was stored at room temperature. Ammonium persulphate solution was prepared fresh each time.

7. Stain stock

Coomassie blue (1% R-250) – 0.5 g

Water - 50 ml

Staining solution

Stain stock - 31.25 ml

Methanol - 125 ml

Acetic acid - 25 ml

Water - make up to 250 ml

8. Destaining solution – 1

Acetic acid - 50 ml

Methanol - 250 ml

Make up to 500 ml with distilled water

9. Destaining solution -2

Acetic acid - 70 ml

Methanol - 50 ml

Make up to l litre

Table 3. Gel Formulations (10 ml)

Percent Gel	Deionised H_20 (ml)	Acrylamide/Bis (30%) (ml)	Gel buffer (ml)
4%	6.2	1.3	2.5
5%	5.8	1.7	2.5
6%	5.5	2.0	2.5
7%	5.2	2.3	2.5
8%	4.8	2.7	2.5
9%	4.5	3.0	2.5
10%	4.2	3.3	2.5

Resolving gel buffer- 1.5 M Tris-HCl pH 8.8
Stackig gel buffer- 0.5 M Tris-HCl pH 6.8

3.3.2.2.2. Preparation of gel

Gel porosity of 7 to10 per cent polymerization was tried. Prepare the monomer solution by mixing all reagents except the TEMED and 10% APS. Stacking and resolving gels were formed by mixing stock solutions at appropriate ratios as described in the above table. Immediately prior to pouring the gel, added 50 µl APS and 5 µl TEMED for resolving gel; 10 µl TEMED for stacking gel. Gel was prepared as mentioned in section 3.3.2.1.2.

3.3.2.2.3. PAGE with Bovine Serum Albumin (BSA)

PAGE was standardized initially with standard protein Bovine Serum Albumin (10mg/ml). The gel was loaded with 10 to 20 microlitre BSA sample for each well. The gel was electrophoressed as mentioned in section 3.3.2.1.4. and stained with Coomassie blue stain. After staining gel was destained with destaining solutions I and II. The destained gel profile was examined for protein pattern and movement.

3.3.2.3. PAGE with Nutmeg leaf protein

PAGE was standardized by testing various extraction buffers (3.3.2.3.1.) and changing gel porosity between seven percent to ten percent (Table 3). Extraction buffer of different composition were tried to extract active protein from leaf tissue. The compositions of different extraction buffer tried are given below.

Extraction buffer 1

Di ethyl dithio carbamate	-	250 mM
Tris HCl	-	100 mM
Sucrose	-	7%
Bovine serum albumin	-	1%
Poly vinyl pyrolidine	-	10%
Sodium meta bi sulphite	-	20 mM
Make up to		1000 ml

Extraction buffer 2

Di ethyl dithio carbamate	-	200 mM
Tris HCl	-	80 mM

Sodium metabi sulphite	-	20 mM
β – mercaptoethanol	-	0.0302 g
Sucrose	-	7%
Bovine serum albumin	-	1%
Make up to		1000 ml
pH		7

Extraction buffer 3

Di ethyl dithio carbamate	-	250 mM
Tris HCl	-	100 mM
Sucrose	-	5%
Bovine serum albumin	-	0.5%
Poly vinyl pyrolidine	-	5%
Sodium metabi sulphite	-	20 mM
β – mercaptoethanol	-	14mM
Ascorbic acid	-	25 mM
Make up to		1000 ml
pH		6

Extraction buffer 4

Di ethyl dithio carbamate	-	250 mM
Tris HCl	-	80 mM
Sucrose	-	4%
Poly vinyl pyrolidine	-	8%
Sodium metabi sulphite	-	10 mM

β – mercaptoethanol	-	14 mM
L- cystein HCl	-	25 mM
Make up to		1000 ml
pH		6.5

Extraction buffer 5

Tris HCl	–	28.8 g
Citric acid	–	2.626 g
Ascorbic acid	–	0.523 g
L- Cystein HCl	–	0.526 g
Insoluble PVP	–	0.50 g
β - mercapto ethanol	–	0.390 g
Made up to	–	500 ml
pH	-	7

Extraction buffer 6

Di ethyl dithio carbamate	-	250 mM
Tris HCl	-	100 mM
Sucrose	-	7%
Bovine serum albumin	-	1%
Poly vinyl pyrolidine	-	10%
Sodium metabi sulphite	-	20 mM
β – mercaptoethanol	-	10 mM
Ascorbic acid	-	25 mM
PMSF	-	0.02 M

Make up to	1000 ml
pH	7.5

Gel was prepared as mentioned in section 3.2.2.2. After polymerization the gel was transferred to electrophoresis unit having upper and lower tanks. The tanks were filled with pre cooled electrode buffer. Diluted the leaf sample at 1:1 ratio with sample buffer, bromophenol blue acted as tracer dye. Ten to twenty micro litre samples were loaded to each well with micro pipette. Upper tank was connected to the cathode and lower one to the anode. The analyses were carried out. A constant current of 20 mA was applied for one and half hour till end of gel running. A cooling system was attached to the electrophoresis unit for heat dissipation and electrophoresis was carried out at 4^0C for 1-2hrs. After electrophoresis the gel was stained and destained as mentioned in section 3.3.2.2.3. The gel was examined for protein pattern and movement.

3.4. Acid phosphatase

Polyacrylamide gel electrophoresis of acidphosphatase was carried out as mentioned in section 3.3.2.3. Staining and developing of isozyme band was done as mentioned below.

3.4.1.1. Staining the gel

Staining of gel was done using Malik and Singh protocol (1994)

Reagents

0.1 M Acetate buffer	50 ml
1-naphtyl-phosphate	50 mg/ml
Fast blue RR salt	50 mg/ml
NaCl	20 mg/ml
10% $MgCl_2$	few drops
pH	5

Staining solution was prepared fresh each time. Before incubating the gels in staining solution, the gels were washed 3 to 4 times in 0.1 M acetate buffer (pH 5.0), by changing the buffer every 20 min. Then incubated the gels for 2 to 3 hours in a solution

containing 1-naphthyl-phosphate (1mg/ml), Fast blue RR salt(1mg/ml), NaCl (1mg/ml) and few drops of 10% $MgCl_2$

in 0.1 M acetate buffer, pH 5.0. After this, the gel was destained with acetic acid, methnol and deionised water (1:3:6) and was observed for banding pattern.

3.4.2. Glutamate oxaloacetate transaminase (GOT)

Polyacrylamide gel electrophoresis of Glutamate oxaloacetate transaminase (GOT) was carried out as 3.3.2.3. in nutmeg protein electrophoresis. Staining and developing of isozyme band was done as mentioned below.

3.4.2.1. Staining the gel

Staining of gel was done using reported protocol (Yagi *et al.*, 2004)

Reagents

AAT substrate solution	–	50 ml
Fast Blue BB salt	–	50 mg

AAT Substrate solution

α- Ketoglutaric acid	–	292 mg
L- Aspartic acid	–	1.07 g
PVP – 40	-	4.00 g
EDTA – Na2 salt	–	400mg
Sodium phosphate, dibasic	–	11.36 g

Deionised water – 800 ml

Added Fast Blue BB salt to substrate solution and incubated at room temperature in the dark. After staining the gel was destained with Acetic acid, methnol and deionised water (1:3:6) and examined for banding pattern.

Results

4. RESULTS

The study on "Sex determination in nutmeg (*Myristica fragrans* Houtt.) through molecular and biochemical markers" was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara during the period 2008-2010. The results of different experiments are described in this chapter.

4.1.1. Standardization of genomic DNA isolation

Electrophoresis of DNA isolated by Roger and Bendich protocol with 2X CTAB extraction buffer and 10% CTAB solution showed no DNA in mature leaf sample but little quantity of DNA in immature leaf sample (Plate 5). So, further DNA isolation was done with immature leaves. The extraction with Roger and Bendich (1994) protocol when modified with 3X CTAB and 5% CTAB solution improved the quality and quantity of DNA (Plate 6). Clear DNA band of 15 kb was observed in all samples. Gel also showed RNA contamination and were free of protein contamination. Extraction with 4X CTAB extraction buffer and 5% CTAB solution didn't yield DNA.

4.1.2. Purification of DNA

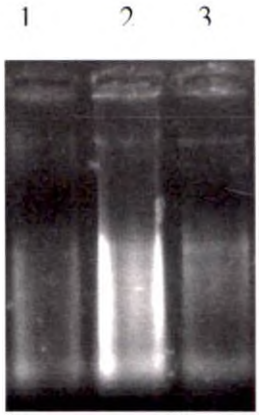
The genomic DNA isolated by modified Roger and Bendich (1994) method showed high degree of RNA contamination. The RNA presence may interfere with the preceeding PCR reactions. So the DNA isolated by modified Roger and Bendich (1994) method was purified by RNase treatment. RNase removed RNA contamination in all samples (Plate 7).

4.1.3. Assesment of the DNA quality and quantity

The quantity and quality of DNA was analyzed using NanoDrop® ND-1000 spectrophotometer and the results are presented in Table 4. The ratio of UV absorbance ($A_{260}/_{280}$) ranged between 1.80-1.84. In males it ranged from 1.80 to 1.83 and in females 1.81 to 1.84. Since ratio of UV absorbance ($A_{260}/_{280}$) comes between 1.80- 2.0,

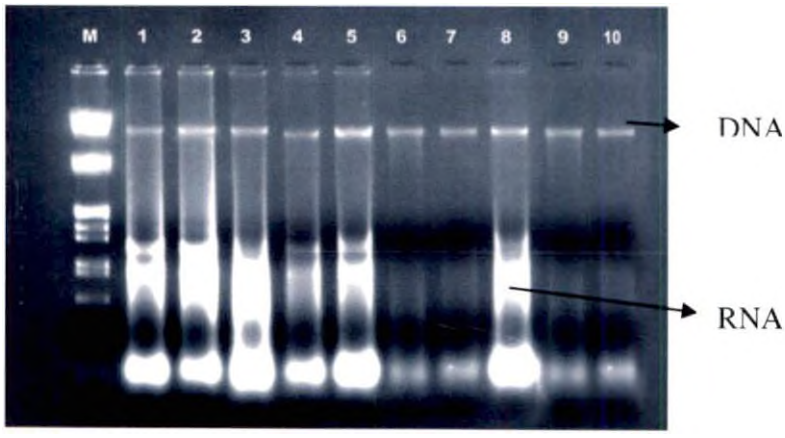
Table 4. Quality and quantity of DNA isolated from nutmeg genotypes as assesed by Nano Drop method

Genotype	UV absorbance at 260 nm (A_{260})	UV absorbance at 280 nm (A_{280})	$A_{260}/_{280}$	Quantity (ng/µl)	Quality
M_1	0.326	0.176	1.83	329.0	Good
M_2	0.291	0.160	1.82	248.0	Good
M_3	0.301	0.167	1.80	235.0	Good
M_4	0.294	0.163	1.80	240.8	Good
M_5	0.340	0.187	1.82	235.0	Good
F_1	0.310	0.170	1.82	255.7	Good
F_2	0.311	0.171	1.83	305.9	Good
F_3	0.298	0.165	1.81	231.0	Good
F_4	0.285	0.156	1.82	244.8	Good
F_5	0.317	0.172	1.84	312.9	Good



1 - Mature dark green leaves
2 & 3 - immature pale green leaves

Plate 5. DNA isolated by Rogers and Bendich method (2X & 10%)

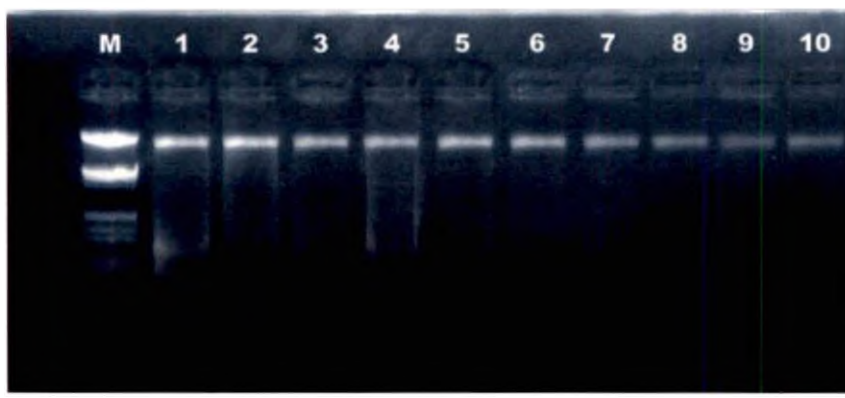


M: Marker Lambda DNA / *Eco* RI/ *Hind* III

1-5: five individual male samples

6-10: five individual female samples

Plate 6. DNA isolated by modified Rogers and Bendich method (3X & 5%)



M: Marker Lambda DNA / *Eco* RI/ *Hind* III

1-5: five individual male samples

6-10: five individual female samples

Plate 7. DNA isolated by Rogers and Bendich method after RNase treatment

quality of DNA in all samples was rated as good. The quantity of DNA in all samples ranged from 231 ng/µl to 329 ng/µl of diluted sample. In males it ranged from 235 ng/µl to 329 ng/µl and in females 231 ng/µl to 312.9 ng/µl. The DNA, thus isolated, after appropriate dilutions were used as template for RAPD and SCAR analyses.

4.1.4. RAPD (Random Amplified Polymorphic DNA) analysis

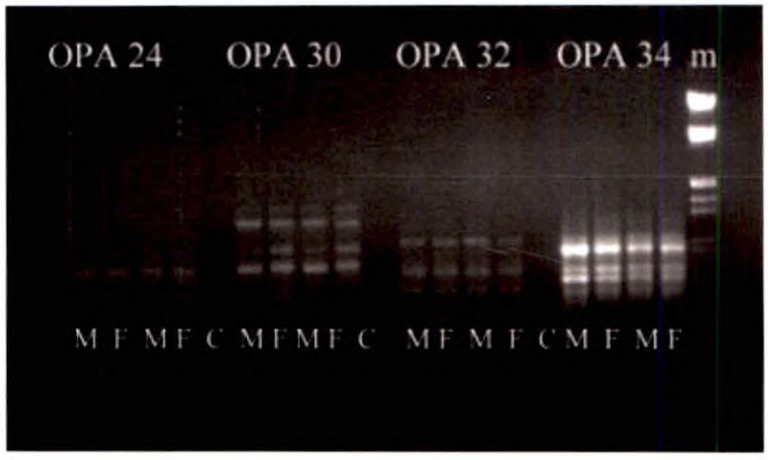
Good quality genomic DNA from the nutmeg genotypes were subjected to RAPD assay. The various experiments carried out under this included screening of random primers and RAPD with selected primers.

4.1.4.1. Screening of primers for RAPD analysis

Sixty-seven primers belonging to series OPA, OPS, OPY, OPN, OPF, OPAH, OPE, OPK, OPD, OPO, OPG and OPC were tested for amplification of genomic DNA of nutmeg. DNA Samples from single male and female were used for screening. PCR amplified products were electrophoresed and gel was examined for polymorphism between male and female samples.

a) Screening with OPA series

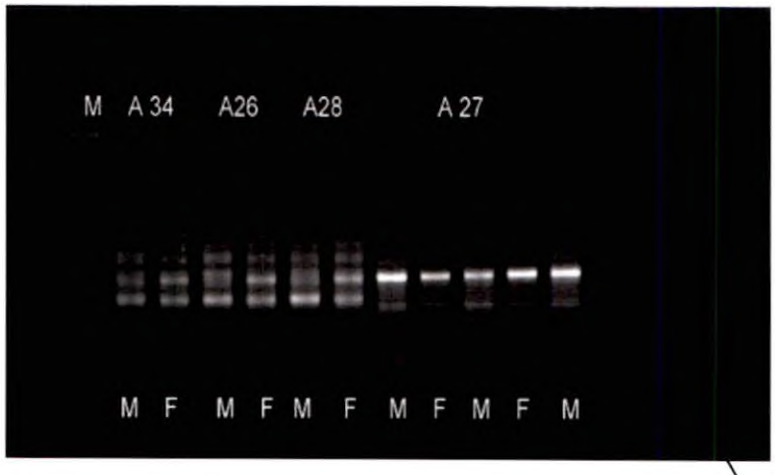
Sixteen primers (OPA 6, 8, 10, 12, 17, 22, 23, 24, 26, 27, 29, 30, 34, 36, 39 and 40) in this series were screened. The amplification pattern obtained for the primers in OPA series is as given in Table 5. The number of bands in the tested male and female sample ranged from zero to twelve and molecular weight of bands varied from 0.58 to 2 kb. Ten primers (OPA 6, 8, 10, 22, 23, 26, 27, 28, 30 and 34) of the primer series showed good amplification with more number of discrete bands (Plate 8). Poor amplification was obtained for the primers OPA 12, 17, 29, 36 and 39. The primer OPA 27 showed polymorphism (Plate 9) and amplified the 1.8 kb polymorphic band in male which was absent in female. This primer was selected for further analysis.



m: Marker Lambda DNA / *Eco* RI/ *Hind* III

C: Negative control

Plate 8. Screening with OPA series primers



M: DNA of single male

F: DNA of single female

Plate 9. Screening with OPA series primers

b) Screening with OPN series

Six primers (OPN 1, 3, 4, 5, 6, 12) in this series were screened and results are given in Table 6. The number of amplicons obtained using the primers in this series ranged from one to twelve and the molecular weight of bands varied from 0.83 to 5 kb. Six primers (OPN 01, 03, 04, 05, 06, 12) showed good amplification (Plate 10 and 11). No polymorphism was observed for the gender identity.

c) Screening with OPY series

Twenty primers (OPY 1-20) in this series were screened and results are given in Table 7. The number of amplicons obtained using the primers in this series ranged from zero to seven and the molecular weight of bands varied from 0.83 to 5 kb. Among twenty primers, sixteen primers (OPY 2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 16, 17, 18, 19, and 20) showed poor amplification (Plate 12). Four primers OPY 1, 3, 14 and 15 had given good amplification. None of the primer showed polymorphism at all.

d) Screening with OPF series

Four primers (OPF 3, 5, 14, 16) in this series were screened and results are given in the Table 8. The number of amplicons obtained using the primers in this series ranged from zero to seven and the molecular weight of bands varied from 0.83 to 5 kb. OPF 5, 3, and 16 primers gave good amplification with more number of discrete bands. OPF 14 gave no amplification. The primer OPF 5 showed polymorphism (Plate 10). It amplified 1.8 kb polymorphic band in male which was absent in female and was selected for further analysis.

e) Screening with OPS series

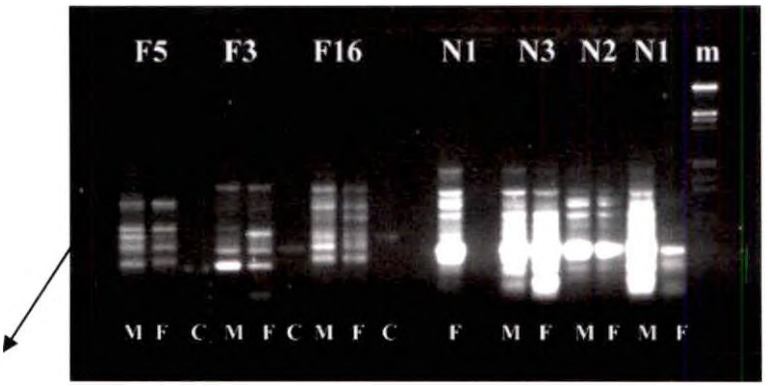
Five primers (OPS 8, 9, 10, 11, 18) in this series were screened. The amplification pattern obtained for different primers in OPS series is as given in Table 8. The number of bands ranged from three to five and molecular weight of bands varied

Table 5. Amplification pattern of DNA of nutmeg genotypes with OPA series Primers

S.No	Primer	No. of amplicons		Quality of amplification
		Male	Female	
1	OPA 06	12	12	Good
2	OPA 08	12	12	Good
3	OPA 10	8	8	Good
4	OPA 12	0	0	Poor
5	OPA 17	0	0	Poor
6	OPA 22	7	7	Good
7	OPA 23	8	8	Good
8	OPA 26	7	7	Good
9	OPA 27	7	6	Good
10	OPA 28	7	7	Poor
11	OPA 30	6	6	Good
12	OPA 24	3	3	Average
13	OPA 34	6	6	Good
14	OPA 36	0	0	Poor
15	OPA 38	0	0	poor
15	OPA 39	0	0	Poor
16	OPA 40	3	3	Average

Table 6. Amplification pattern of DNA of nutmeg genotypes with OPN series primers

S.No	Primer	No. of amplicons		Quality of amplification
		Male	Female	
1	OPN 01	12	12	Good
2	OPN 03	10	10	Good
3	OPN 04	8	8	Good
4	OPN05	4	4	Average
5	OPN 06	5	5	Good
6	OPN 12	7	7	Good



1.8 kb polymorphic band

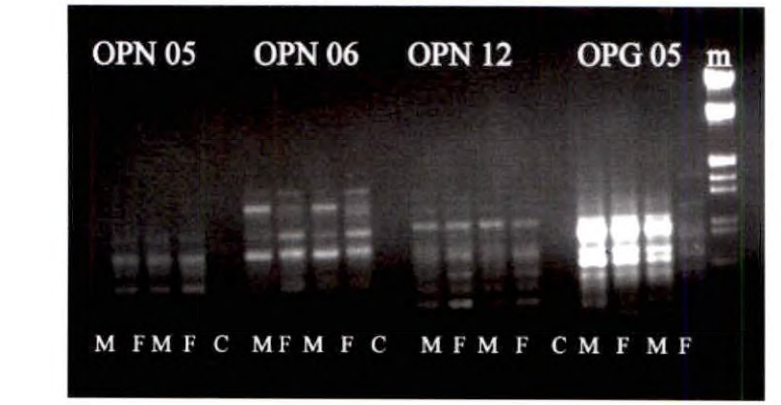
m: Marker Lambda DNA / *Eco* RI/ *Hind* III

M: DNA of single male

F: DNA of single female

C: Negative control

Plate 10. Screening with OPN and OPF series primers



m: Marker Lambda DNA / *Eco* RI/ *Hind* III

M: DNA of single male

F: DNA of single female

C: Negative control

Plate 11. Screening with OPN and OPG series primers

from 0.83 to 2 kb. OPS 9 gave good amplification. OPS 8, 10, 11 and 18 showed poor amplification (Plate 13). None of the primer showed polymorphism at all.

f) Screening with OPK 01 primer

Results of screening are given in the Table 10. The number of amplicons obtained using the primer OPK 01 ranged from seven to nine and molecular weight of band ranged from 0.3 to 4kb. The primer gave good amplification with discrete bands and has shown Polymorphism (Plate 13). The primer OPK 01 amplified 1.1 kb polymorphic band in female which was absent in male and was selected for further analysis

g) Screening with OPAH series

Two primers (OPAH 9, 12) in this series were screened. The amplification pattern obtained for two primers in OPAH series is as given in Table 9. Primers showed very less amplification. No polymorphism was obtained for gender identity.

h) Screening with OPE series

Two primers (OPE 7, 11) in this series were screened. The amplification pattern obtained for different primers in OPE series is as given in Table 9. The number of bands ranged from one to nine and molecular weight of bands varied from 0.28 to2 kb. OPE 7 amplified more concentrated bands and OPE 11 gave good amplification and distinct band (Plate 14). None of the primer showed polymorphism for sex identity.

i) Screening with OPD series

Three primers (OPD 10, 15, 20) in this series were screened. The amplification pattern obtained for different primers in OPD series is as given in Table 9. The number of bands ranged from one to eight and molecular weight of bands varied from 0.28 to 2 kb. The primer OPD 15 showed good amplification pattern. Primer showed

Table 7. Amplification pattern of DNA of nutmeg genotypes with OPY series Primers

S.No	Primer	No. of amplicons		Quality of amplification
		Male	Female	
1	OPY 1	0	0	Poor
2	OPY 2	0	0	Poor
3	OPY 3	2	2	Poor
4	OPY 4	4	4	Average
5	OPY 5	3	3	Average
6	OPY 6	3	3	Average
7	OPY 7	2	2	Poor
8	OPY 8	0	0	Poor
9	OPY 9	4	4	Average
10	OPY 10	4	4	Average
11	OPY 11	3	3	Average
12	OPY 12	1	1	Poor
13	OPY 13	7	7	Average
14	OPY 14	5	5	Average
15	OPY 15	6	6	Average
16	OPY 16	0	0	Poor
17	OPY 17	2	2	Poor
18	OPY 18	3	3	Average
19	OPY 19	0	0	Poor
20	OPY 20	0	0	Poor

Table 8. Amplification pattern of DNA of nutmeg genotypes with OPF and OPS series primers

S.No	Primer	No. of amplicons		Quality of amplification
		Male	Female	
1	OPF 05	7	6	Good
2	OPF 03	6	6	Good
3	OPF 16	6	6	Good
4	OPF 14	0	6	Poor
5	OPS 08	5	5	Average
6	OPS 09	5	5	Average
7	OPS 10	3	3	Average
8	OPS 11	5	5	Average
9	OPS 18	0	0	Poor

Table 9. Amplification pattern of DNA of nutmeg genotypes with OPE, OPAH, OPD and OPC series primers

S.No	Primer	No. of amplicons		Quality of amplification
		Male	Female	
1	OPAH 12	2	2	Poor
2	OPAH 09	3	3	Poor
3	OPE 07	6	6	Good
4	OPE 11	9	9	Good
5	OPD 10	6	6	Good
6	OPD 20	4	4	Average
7	OPD 15	3	3	Poor
8	OPC 01	0	0	Poor
9	OPC 08	0	0	Poor
10	OPC 09	0	0	Poor
11	OPC 14	0	0	Poor

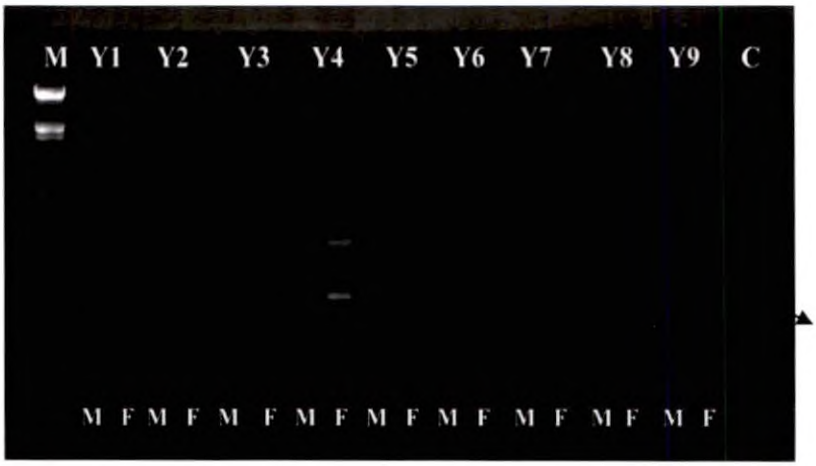


Plate 12. Screening with OPY series

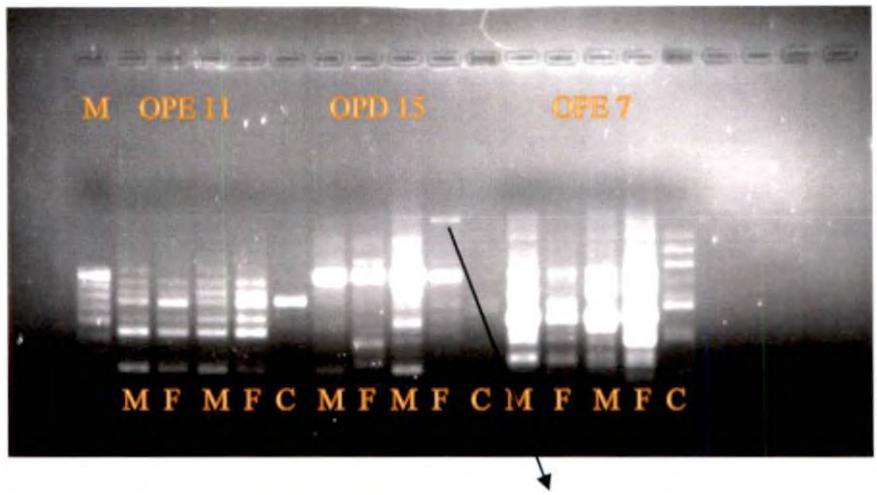
m: Marker Lambda DNA / *Eco* RI/ *Hind* III

M: DNA of single male

F: DNA of single female

C: Negative control

Plate 13: Screening with OPS and OPK primers



m: Marker (100 bp)

1.8 kb polymorphic band

M: DNA of single male

F: DNA of single female

C: Negative control

Plate 14: Screening with OPE and OPD series primers

polymorphism (Plate 14). It amplified 1.8 kb polymorphic band in female which was absent in male and was selected for further analysis.

j) Screening with OPC series

Four primers (OPC 1, 2, 3 and 14) in this series were screened. The amplification pattern obtained for different primers in OPC series was as given in Table 9. No amplification was obtained for these four primers.

k) Screening with UBC 354 primer

Results of screening are given in the Table 10. The number of amplicons obtained using the primer was ranged from one to three and molecular weight ranged from 0.4 to 1.5 kb. The primer gave poor amplification with less concentrated bands. It did not show polymorphism for sex identity.

l) Screening with OPG 05 primer

Results of screening are given in the Table 10. The number of amplicons obtained using the primer ranged from one to four and molecular weight ranged from 0.4 to1.8 kb. It gave good amplification with discrete bands but no polymorphism was obtained at all.

m) Screening with OPO 15 primer

Results of screening are given in the Table 10. The number of amplicons obtained using the primer ranged from one to six and molecular weight ranged from 0.4 to 2.0 kb. The primer gave good amplification with discrete bands but no polymorphism was obtained at all.

4.1.4.2. RAPD with selected primer

Out of sixty-seven, four primers OPF 5, OPD 15, OPA 27 and OPK 01 were selected for further analysis. They were used for amplifying bulk DNA of five males

Table 10. Amplification pattern of DNA of nutmeg genotypes with OPG, OPK, UBC and OPO primers

S.No	Primer	No. of amplicons		Quality of amplification
		Male	Female	
1	OPO 15	6	6	Good
2	OPG 05	4	4	Average
3	UBC 354	3	3	Average
4	OPK 01	8	7	Good

and five females, individual DNA from single male, single female and negative control (without DNA).

1) RAPD with OPF 05

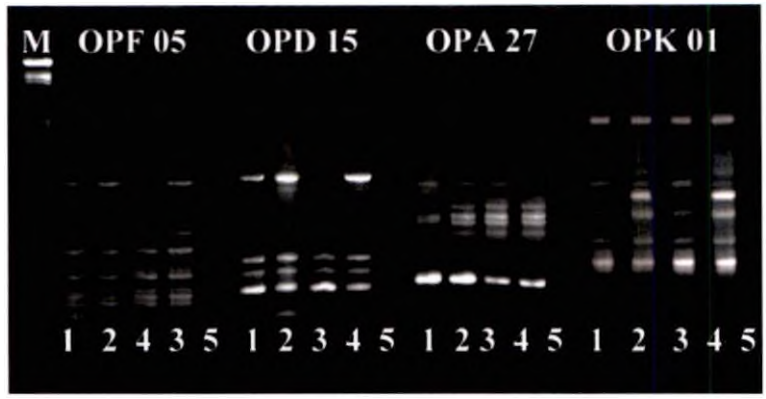
The agarose gel profile for the DNA amplification with the primer OPF 05 is given in plate 15. Ten to twelve amplicons were observed in male and female samples. The molecular weight of the products ranged between 0.3 to 2.0 kb. The primer didn't produce male specific amplicon in DNA of individual and bulk samples as expected. It amplified 1.8 kb size band not only in bulk and individual male samples but also in bulk female. This band was absent in individual female.

2) RAPD with OPD 15

The agarose gel profile for the DNA amplification with the primer OPD 15 is given in plate 15. Five to seven amplicons were observed in male and female samples. The molecular weight of the products ranged between 0.8 to 2.0 kb. The primer did not produce female specific amplicon in DNA of individual and bulk female samples as expected. It amplified 1.8 kb size band not only in bulk and individual female samples but also in bulk males. This band was absent in individual male sample.

3) RAPD with OPA 27

The agarose gel profile for the DNA amplification with the primer OPA 27 is given in Plate 15. Seven to nine amplicons were observed in male and female samples. The molecular weight of the products ranged between 0.8 to 1.9 kb. The primer did not produce polymorphic male specific amplicon in DNA of individual and bulk male samples as expected. It amplified 1.8 kb size band not only in bulk and individual male samples but also in bulk female. This band was absent in individual female sample only.



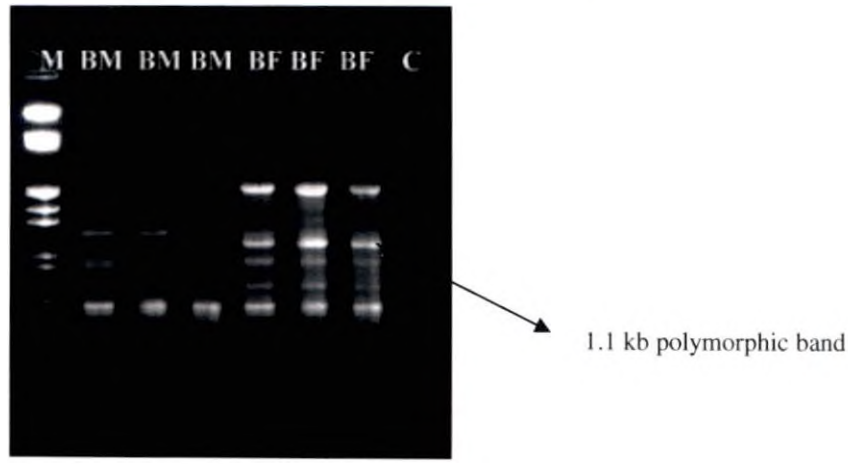
M: Marker Lambda DNA / *Eco* RI/ *Hind* III

1 and 2: DNA of bulk male and female samples

3 and 4: DNA of individual male and female samples

5: Negative control

Plate 15. RAPD analysis with DNA of bulk and individual male and female samples with selected primers OPF 05, OPD 15, OPA27 and OPK 01



M: Marker Lambda DNA / *E co* RI/ *Hind* III

BM: bulk male samples

BF: bulk female samples

C: Negative control

Plate 16. Reproducibility of OPK 01 primer with DNA of bulk male and female samples

4) RAPD with OPK 01

The agarose gel profile for the DNA amplification with the primer OPK 01 is given in plate 15. Eight to nine amplicons were observed in male and female samples. The molecular weight of the products ranged between 0.71 to 5.0 kb. The primer amplified 1.1 kb size female specific amplicon in DNA of individual and bulk female samples as expected. This band was absent in DNA of individual and bulk male samples. So reproducibility of the primer was good and was able to differentiate male and female individuals. The OPK 01 primer was selected for further RAPD analysis.

4.1.4.3. Reproducibility of OPK 01 polymorphic amplicon

i) Amplification of bulk DNA with OPK 01

The primer OPK 01 was rechecked with three replications of DNA of bulk male and female samples. It amplified 1.1 kb polymorphic band in DNA of three bulk female samples which was absent in respective male bulk samples (Plate 16).

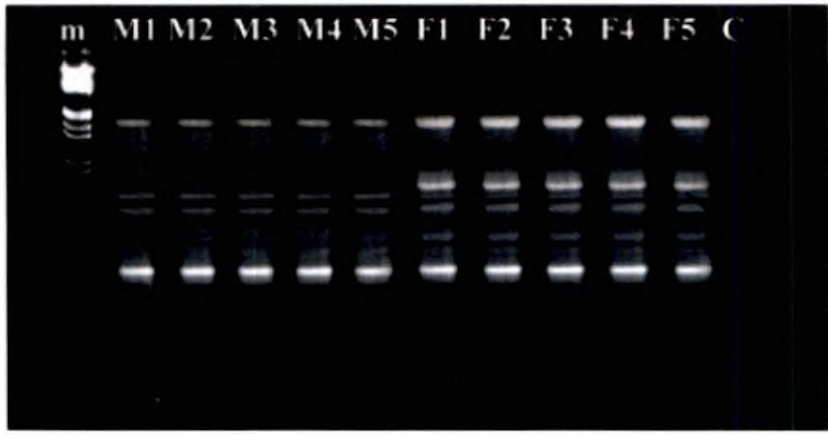
ii) Amplification of individual DNA samples with OPK 01

The primer OPK 01 was again rechecked with five individual male and female samples. It amplified 1.1 kb polymorphic band in all five individual female samples which was absent in respective five individual male samples (Plate 17).

4.1.4. Elution of specific PCR amplicon

The genomic DNA of five individual male and females were amplified by OPK 01primer for eluting female specific 1.1 kb band. The RAPD products were resolved on 1.2 per cent agarose gel and the band of interest was cut from five specific female lanes (Plate 18). The products were pooled and DNA was eluted from the agarose as mentioned in section 3.2.1.6.

The quality of eluted product (2 µl) was checked by using NanoDrop^{R} ND-1000 spectrophometer and showed UV absorbance ratio (A_{260}/A_{280}) of 1.84. So the



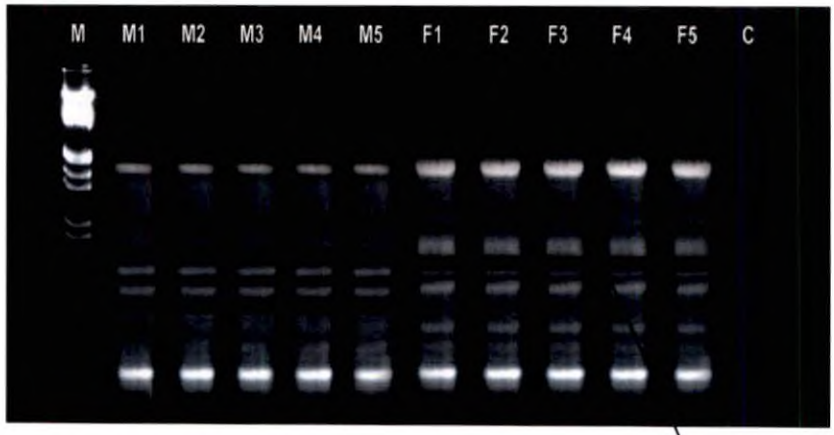
M: Marker Lambda DNA / *E co* RI/ *Hind* III

M1 to M5: five individual male samples

F1 to F5: five individual female samples

C: Negative control

Plate 17. Reproducibility of OPK 01 primer with DNA of individual male and Female samples



M: Marker Lambda DNA / *E co RI/ Hind III*

M1 to M5: five individual male samples

F1 to F5: five individual female samples

C: Negative control

Plate 18. Gel for elution of specific OPK 01amplicon of 1.1kb

eluted product was of good quality. This eluent was used for cloning and sequencing studies.

4.1.5. Cloning of amplicon in *E. coli*

4.1.5.1. Preparation and screening of competent cells

Competent cells were prepared from *E. coli* JM 109 strain as per the procedure mentioned in section 3.2.1.7.1. The competency of *E. coli* cells was checked by transformation with pUC 18 ampicillin resistant plasmid. Profuse blue colonies were observed in LBA ampicillin plates after overnight incubation of (Fig 1). The presence of blue colonies confirmed the competency of *E. coli* JM 109 for transformation.

4.1.5.2. Transformation of DNA

The eluted DNA was ligated into pDrive vector (Fig 2). After confirmation of competence, the ligated product was transferred into competent *E. coli* JM 109 cells using heat shock method at 42^0C. When the transformed *E. coli* cells were incubated overnight with LBA ampicillin plates overlaid with X-gal and IPTG, blue and white colonies were obtained. Presence of white colonies confirmed successful transformation (Fig 3).

4.1.5.3 Confirmation of presence of insert using colony PCR

The white colony was checked for the presence of insert by colony PCR conformation. The DNA from white and blue colonies were amplified using specific SP6 and T7 universal primers. The PCR products were resolved on 1.2 per cent agarose gel (Plate 19). The amplified product of white colony had 1.1kb high molecular weight band and blue colony had less molecular weight 500 bp band. The higher molecular weight amplicon of white colony confirmed the presence of DNA insert.

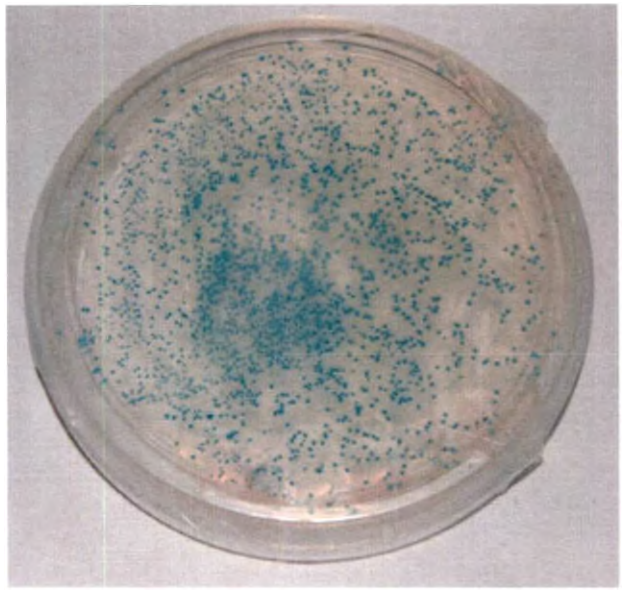


Fig 1: Competent cell colonies growing in amipicillin medium

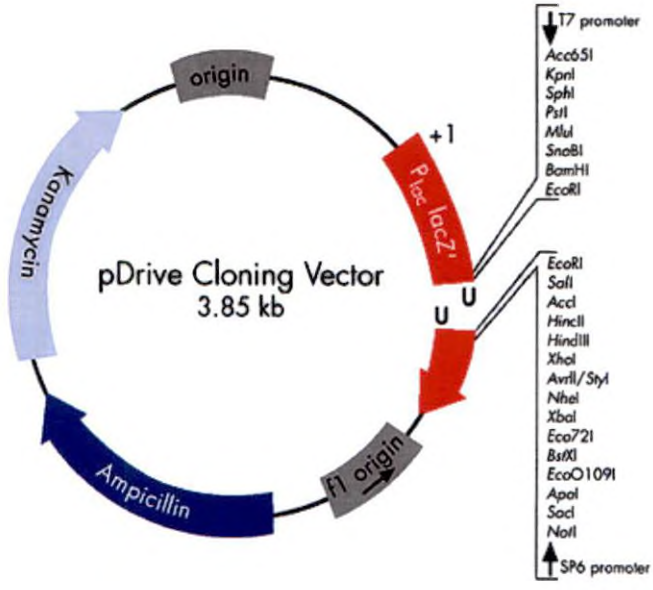


Fig 2: pDrive vector used in cloning of amplicon

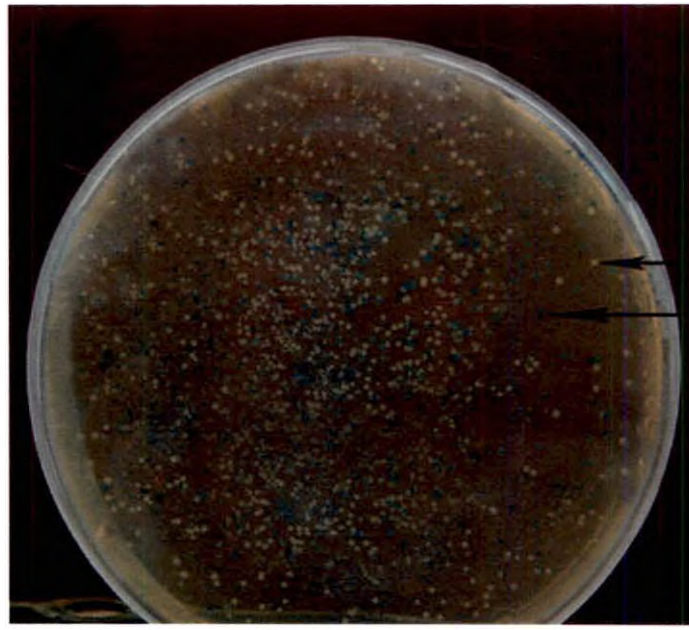
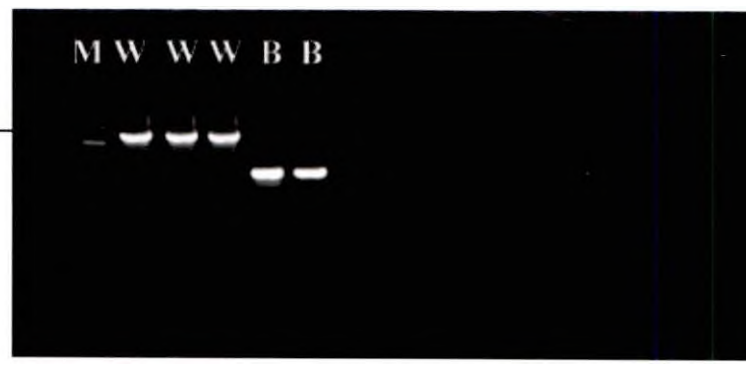


Fig 3: Blue and white colonies in the transformed plate



M: Marker (100 bp)

W: DNA of white colony

B: DNA of blue colony

Plate 19. Confirmation of DNA insert by colony PCR

4.1.6. Sequencing of DNA clones

Pure culture of white colony was raised in culture tubes. These stabs were sent for sequencing. Sequencing was done at Bioserve Biotechnologies Pvt. Ltd. Hyderabad using SP6 and T7 primers with automated sequencer. The sequence information for OPK 01 amplicon obtained by both forward and reverse primers were merged by DNA merger tool. The sequence information thus obtained for the OPK 01 amplicon was 1100 bp (referred as OPK_{1100}) is given in Fig 4. The first ten bases of sequence (OPK_{1100}) represent the RAPD primer OPK 01 sequence (TGGCGACCTT).

4.1.6.1. Vector screening

The sequence obtained after automated sequencing was subjected to Vector screening using the VecScreen tool in NCBI to remove vector regions from the clones (Fig 5). The region starting from 825 to 1028 bases showed similarity with pDrive vector sequence, and it was eliminated from the original sequence by using merge DNA sequence tool. The sequence of 816 bases obtained after vector screening was named as *Nut* seq_{816} is presented in Fig 6.

4.1.6.2. Sequence analysis with various bioinformatic tools

1. **Homology search with Blastn**

The homology search for the sequence with Blastn is as shown in Fig 7. Blastn search showed no homology with published sequences present in public database of NCBI.

2. **Detection of Open Reading Frame (ORF)**

Open reading frame search was made in Nut seq_{816} using ORF finder tool. Three open reading frames (+2, +3 and -3) were observed in the given sequence (Fig 8). The +2 ORF strand encodes two domains for amino asparate transaminase (GOT) and cystathionine beta- lyases with E value 3.6. The other ORFs did not encode any domain.

OPK 01

TGGCGACCTT**AAGTTAACTTATGC**AAGGTAGCTTTTAGCCTGGACTGATCGGGGCATTTAAGGCCGATGCATGCGT
AGAAATCAGTCGACTAGAGTCATCATATATACAAAAGTCATCCATCGGGACTGAGGCTAGAGTACCAGTCCCAGTA
CAAGATACCGACATAGCTGCGAGATTATTTAACCGTACTGTCAGCATGGGACTTAGCAGGTCCCCATACGGACTAT
GATCGATCGGACCTAGTAGGAACCTAGCTACGAGGCCAATTCCGGGTGCGACGTAACAGTCTTTTAAAATCGGAAG
CCCCCGTACCGGGTGGGAAGTTAGCCTAAGCTACGTACTAGAAGGTTAATGGTCACCCTAAGCAATTGGACTAGCT
GGAATCTCTTGGAAAGTGTTACCAATGCAGTACCTGATCGTACTAGGGAATATCTACGTATAGCTATTGCTGATAG
TGAGTCGGATCATCGCATATACGTGGCGACCTTGGATGTCGATTCTAGTATTAAGCAACCGGAACCTATACGGACG
AAGCCGATTAGCAATGCATACAGCTAGAGAATTCCCGTACAGGTACAAACACCCTAGTAGTGCGATCGAGCTTGGG
AAACTAGCTGGATCGATCTGGGGTACCCTGACTGATCGATCGGTAGTCCGATCGATCGTAGCTAGCTAGCTCGCTC
TGGAAGGGTCCAGATCCGAGTCTCGGAGATCGATCGGATCGATCATGCTAGTACGTAGGATCGGATCGATCGATCA
CCGCGGATTAACCGTGAACCTTAGTTAGCGGGGTAAGCGAATGGGCAAGCGGTTAACTGCAGACGCGTTACGTATC
GGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGTGCACTGCATAATTCTCTTACTGTCATGCCAT
CCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGATAGTGTATGCGGCGACCGAGTTGC
TCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCAGTGCCCAACGTGTGG
TCTAGAGCTAGCCTAGGCTCGAGAAGCTTGTCGACGAATTCAGATCAATTTCATGGCCGTCG

Fig.4. Nucleotide sequence of OPK_{1100}

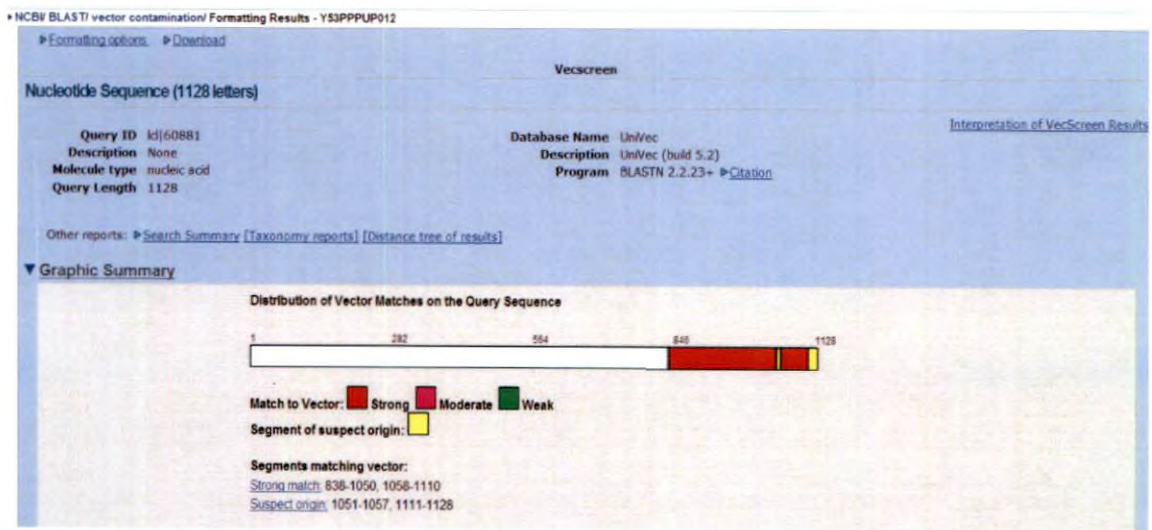


Fig.5. VecScreen output of OPK_{1100}

OPK 01

TGGCGACCTT**AAGTTAACTTATGC**ACGTTGACTGGCCTAAGGTAGCTTTTAGCCTGGACTGATCGGGGCATT
TAAGGCCGATGCATGCGTAGAAATCAGTCGACTAGAGTCATCATATATACAAAAGTCATCCATCGGGACT
GAGGCTAGAGTACCAGTCCCAGTACAAGATACCGACATAGCTGCGAGATTATTTAACCGTACTGTCAGCA
TGGGACTTAGCAGGTCCCCATACGGACTATGATCGATCGGACCTAGTAGGAACCTAGCTACGAGGCCAAT
TCCGGGTGCGACGTAACAGTCTTTTAAAATCGGAAGCCCCCGTACCGGGTGGGAAGTTAGCCTAAGCTAC
GTACTAGAAGGTTAATGGTCACCCTAAGCAATTGGACTAGCTGGAATCTCTTGGAAAGTGTTACCAATGC
AGTACCTGATCGTACTAGGGAATATCTACGTATAGCTATTGCTGATAGTGAGTCGGATCATCGCATATAC
GTGGCGACCTTGGATGTCGATTCTAGTATTAAGCAACCGGAACCTATACGGACGAAGCCGATTAGCAATG
CATACAGCTAGAGAATTCCCGTACAGGTACAAACACCCTAGTAGTGCGATCGAGCTTGGGAAACTAGCTG
GATCGATCTGGGGTACCCTGACTGATCGATCGGTAGTCCGATCGATCGTAGCTAGCTAGCTCGCTCTGGA
AGGGTCCAGATCCGAGTCTCGGAGATCGATCGGATCGATCATGCTAGTACGTAGGATCGGATCGATCGAT
CACCGCGGATTAACCGTGAACCTTAGTTAGCGGGGTAAGCGAATGGGCAAGCGGTTAA

Fig.6. Nucleotide sequence after deleting vector sequence (Nut seq_{816})

Fig.7. Blastn output of *Nut* seq_{816}

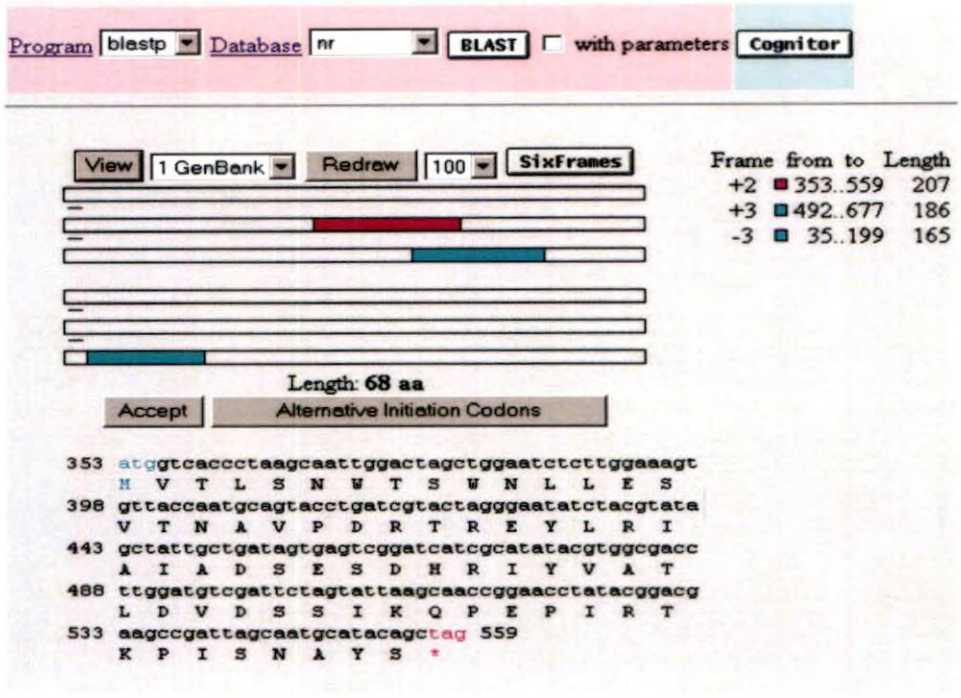


Fig.8. ORF finder output of *Nut seq*$_{816}$

```
GENSCAN 1.0     Date run:  8-Mar-110     Time: 06:13:55


Sequence /tmp/03_08_10-06:13:55.fasta : 816 bp : 49.39% C+G : Isochore 2 (43 - 51 C+G%)


Parameter matrix: HumanIso.smat


Predicted genes/exons:


Gn.Ex Type S .Begin ...End .Len Fr Ph I/Ac Do/T CodRg P.... Tscr..

----- ---- - ------ ------ ---- -- -- ---- ---- ----- ----- ------


NO EXONS/GENES PREDICTED IN SEQUENCE
```

Fig.9. GenScan output of *Nut seq*$_{816}$

3. Exon/gene prediction

Prediction of exon/gene in *Nut* seq_{816} was done using GenScan tool. No exon/gene was predicted in the given sequence in Fig 9.

4. Amino acid sequence analysis

Amino acid sequence was translated from nucleotide sequence using Transeq tool. The translated amino acid sequence of $Nutseq_{816}$ is as shown in Fig 10.

5. Secondary structure prediction

The secondary structure of protein predicted by GOR programme of ExSpacy tool is presented in Fig 11. The translated amino acid sequence from $Nutseq_{816}$ had 86.22 per cent extended strands and random coils 13.78 per cent.

6. Physico- chemical properties

The physico-chemical properties of protein was predicted by Protparam programme of ExSpacy tool. The major amino acids present in $Nutseq_{816}$ were Alanine, Cystine, Glycine and Threonine. The protein had molecular weight 62419.9 with pI 5.01. The total number of atoms (C, H, O, S and N) present was 7922.

4.2. DEVELOPMENT OF SCAR MARKER

4.2.1. SCAR primer designing

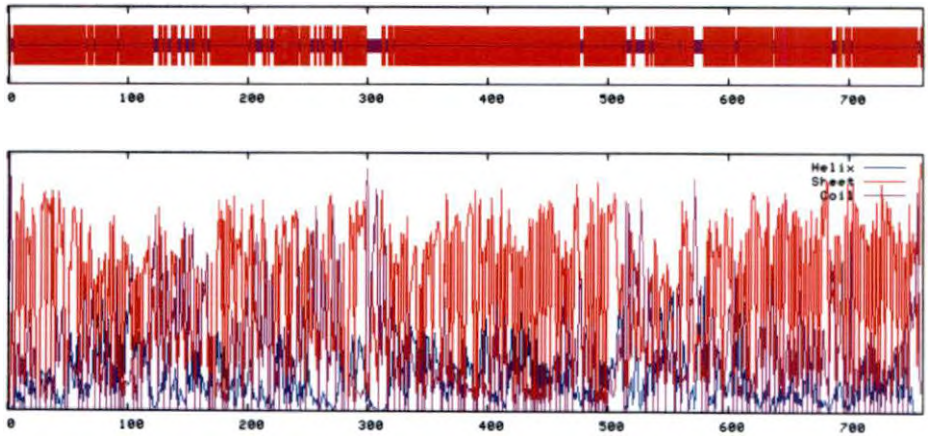
Based on parameters described in section 3.2.2.1. two pairs of SCAR primers SP1 and SP2 were designed using primer3 programme. The input data was sequence information of *Nut* seq_{816}.

Primers SP1 and SP2 were synthesized by Sigma- Aldrich Pvt. Ltd. Bangalore. Primer sequences and length of primers are provided in Table 11. These four primer sequences had no palindromic sequences.

EMBOSS_001_1

WRPYVDWPKVAFSLDSGHLRPMHAKSVDSHHIYKSHPSGLRLEYQSQYKIPTLRDY
LTVLSAWDLAGPHTDYDRSDLVGTLRGQFRVRRNSLLKSEAPVPGGKLAATYKVNGH
PKQLDLESLGKCYQCSTSYGISTYSYCVGSSHIRGDLGCRFYATGTYTDEAD
QCIQLENSRTGTNTLVVRSSLGNLDRSGVPLIDRSDRSLASSLWKGPDPSLGDRS
DRSCYVGSDRSITADPTLVSGVSEWASG

Fig.10. Translated amino acid sequence of *Nut seq*$_{816}$



Sequence length : 762

GOR4 :

Alpha helix	(Hh)	:	0 is	0.00%
3_{10} helix	(Gg)	:	0 is	0.00%
Pi helix	(Ii)	:	0 is	0.00%
Beta bridge	(Bb)	:	0 is	0.00%
Extended strand	(Ee)	:	657 is	86.22%
Beta turn	(Tt)	:	0 is	0.00%
Bend region	(Ss)	:	0 is	0.00%
Random coil	(Cc)	:	105 is	13.78%
Ambigous states	(?)	:	0 is	0.00%
Other states		:	0 is	0.00%

Fig.11. The secondary structure of protein predicted by GOR tool

The SP1 had forward and reverse primers of 24 bases and each with 10 bases represent of RAPD primer OPK 01 at the beginning. SP1 primers had annealing and melting temperatures of 65^{0}C, 63^{0}C and 68^{0}C, 70^{0}C and GC per cent of 48.3 and 41.6. The SP2 had forward and reverse primers of 24 bases and without RAPD primer sequence representation. SP2 primers had annealing and melting temperatures of 68^{0}C, 65^{0}C and 72^{0}C, 70^{0}C and GC per cent of 44 and 40. Physical properties of primers viz., annealing temperature, melting temperature and GC per cent are given in Table 12.

4.1.2.2. PCR amplification using SCAR primers

4.1.2.2.1. Standardization of PCR conditions for SCAR analysis

Varying quantities of DNA 15 and 30 ng were amplified at different annealing temperatures 61, 63, 64, 65, 66, 67 and 68^{0}C. Distinct single band of size 300 bp was generated only at 68^{0}C with 15 ng of DNA. Other annealing temperatures did not amplify SP1 primers at 15 ng and 30 ng DNA.

4.1.2.2.2. Expression of SCAR in mature trees

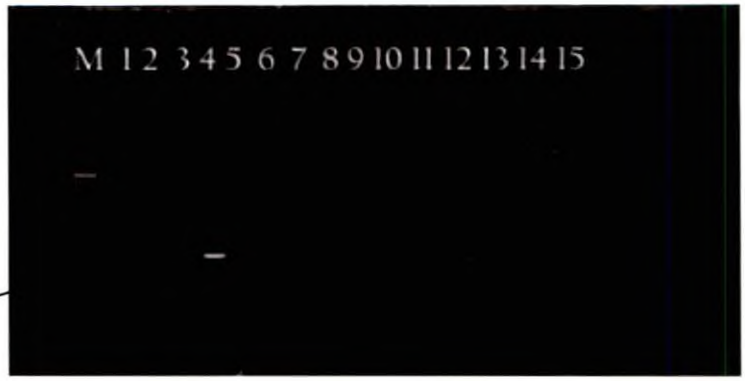
The DNA of five male, five female and four occasional fruiting male trees was amplified with SP1 primers with specific PCR conditions as mentioned in section 3.2.2.2.1. The specific conditions standardized for SCAR amplification were genomic DNA quantity 15 ng and annealing temperature 68^{0}C. The products electrophoresed with molecular weight marker (100bp). The gel profile revealed an amplicon of 300 bp in all females and one occasional fruiting male (Plate 20). The selected male, female and occasional fruiting males were also amplified with SP2 primers with specific PCR conditions as mentioned in section 3.2.2.2.1. The quantity of DNA used was 15 ng and annealing temperature was 68^{0}C. No amplification was obtained in any one of the samples with SP2 primers.

Table 11. Details of SCAR primers used in SCAR analysis

Sl.No	Primer		Primer sequence (5'-3')	Length (bp)
1	SP1	F1	TGGCGACCTTAAGTTAACTTATGC	24
		R1	ACCGCTGGAACTTGACAATATATC	24
2	SP2	F2	TAGCTGCGAGATTATTCAACCGTC	24
		R2	CATTGCTAATCTGCTTCATCCTAG	24

Table 12. Details of annealing temperatures, melting temperatures and GC content of SCAR primers

Sl.No	Primer	Annealing Temperature (^{0}C)	Tm (^{0}C)	GC%
1	SP1- Forward primer	65	70	48.3
2	SP1- Reverse primer	63	68	41.6
3	SP2- Forward primer	68	72	44.0
4	SP2- Reverse primer	65	70	40.0



M: Marker (100 bp)

1-5: DNA of five female individuals

6-10: DNA of five male individuals

11-14: DNA of four occasional fruiting males

15: Negative control

Plate 20. SCAR gel profile in bearing trees

M: Marker (100 bp)

1-10: DNA of ten seedlings

C: Negative control

m: Marker Lambda DNA / *E co RI/ Hind III*

Plate 21. SCAR gel profile in seedlings

4.2.2.3. Expression of SCAR primers in seedlings

DNA of two to four year old ten seedlings were amplified using SP1 primers. The products were electrophoresed with molecular weight marker (100bp). The gel profile revealed an amplicon of around 300 bp in four seedlings out of ten samples (Plate 21).

4.3. Biochemical marker analysis

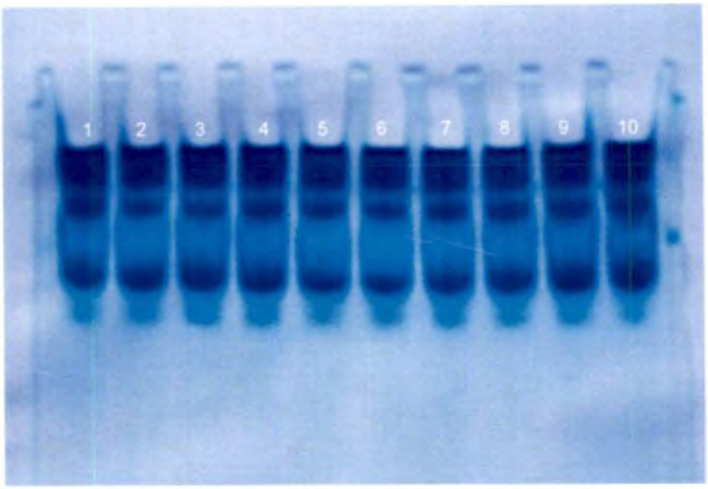
4.3.1. Standardization of polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis system was adopted to carry out isozyme analysis for acid phosphatase and glutamate oxaloacetate transaminase. Initially PAGE protocol as reported by Thomas (1997) was followed but there was no protein banding in gel. So BIO-RAD manual protocol was tried.

Polyacylamide gel electrophoresis by BIO-RAD was standardized with standard protein Bovine serum albumin. The sample of standard protein used was 10mg/µl. The sample was electrophoressed as mentioned in section 3.3.2.1.4. After electrophoresis the gel was stained with Coomassie blue and then destained with destaining solutions I and II (Section 3.3.2.2.3). The destained PAGE gel showed movement of protein and separation of bands according to molecular weight (Plate 22).

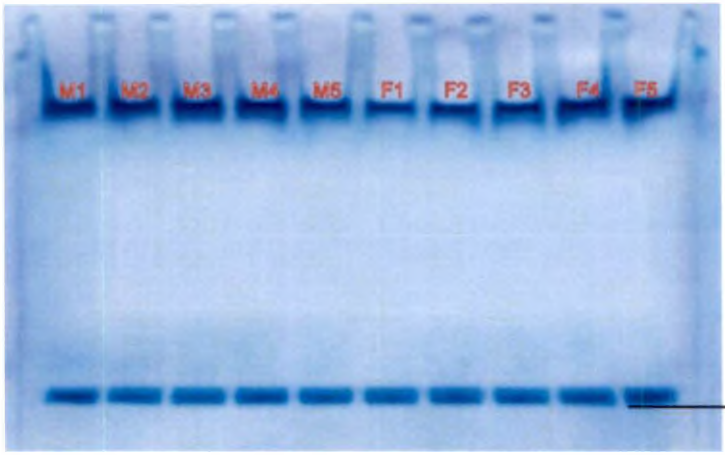
4.3.2. Nutmeg leaf protein pattern by electrophoresis

The nutmeg leaf protein was electrophoresed in polyacrylamide gel, which was standardized with standard protein. Five male and female nutmeg leaf samples were used. Leaf samples were ground with and without liquid N_2 and the leaf protein was extracted with six different extraction buffers (Section 3.3.2.3.) at 4^0C. The leaf sample homogenized in liquid N_2 and extracted with Extraction buffer -6 only expressed protein band in polyacrylamide gel. Other extraction buffers didn't show any band (Table 13). The homogenized sample without liquid N_2 didn't express any protein band in any other extraction buffers tested.



1-10: BSA samples

Plate 22. PAGE gel profile for Bovine Serum Albumin (BSA)



M1-M5: Five individual male leaf samples

F1-F5: Five individual female leaf samples

Plate 23. PAGE gel profile for Nutmeg leaf protein

The leaf extract obtained with Extraction buffer- 6 was electrophoresed (Section3.3.2.3.). After electrophoresis the gel was stained with Coomassie blue and was destained with destaing solutions I and II (Section 3.3.2.2.3). The destained PAGE gel showed single protein band in both male and female samples (Plate 23). The relative mobility of band was 0.77 in both male and female samples (Table 14).

4.3.3. Isozyme analysis

4.3.3.1. Acid phosphatase

Samples used were leaves of four male and five female nutmeg trees. The samples were homogenized in liquid N_2 and extracted with Extraction buffer -6 of pH 7.0 containing Tris. The supernatant was electrophoresed as described in section 3.3.2.3. After electrophoresis the gel was washed 3 to 4 times in 0.1 M acetate buffer to reduce the gel pH from 8.5 to 5.0. There after, the gel was incubated in fast blue RR salt staining solution at room temperature for 3 to 5 hours and destained with destaining solution of acetic acid and methanol mixture. The destained gel showed four bands with different mobilities. The four bands were monomorphic as was seen in both male and female samples (Plate 24). The relative mobility of bands was same in both male and female samples. The relative mobility fractions for four monomorphic bands were 0.16, 0.22, 0.50 and 0.72. is given in Table 14.

4.3.3.2. Glutamate oxaloacetate transaminase

Samples used were leaves of four male and five female nutmeg trees. The samples were homogenized in liquid N_2 and extracted with extraction buffer 6 of pH 7.5 containing Tris. The supernatant was electrophoresed as described in section 3.3.2.3. After electrophoresis the gel was incubated in fast blue BB salt staining solution at room temperature for overnight. After staining gels were destained. The destained gel showed single band in female samples with RF value 0.26 and some scar bands in males (Plate 25). Refinement of the procedure is needed for the assessment of this enzyme.

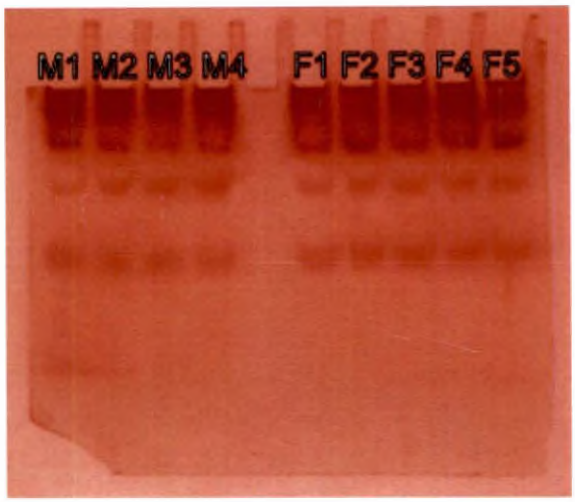
Table 13. Result of different extraction buffers

Buffer	Protein movement
Extraction buffer - 1	Unsatisfactory
Extraction buffer - 2	Unsatisfactory
Extraction buffer - 3	Unsatisfactory
Extraction buffer - 4	Unsatisfactory
Extraction buffer - 5	Unsatisfactory
Extraction buffer - 6	Satisfactory

Table 14. Number of bands and relative mobilities (RF) of nutmeg leaf protein and different enzymes

Sample	No of bands		RF	
	M	F	M	F
Protein	1	1	0.77	0.77
Acid phosphatase	4	4	0.16	0.16
			0.22	0.22
			0.50	0.50
			0.72	0.72
GOT	1	0	0.26	-

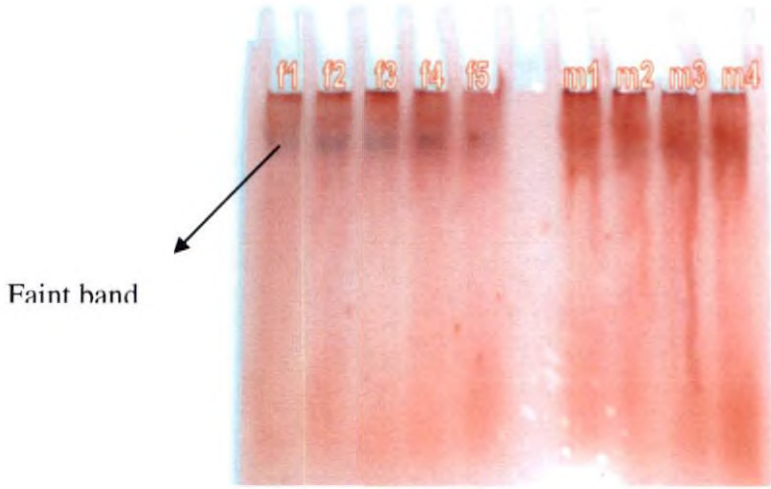
RF – Relative mobility; GOT – Glutamate oxaloacetate transaminase



M1-M4: Four individual male leaf samples

F1-F5: Five individual female leaf samples

Plate 24. PAGE gel profile of Acid phosphotase isozymes of nutmeg leaf



M1-M4: Four individual male leaf samples

F1-F5: Five individual female leaf samples

Plate 25. PAGE gel profile of GOT enzyme of nutmeg leaf

Discussion

5. DISCUSSION

Nutmeg (*Myristica fragrans* Houtt.) is an important tree spice, yielding two products of commercial value, the 'nutmeg' and the 'mace'. Both nutmeg and aril have stimulative, carminative, astringent and aphrodisiac properties and are used as spice and medicine. In this crop, apart from long pre bearing period, dioecy is recognized as one of the major problem in crop management. Both male and female plants remain indistinguishable until the flowering stage which normally extends 5 to 7 years after planting. Dioecism is found only in a small number of plant species. Yampolsky and Yampolsky (1922) noted that while only 5% of plant genera are wholly dioecious, 75% of flowering plant families have some dioecious species. Dioecy (separate male and female individuals) is well established in animals, but occurs sporadically in plants. Charlesworth (1985) extended the compilation of Yampolsky and Yampolsky (1922) and identified 1303 genera and 170 families in which dioecy is well established. Understanding the genetic and epigenetic factors controlling sex determination in dioecious plants is still at a preliminary stage. Chromosomal determination of sexual phenotype has been detected in only five dioecious plant families (Parker 1990).

Presently, the only option to overcome dioecy in nutmeg is the use of vegetatively propagated material or top working. Even though several vegetative methods have been reported with varying degree of success, the large scale adoption of these methods is constrained due to non availability of orthotrophs in sufficient numbers. The orthotropic scions are required for the production of plants with normal erect growth habit. Hence seedling continues to be the major propagating material. If sex of the seedling is identified at the early stage itself it will be of immense use to the farming community. In the present study the possibility of using molecular and biochemical markers to identify sex at seedling stage is explored.

Several attempts have been made in the past to identify the sex of nutmeg plants at the seedling stage on the basis of morphological variations in mature male and female

plants (Flach, 1966; Nayar *et al.*, 1977). However, suggested morphological markers were not accurate and repeatable. Biochemical methods were also adopted with limited success. (Phadnis and Chowdary, 1971 and Thomas, 1997).

Sex of many species cannot be deduced from external morphology. One effective solution is to exploit DNA markers to diagnose sex. Such markers are present in the genome whenever sex determination is genetically controlled. In many organisms, sexual differentiation is governed by chromosomal sex determination, where the sex-determinant genes are carried on a specialized pair of sex chromosomes. The two main forms are male heterogametic, where the male has X and Y-chromosomes and the female is XX, and female heterogametic, where the female is WZ and the male is ZZ. The Y or W chromosomes are, thus, unique to one sex, so their presence or absence in a sample of genomic DNA is indicative of sex.

Sex identification at the DNA level is a well-established technique that has shown to be fast and accurate. It requires only minimal tissue samples and can be collected at all stages of crop growth and season. The extracted DNA can be preserved under ambient conditions for long periods prior to analysis. The technique can be more cost effective by choosing high value crops. The only serious drawback is the difficulty of obtaining Y- or W-linked markers.

Several dioecious plants, white campion (*Silene latifolia*), papaya (*Carica papaya*), and asparagus (*Asparagus officinalis*) have an active X-Y system of sex determination with heterogametic males (XY) and homogametic females (XX). In some other plant hop (*Humulus lupulus*), ratio of X to autosome operates sex determination. But in the case of *Myristica fragrans*, no such lucid information at chromosome or DNA level is available. According to Flach (1966) females of nutmeg possess a chromosome that had facultative nuclear property and is holokinetic in nature. The use of DNA markers to discriminate two sexes has been advocated if the genetic mechanism of sex determination is not known (Banerjee *et al.*, 1999; Xu *et al.*, 2004).

In the present study two types of markers were taken with a vision to identify sex at seedling stage in nutmeg. DNA of identified male and female trees were

characterized by molecular and biochemical markers. Molecular marker used was RAPD and SCAR and biochemical markers were isozyme analysis of acid phosphatase and glutamate oxaloacetate transaminase (GOT). The methodology included

A) Isolation of good quality DNA

B) RAPD with selected primers

C) Elution, cloning and sequencing of RAPD polymorphic amplicon

D) Sequence analysis with bioinformatic tools

E) SCAR primer designing and testing the SCAR primers in sex identification.

F) Analysis of enzyme acid phosphotase and glutamate oxaloacetate transaminase

The results obtained on various aspects are discussed here under.

5.1. Molecular marker analysis

Molecular characterization of mature nutmeg male and female trees for identification of sex was done with RAPD and SCAR markers.

5.1.1. Standardization of DNA isolation

The samples were leaves from five mature male and female trees. The protocol suggested by Roger and Bendich (1994) modified with 3X CTAB extraction buffer and 5% CTAB solution yielded good quality DNA from tender immature, pale green third leaf from apex of the shoot. The mature dark green leaves were not suitable for DNA isolation. The electrophoresed DNA showed distinct bands without shearing. The samples showed RNA contamination and were free of protein contamination (Plate 6). The RNA contamination was removed by treatment with Ribonuclease A. The UV absorbance ratio OD_{260}/OD_{280} as assessed by NanoDrop® ND-1000 spectrophometer ranged between 1.80-1.84 which indicated that quality of DNA was good (Table 2).

Sheeja *et al.* (2008) has reported that immature, pale green third leaf from apex was good for DNA isolation with Roger and Bendich (1994) protocol. She has used 3X CTAB extraction buffer with 10% CTAB solution. But in the present study extraction with 3X CTAB extraction buffer and 5% CTAB solution was found good.

Isolation of good quality genomic DNA is one of the most important pre requisites for doing RAPD and SCAR analysis. Nutmeg is rich in polyphenols and getting good quality DNA is a difficult task. Polyphenolic contamination was removed by collecting the sample in ice and preserving the same at -20^0C prior to grinding. Addition of 50 µl β- mercapto ethanol and pinch of polyvinyl pyrolidine while grinding was also done. The use of tender immature leaf samples also might have reduced the polyphenol interference in the DNA isolation. Tender immature leaves contain actively dividing cells with lesser concentration of extra nuclear materials like protein, oil, carbohydrates, fats and other metabolites that interfere with the isolation of nucleic acids.

Micheli *et al.* (1994) reported that RNA in the genomic DNA preparation often influences the reproducibility of RAPD patterns. So RNA contamination was removed. Good quality DNA with the UV absorbance ratio OD_{260}/OD_{280} 1.80-1.84 was used for all RAPD and SCAR analyses.

5.1.2. RAPD (Random Amplified Polymorphic DNA) analysis

The RAPD technique was developed by Williams *et al.* (1990) and the technique relies on the differential enzymatic amplification of small DNA fragments using PCR with arbitrary decamer oligonucleotide primers. In RAPD markers, polymorphism results from the changes in the sequence of the primer binding site. Usually RAPD markers are dominant in nature (Waugh *et al.*, 1992) because polymorphisms are detected as the presence or absence of bands.

The RAPD technique (Williams *et al.*, 1990) is a simple identifier of polymorphism and has been used to develop markers of sex determination in several

plants, i.e. *Salix viminalis* L. (Alstrom *et al.,* 1998), *Actinidia chinensis* (Harvey *et al.,* 1997; Gill *et al.,* 1998), *Asparagus* (Jiang and Sink 1997), *Cannabis sativa* L. (Mandolino *et al.,* 1999), *Eucommia ulmoides* Oliv. (Xu *et al.,* 2004), *Encephalartos natalensis* (Prakash and Staden 2006), *Carica papaya* (Urasaki *et al.,* 2002; Chaves and Bedoya 2007). Xiao *et al.,* (2009) attempted to develop AFLP and RAPD markers linked to sex in *Actinidia* by bulk segregant analysis. More than 33,000 well-separated bands, obtained from 520 RAPD primers and 64 AFLP primer combinations, were analysed in the male and female bulks of the 3 populations together with the parent plants. Two RAPD primers, OPA9 and OPAI12, each produced one male-related band in P2.

5.1.2.1. Screening of Primers

The DNA of individual male and female samples was initially screened with sixty seven Operon decamer primers belonging to series OPA, OPY, OPN, OPF, OPS, OPAH, OPE, OPC, OPD, OPG, OPO and OPK (Table 1) along with marker. Out of sixty-seven, thirteen were sex specific primers reported in other crops as given in table 2. Among total primers tested, only four OPA 27, OPF 05, OPD 15 and OPK 01 have showed polymorphism between male and female individual samples. The primers OPA 27 and OPF 05 amplified male specific band of 1.8 kb. The primers OPD 15 and OPK 01 amplified female specific band of 1.8 kb and 1.1 kb respectively. These four polymorphic primers (OPA 27, OPF 05, OPD 15 and OPK 01) were further used in RAPD with bulk and individual samples. Initial screening with sixty-seven primers with individual male and female DNA samples minimized the labour and inputs required for the total RAPD analysis.

The standardized annealing temperature for the PCR amplification was 35^0C and GC content varied 50 to 60 per cent according to primer. Shibu *et al.* (2008) also used the same condition for amplifying nutmeg DNA in RAPD analysis for sex determination.

The primers OPK 01 and OPD 15 which were identified as male specific markers for date palm and rumex respectively (Younis *et al.*, 2008; Sink and Jiang, 1997) have given polymorphism for nutmeg with female specificity. The sex determination system in date palm and rumex was XX-XY (XX –female; XY- male) chromosome system (Parker, 1990). In nutmeg also it appears that sex is genetically determined with a strong possibility of a mono-factorial sex-determining mechanism (Flach, 1966). The primers OPK 01 and OPD 15 has the capacity to identify the DNA material of the sex chromosomes. Since these primers had amplified male specific band in date palm and rumex while female specific band in nutmeg, the DNA material coding for sex is situated very much closer and may not be varying very much in base pairs. It can also be assumed that in dioecious species certain DNA material related to gender may be conserved over evolution.

The male sex specific primers UBC354, OPO18, OPA08, OPD10, OPN 01, OPG05, OPA06, OPA17 and OPY07 identified sex in basket willow, pandanus, hemp, asparagus, atriplex, jojoba, palmyrah, *Schisandra nigra* and papaya but did not produce polymorphism between male and female nutmeg plants. Williams *et al.* (1990) reported that even a single base change in the primer sequence could cause a complete change in the set of amplified DNA segments.

5.1.2.2. RAPD with selected primers

The DNA samples from the bulk male, bulk female, individual male, individual female and negative control were amplified with selected polymorphic primers. Only the primer OPK 01 amplified polymorphic female specific band of 1.1 kb in bulk and individual samples (Plate15). The reproducibility of OPK 01 was again checked by amplifying DNA from five individual male and females and has given positive results (Plate 17). The sequence information generated by OPK 01 amplicon of 1.1 kb was used for SCAR primer design and analysis.

The results produced by OPA 27, OPF 05 and OPD 15 in primer screening were also not reproduced in RAPD analysis with bulk and individual samples. In the past,

RAPD has been found to be a very effective technique for the identification of sex-linked molecular markers in several dioecious plant species, e.g. *P. longum* (Banerjee *et al.*, 1999). Manoj *et al.*, (2008) identified two male sex-associated RAPD markers, $OPA10_{827}$ and $OPA15_{744}$, that were amplified with OPA10 and OPA15, respectively, from the genomic DNA of only male plants of *Piper longum*.

In RAPD, DNA amplification is highly influenced by annealing temperatue, GC content, single nucleotide change, concentration of polymerase and quality and quantity of DNA (Williams *et al.*, 1990; Fakuoka *et al.*,. 1992 and Murray and Thompson, 1994). Williams *et al.*, (1990) found that the GC content in the 10- mer primer influenced the amplification and a GC content of 40 per cent or more in the primer sequence was needed to generate detectable levels of amplified products. Fakuoka *et al.*, (1992) reported that in rice, increased GC content in the range of 40 to 60 per cent tended to increase the number of amplification products. Annealing temperatures above 40^0C in the thermal cycling profile prevented amplification by many of the 10 base oligonucleotides tested (Murray and Thompson, 1980). Non-discrete band of amplification products, appearing as a 'smear' in gel could be converted to discretely sized bands by reducing the concentration of either the polymerase or the genomic DNA.

5.2. Elution of specific PCR amplicon

The genomic DNA of five individual male and females were amplified by OPK 01primer and the specific band of 1.1 kb was eluted from the female specific lanes (Plate 14) using gel elution kit (Axygen). The eluted amplicon recorded UV absorbance ratio (A_{260}/A_{280}) of 1.84 and was of good quality. The gel elution kit by Axygen was suitable for elution of DNA from agarose gel.

5.2.1. Cloning of amplicon in *E. coli*

Cloning of amplicon was done in *E. coli* to get multiple copies of amplicon for further sequence analysis. Competent cells of *E. coli* JM 109 were prepared by $CaCl_2$ treatment and genetic transformation of the same was done with pDrive vector. For this

the eluted specific amplicon of 1.1 kb by primer OPK 01 was ligated with pDrive vector (Fig 2) using Qiagen PCR cloning kit. The ligated pDrive vector containing the specific amplicon was transformed into competent *E. coli* cells by giving a shock treatment which consisted of increasing the temperature from zero to 42^0C for a short period of 90 seconds. The *E. coli* cells after transformation when plated on LBA ampicillin plates overlaid with X-gal/IPTG produced blue and white colonies after overnight incubation (Fig 3).

The presence of insert in white colonies was checked by colony PCR. The DNA of white and blue colonies were amplified using T7 and SP6 primers at standard PCR conditions and was electrophoresed (section 3.2.1.7.5). The DNA of white colonies recorded 1.1 kb higher molecular weight band and the blue colonies only 500bp, confirming the presence of insert in white colony (Plate 19).

The pDrive vector contained T7 and SP6 polymerase promoters and were equipped with ampicillin resistance marker (*amp*^{r}) and *Lac Z* gene as reporter. *Lac Z* gene region also contained multiple cloning site for insertion of insert. This facilitates the blue white screening or recombination by insertional inactivation of β-galactosidase. The insertion of eluted DNA fragment into the plasmid destroyed the integrity of the *lacZ* gene present on the molecule and β-galactosidase was not synthesized. Transformed *E. coli* cells are capable to grow in ampicillin containing media due to presence of ampicillin resistance gene in pDrive vector. The vector supplied was custom made by cutting with *Eco* RV and adding 3' terminal uracil to both ends. These single 3' – U overhangs at the insertion sites improved the efficiency of ligation of a PCR product into plasmid by preventing recircularization of the vector and providing a compatible overhang for the product generated by *Taq* polymerase. The thermostable Taq polymerase enzyme often adds a single deoxyadenosine, in a template in serial fashion, to the 3' end of amplified fragments (Clark, 1998).

Multiple copies of OPK 01 amplicon was made easily by fast dividing transformed *E. coli* cells. The white colony stabs were prepared for sequencing of the amplicon.

5.3. Sequencing of the cloned fragment

The prepared white colony stabs were sequenced at Bioserve Biotechnologies Pvt. Ltd. Hyderabad by automated sequencing with SP6 and T7 primers and provided 5'-3' sequence data in both forward and reverse directions with precise order of nucleotides. Sequence information obtained for the fragment with OPK 01 primer was referred as OPK_{1100} and had a size of 1100 bp (Fig 4). It was further analysed by various bioinformatics tools for getting more sequence information.

5.3.1. Sequence analysis with various bioinformatic tools

The sequence obtained after automated sequencing were subjected to vector screening using the VecScreen tool in NCBI to remove vector regions and the bases region 825 to 1128 bases showed similarity with the pDrive vector was deleted from the original sequence. The remaining 816 bases were named as *Nut* seq_{816} and is presented in Fig 6.

Homology of the sequence obtained from the cloned product of nutmeg (*Nut* seq_{816}) with the other reported sequences was analyzed using blastn search tool. It has shown no significant similarity for the given sequence (Fig 7). Liu *et al.* (2004) identified the male specific (MSY) region in hermaphrodite and male papaya plants. However, no sequence similarities were found in BLASTN with sequences of the MSY region to any other gene in the database.

Open reading frame was identified in the sequence *Nut* seq_{816} using NCBI tool 'ORF Finder'. The sequence contained three ORFs from 353 bases region to 677 bases (Fig 8). The +2 ORF strand encodes two domains for amino asparate transaminase (GOT) and cystathionine beta- lyases with E value 3.6. The other ORFs didn't encode any domain. The GenScan tool was used to predict the gene/exon present in the sequence but no exon/gene predicted in the given sequence search (Fig 9).

GenScan tool didn't predict any gene/ exon in *Nut* seq_{816} and this may be due to the smallness of the fragment. The ORF finder identified three ORFs suggesting that the sequence code for certain traits and the sex of nutmeg can be genetically controlled.

In date palm amino asparate transaminase (GOT) showed difference between male and female palms (Beksheet *et al.,* 2008). This fact supports the assay of GOT as biochemical marker for sex identification.

The nucleotide sequence was translated into amino acid sequence using Transeq programme of ExSpasy tools (Fig 10). The physico chemical properties of amino acid sequence were predicted using Protparam of ExSpasy tools. The major amino acids deduced from the cloned fragment *Nut* seq_{816} were Alanine, Cystine, Glycine and Threonine. The secondary structure of protein predicted by GOR programme of ExSpasy tool is presented in Fig 11. The amino acid sequence of *Nut* seq_{816} had 86.22 per cent extended strands and 13.78 per cent random coils. Suitable bioinformatics tools may give precise information about proteins/enzymes coded by *Nut* seq_{816}.

5.4. Development of SCAR marker

The accuracy and reliability of RAPD characterization can further be increased by sequencing the termini of specific RAPD markers and designing longer primers for more specific amplification (Paran and Michelmore, 1993). The technique SCAR (Sequence Characterized Amplified Regions) uses longer primers of 18 to 24 bases in place of ten bases long RAPD primer to amplify specific region as single band in gel document. By increasing the specificity of the primers, the results become more reproducible and more specific (Hernandez *et al.,* 1999). Several of the sex-linked random amplified polymorphic DNA (RAPD) markers have been converted into SCAR markers i.e., in *Salix viminalis* (Gunter *et al.*, 2003), in *Actinidia chinensis* (Gill *et al.*, 1998) in Papaya (Urasaki *et al.*, 2002). Several disease resistance RAPD markers were also converted to SCAR (Paran and Michelmore 1993, Arnedo *et al.*, 2002). SCAR marker has been developed as a marker of sex determination in several dioecious crops, *Atriplex garretti* (Kafkas, *et al.,* 1998), *Schisandra nigra* (Jung *et al.,* 2001), *Carica papaya* (Urasaki *et al.,* 2002), *Mercurialis annua* (Khadka *et al.,* 2002) *Eucommia ulmoides* Oliv. (Xu *et al.,* 2004), *Carica papaya* (Chaves and Bedoya, 2007), *Pandanus*

fascicularis (Vinod *et al.*, 2007), *Pueraria tuberose* (Devaiah and Venkatasbramanian, 2008).

5.4.1. SCAR primer designing

Two pairs of SCAR primers SP1 and SP2 were designed. For designing longer primers of 24 bp length the sequence of RAPD fragment OPK 01(*Nut* seq_{816}) was used. Each SCAR primer contained the original 10 bases of RAPD primer plus then next 14 internal bases from the 5' end (Masuzaki *et al.*, 2007). Devaiah and Venkatasubramanian (2008) used intermediate sequences of the RAPD amplicon to design a pair of SCAR primers (22 and 26 mer) and amplified 320 bp male specific expected amplicon in *Pueraria tuberose*. Chaves and Bedoya (2004) have designed a pair of 18 mer and 20 mer SCAR primers based on end sequence information of RAPD amplion and amplified male specific amplicon of 369 bp in papaya

Vinod *et al.* (2007) has designed forward and reverse primers of 18 and 20 mer based on end sequence information of RAPD amplicon and amplified male specific amplicon of 976 bp in pandanus. The factors considered for designing primers were sequence length (18-24 bases), GC content (40-50%), Tm (55-65^{0}C), non complementarity between the primers and absence of secondary loop structures. In the present study based on the requisites two sets of primers (SP1 and SP2) were synthesized considering these factors.

5.4.1.1. PCR amplification using SCAR primers

5.4.1.2. Standardization of PCR conditions for SCAR analysis

Varying quantities of DNA (15 and 30 ng) were amplified at different annealing temperatures (61, 63, 64, 65, 66, 67 and 68^{0}C). Distinct single band of size 300 bp was generated only at 68^{0}C with 15 ng of DNA. Other annealing temperatures didn't amplify SP1 primers at 15 ng and 30 ng of DNA. According to Devaiah and Venkatasbramanian (2008) changing the annealing temperature conditions may generate distinct SCAR band suppressing other fragments. In the present study the annealing temperature was changed from 60^{0}C to 68^{0}C.

The chance of amplifying specific DNA region linked to a trait depends upon number of nucleotide bases in primer, annealing temperature, melting temperature and GC content (Fakuoka *et al.*, 1992). The result of the present study emphasise that the quantity of DNA and annealing temperature very much influence the amplification of specific DNA region by the long SCAR primer of 24 bases.

5.4.1.3. Expression of SCAR marker in mature trees and seedlings

Expression of SCAR primers in mature trees was analysed. The PCR amplification was done with five males, five females and four occasional fruiting males. The gel document revealed an amplicon of around 300 bp in all females and one occasional fruiting male (Plate 20). There was polymorphism between male and females. The selected male, female and occasional fruiting males were also amplified with SP2 primers with specific PCR conditions as mentioned in section 3.2.2.2.1. The quantity of DNA used was 15 ng and annealing temperature was 68^0C. No amplification was obtained in any one of the sample with SP2 primers.

Expression of SCAR primers was again checked in leaf samples of ten seedlings of 2 to 4 years age seedlings. The gel image revealed around 300 bp amplicon in four out of ten seedlings (Plate 21).

In the present investigation of sex determination, a characteristic female specific band was amplified suggesting that in nutmeg the female may be heterogametic. This is supported by RAPD analysis by Shibu *et al.* (2008) in which the primer OPE 11 also amplified a female specific band.

These results seem to support earlier karyological studies in nutmeg (Flach 1966). Though cytological investigations did not reveal heteromorphic chromosomes in the sexes (as an indication of heterogametic condition), females were found to possess chromosomes that had facultative nucleolar property. Flach (1966) argued that this may be due to the heterogametic nature of the females. An additional but indirect support for this view is drawn from the fact that *Myristica fragrans* is apparently the first dicotyledonous plant known to possess a holokinetic chromosome (a condition wherein

the chromosomes occur without a localized centromere which is diffused all along the length of the chromosome). A holokinetic state is relatively rare but has been reported to occur in the insect orders Lepidoptera and Trichoptera, where the female is heterogametic. It appears that sex in *M. fragrans* is genetically determined with a strong possibility of a mono-factorial sex-determining mechanism, such as a XX-XY system, with females being heterogametic (Flach, 1966).

The expression of SCAR primer SP1 as single band of 300 bp in mature females and some seedlings suggest that it could be used as molecular marker for sex identification of nutmeg at seedling stage. But the accuracy of primer as a female specific molecular marker has to be validated by characterizing more mature plants of known sex. The gender expression of characterized seedlings on flowering can also be verified.

5.5. Biochemical marker analysis

Biochemical characterization of nutmeg male and female trees for identification of sex was done with isozyme analysis of acid phophatase and glutamate oxaloacetate transaminase. In the study leaf samples from four male and five female trees were used.

Thomas (1997) reported that peroxidase did not show any difference between male and female nutmeg trees.

5.5.1. Standardization of Polyacrylamide gel electrophoresis

Initially PAGE protocol reported by Thomas (1997) for nutmeg showed no protein movement. PAGE protocol was standardized with BIO-RAD manual protocol (2004) and it was standardized with standard protein Bovine Serum Albumin (BSA). The gel revealed protein movement and separation of protein according to the molecular weight (plate 22). The resolving power of standardized PAGE was again tested with nutmeg leaf protein (plate 23) and the same was used for acid phosphotase and glutamate oxalo acetae transaminase (GOT) assay.

In polyacrylamide gel electrophoresis (PAGE), multiphasic buffer systems employ two kinds of gel in one run: lower (analysing) and the upper (stacking) gel (Wendel., 1989). The pH of electrophoretic buffers may be manipulated within range to

optimize the resolution of bands of proteins being electrophoresed. Electrophoresis operates on two fundamental and inter related electrical principles i.e voltage and power of the electric current used. Usually the electric current will be supplied in a voltage of 10 to 50 V and the power in the range of 10 to 20 mA. The heat generated during the electrophoretic process must be dissipated because excessive heat decreases enzyme activity, so a cool environment of 4 to 6^0C was given during electrophoresis (Andrews, 1986). The facts mentioned above combined with the duration of electrophoresis, protein concentration, quality and size of the sample, procedures of staining gels (Vallejos, 1983) and protocols strongly influence the results.

The banding pattern generated after PAGE is an electrophoretic phenotype (Wendel., 1989), which usually consist of one or more colored bands for each individual analysed. In some cases, it may be simple and consist of a single invariant band in the whole sample. In contrast, some enzymes may display complex phenotypes with 15 or more bands per individual. So a correct interpretation of banding patterns in genetic terms requires referred electrophoresis with the proper determination of the pertinent factors that influence the electrophoretic phenotype.

5.5.2. Acid phosphatase isozyme analysis

Acid phosphatase assay was done as per the Standardized PAGE protocol. The staining with fast blue GBG salt stain did not generate any protein band. Staining with fast blue RR salt generated four zones of enzymatic activity (Plate 24). The zone of activity was same in both male and female samples. All bands had the same Relative mobility values in both male and female and there was no polymorphism between them.

Marino *et al* (2002) has reported staining procedure with fast blue RR salt for acid phosphatase analysis in cauliflower. Torres and AlJibouri, (1989) has reported that fast blue GBG salt showed band pattern in hemp. Truta *et al.* (2002) reported the solution for incubation 0.15 M acetate buffer (pH 7), 1% -naphthyl acetate and Fast blue RR stain (2 mg/ml) was good in acid phosphatase staining. In the present study 0.1 M acetate buffer, 1% -naphthyl acetate and fast blue RR stain (1 mg/ml) showed distinct band pattern. Maestri *et al.* (1990) reported that acid phosphatase showed greater

intensity of bands in females than male phylloclade of *Asparagus officinalis*. Saker *et al.* (2002) reported that ten percent gel porosity has given better resolution than seven and eight percent gel porosity.

Beksheet *et al.* (2008) has reported that acid phosphatase has shown distinct band in male date palms. Maestri *et al.* (1990) has reported that acid phosphatase shown distinct banding pattern in male progenies of asparagus. Gulati, (1989) higher acid phosphatase activity was noted in the explants from male plants over those from the female plants. In the present investigation there was no significant difference in acid phosphatase enzyme activity between male and female leaf samples. So, acid phosphatase isozyme cannot be used as a biochemical marker for sex determination in nutmeg.

Joshi *et al.* (2008) tested isozymes of nine enzymes viz. Esterase (EST), Glucose-6-phosphate dehydrogenase (G6PDH), Malate dehydrogenase (MDH), Malic enzyme (ME), 6-phosphogluconate dehydrogenase (6PGDH), phosphoglucoisomerase (PGI), phosphoglucomutase (PGM), menadione reductase (MR), shikimic acid dehydrogenase (SKDH) for the development of biochemical markers for polygamodioecious character in *Simarouba glauca*. Out of nine enzyme systems tried, five enzymes viz. G6PDH, 6PGDH, SKDH, MR and PGI showed good resolution and the number of putative loci for the five enzymes ranged from 1 to 2. However, no polymorphism could be found in any of these enzymes. .

5.5.3. Glutamate Oxaloacetate Transaminase (GOT) isozyme analysis

The GOT assay in leaf samples, specific to male and females were done as per the section 3.4.2. The gel profile (Plate25) has revealed a low intensity band in five female samples and scar bands in four male samples. The relative mobility of band in female samples was 0.26. Afterwards different extraction buffers with pH gradient 6 to 8 were tested for increasing the band intensity. But there was no band display after staining. So refinement for this analysis can be made. In the enzyme assay as mentioned earlier band display is influenced by many factors such as pH of the buffer, porosity of the gel, voltage, power of electrophoresis and staining procedure etc. Refinement of the

present PAGE protocol by changing the aforesaid factors may yield distinct bands in different genotypes.

Stejskal (1994) reported that L-cysteine sulfmic acid and phenazine methosulfate more sensitive staining than L-aspartic acid and Fast Blue BB salt. In the present study L-aspartic acid and Fast Blue BB salt showed banding pattern in nutmeg sample. Beksheet *et al.* (2008) has reported that glutamate oxalo acetate enzymes gave strong difference between male and female date palms. Maestri *et al.* (1990) has reported that polymorphism was observed in the three zones of GOT activity in female asparagus. In the present study there was polymorphism between male and female nutmeg plants with respect to GOT. The possibility of using GOT as biochemical marker can be explored by detailed investigation.

Conclusion

In the molecular marker analysis, the primer OPK 01 from Operon Technologies, USA has amplified a female specific polymorphic band of 1.1 kb in bulk and individual DNA of female nutmeg plants. SCAR primer SP1 was developed from eluted and sequenced amplicon of OPK 01_{1100}. In the PCR amplification, the SP1 amplified single band of 300 bp in females. Seedlings of two to four years have also shown SCAR amplicon of 300 bp randomly. There was no polymorphism between male and female nutmeg plants in biochemical marker analysis with respect to acid phosphatase. The enzyme Glutamate Oxaloacetate Transaminase (GOT) has shown polymorphism between male and female nutmeg plants. The investigation opens up the possibility of using molecular markers RAPD and SCAR in sex identification of nutmeg seedlings at early stage.

Future line of work

1. Validate SCAR primer with more number of samples
2. Explore the possibility of practical application of SCAR in sex identification of nutmeg seedlings
3. Refine the assay of GOT in male and female samples for verifying polymorphism.


Summary

6. SUMMARY

The study on "Sex determination in nutmeg (*Myristica fragrans* Houtt.) through molecular and biochemical markers" 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 distinguish male and female nutmeg trees by molecular and biochemical markers and to develop a SCAR marker to distinguish male and female trees.

The salient findings of the study are summarized below.

1. The protocol suggested by Rogers and Bendich (1994) was tested for extraction of genomic DNA from nutmeg. DNA was isolated from mature dark green and immature pale green leaves of five individual male and females. Dark green leaves did not yield DNA, immature pale green leaves yielded a little quantity of DNA. The extraction with Roger and Bendich (1994) protocol when modified with 3X CTAB and 5% CTAB solution improved the quality and quantity of DNA.

2. The quality and quantity of DNA was analyzed by NanoDrop® ND-1000 spectrophotometer. The absorbance ratio ranged from 1.80-1.84, which indicated good quality DNA.

3. The RNA contamination was completely removed through RNase treatment. This yielded DNA with no impurities and was suitable for RAPD and SCAR analyses.

4. Sixty seven random decamer primers belonging to eleven operon series including thirteen sex specific primers were used for primer screening. Out of 67, four primers (OPA 27, OPD 15, OPF 05 and OPK 01) showed polymorphism between individual male and female trees.

5. These four primers were selected for further RAPD analysis with bulked and individual samples. Out of four, only OPK 01 amplified 1.1 kb polymorphic band in both bulk and individual female samples.

6. The polymorphic fragment 1.1 kb obtained through RAPD reaction with OPK 01 primer was eluted and cloned into pDrive vector and the product was transformed into the competent cells of *E coli* JM 109

7. Recombination of the insert was confirmed through colony PCR with SP6 and T7 primers. The higher molecular band (1.1 kb) of white colony confirmed the presence of insert. The blue colony recorded only 500 bp band due to absence of insert.

8. Sequencing of cloned fragment with universal SP6 and T7 primers gave the sequence data for 1128 bases and named as OPK_{1100}. Vector screening was performed to remove the vector sequence from OPK_{1100} using VecScreen tool and insert sequence 816 bases was obtained. This sequence was named as *Nut* seq_{816}.

9. *Nut* seq_{816} was subjected to nucleotide Blast search and revealed no significant homology with published sequences of NCBI database.

10. Three open reading frames were identified in the *Nut* seq_{816} using NCBI tool "ORF Finder". The +2 ORF strand encodes two domains for amino asparate transaminase (GOT) and cystathionine beta- lyases with E value 3.6. The other ORFs didn't encode any domain. GenScan predicted no gene in the *Nut* seq_{816}.

11. The amino acid composition of the $Nutseq_{816}$ was analysed using Transeq tool and the major amino acids present were Alanine, Cystine, Glycine and Threonine.

12. The secondary structure predicted for *Nutseq$_{816}$* contained 86.22 per cent extended strands and random coils 13.78 per cent and showed that the extended strands are the major components.

13. Two pairs (SP1 and SP2) SCAR primers were designed based on sequence information of *Nutseq$_{816}$*. Forward and reverse primers of SP1 and SP2 had melting temperatures 68^{0}C, 70^{0}C, 72^{0}C and 70^{0}C and sequence length of 24 bases.

14. The amplification cycle was altered to get specific amplification pattern with SCAR primers. The specific conditions standardized for SCAR amplification were genomic DNA quantity 15 ng and annealing temperature 68^{0}C. The SCAR primer SP1 amplified 300 bp in all females and not in males. So, there is polymorphism between male and female trees. No amplification was obtained in any one of the samples with SP2 primers. SCAR primer was again checked with four occasional fruiting males and it amplified 300 bp band in one occasional fruiting male.

15. DNA of two to four year old ten seedlings was amplified with SP1 primers. The primers amplified approximately 300 bp amplicon in four samples.

16. The expression of SCAR primer SP1 as single band of 300 bp in mature females and some seedlings suggest that it could be used as molecular marker for sex identification of nutmeg at seedling stage. But the accuracy of primer as a female specific molecular marker has to be validated by characterizing more mature plants of known sex.

17. Isozyme of acid phosphotase and glutamate oxaloacetate transaminase (GOT) were studied. Polyacrylamide gel electrophoresis (PAGE) was carried out for protein banding. PAGE was standardized as per BIO-RAD manual protocol with standard protein Bovine serum albumin (BSA) and nutmeg leaf protein.

18. There was no polymorphism between male and female with respect to acid phosphatase assay and four zones of activity were present in both samples.

19. GOT assay showed polymorphism and need protocol refinement.

20. Future research can be done on validation of SCAR marker with more number of samples of known sex and the possibility of practical application in sex determination of nutmeg seedlings can be explored.

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Appendices

APPENDIX-I

List of Laboratory Equipments Used For the Study

High speed refrigerated centrifuge	Kubota, Japan
Horizontal electrophoresis system	BIO-RAD
Thermal cycler	Master cycler personal, Eppendrof
Gel documentation system	Gel DOC- It ^{TM} Imaging system UVP (USA)
PAGE system	Mini PROTEAN® 3 cell BIO-RAD

APPENDIX-II

Composition of Reagents Used for DNA Isolation

1. **Rogers and Bendich (CTAB) method**

3X CTAB Extraction Buffer

1M Tris (pH 8.0)

0.5 M EDTA (pH 8.0)

5 M NaCl

PVP (40.000 MW)

5% CTAB Solution

5% CTAB (w/v)

0.7 M NaCl

TE Buffer

10 mM Tris (pH 8.0)

10 mM EDTA (pH 8.0)

APPENDIX-III

Composition of Buffers and Dyes used for Gel electrophoresis

1. **TAE Buffer 50X**

   242 g Tris base

   57.1 ml glacial acetic acid

   100 ml 0.5 M EDTA (pH 8.0)

2. **TBE Buffer 10X**

   54 g Tris base

   27.5 g boric acid

   20 ml 0.5 M EDTA (pH 8.0)

3. **Loading Dye (6X)**

   0.25% bromophenol blue

   0.25% xylene cyanol

   30% glycerol in water

4. **Formamide Dye**

   Formamide – 10 ml

   Xylene cyanol – 10 mg

   Bromophenol blue – 10 mg

   0.5 M EDTA (pH 8.0) – 200 µl

APPENDIX-IV

Composition of reagents used for cloning and transformation studies

Reagents used for competent cell preparation

1. **Solution A**

   Ice- cold 100 mM CaCl2

2. **Luria Bertani (LB) broth**

   Tryptone - 10 g

   Yeast extract - 5 g

   NaCl - 5 g

   pH adjusted to 7

   Distilled water - 1 L

3. **Luria Bertani Agar medium**

   Tryptone - 10 g

   Yeast extract - 5 g

   NaCl - 5 g

   Agar - 20 g

   pH adjusted to 7

   Distilled water - 1 L

SEX DETERMINATION IN NUTMEG (*Myristica fragrans* Houtt.) THROUGH MOLECULAR AND BIOCHEMICHAL MARKERS

By

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ABSTRACT OF THE THESIS

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ABSTRACT

Nutmeg (*Myristica fragrans* Houtt.) is an important tree spice of southern India, Malaysia and Indonesia yielding two products of commercial value, 'nutmeg' and 'mace'. Nutmeg of commerce is the dried seed and mace is the dried aril. It is a spreading evergreen tree of the family Myristicaceae and is dioecious with long pre bearing period of 5 to 7 years. Presently, the dioecy in nutmeg is overcome by vegetative propagation or top working. Even though several vegetative methods have been reported, the large scale adoption of these methods is constrained due to insufficient number of orthotrops. So seedling continues to be the major propagating material. In the present study, an attempt was made to identify sex in nutmeg through molecular and biochemical markers so as to identify sex at seedling stage itself.

Molecular marker used was RAPD and biochemical marker was isozymes of acid phosphatase and glutamate oxaloacetate transaminase (GOT).

Based on bearing pattern and floral morphology, five typical male and female plants of age 10-15 years were selected for RAPD analysis. DNA isolation technique was standardised using CTAB method. Good quality DNA with UV absorbance ratio (A_{260}/A_{280}) 1.80- 1.84 was used for analysis. Sixty seven decamer primers were screened and four primers showing the polymorphism has been selected for further RAPD analysis. PCR amplification with selected primers *viz.* OPD 15, OPA 27, OPF 05 and OPK 01 was carried out with samples of bulk DNA from five male and female, DNA of individual male and female and negative control. Among them OPK 01 amplified reproducible female specific band (1.1 kb) in bulked and individual samples.

The polymorphic female specific band amplified by OPK 01 primer was eluted and cloned in pDrive vector and transformed into *E. coli* JM 109 cells. Cloned cells were subjected to blue-white screening and transformed white clones were sequenced at Bioserve, Hyderabad.

The sequence obtained after VecScreen (*Nut seq* 816 bp) was analysed with various bioinformatic tools like Blastn, Blastp, GenScan, ORF Finder, Transeq, GOR and Protparam. Three open reading frames identified in the sequence *Nut seq*$_{816}$

sing NCBI tool "ORF Finder". The +2 ORF strand encodes two domains for amino asparate transaminase (GOT) and cystathionine beta- lyases with E value 3.6. The other ORFs didn't encode any domain. GenScan predicted no gene in the *Nut seq*$_{816}$.

Based on sequence information two pairs (SP1 and SP2) of SCAR primers (24 bp) were designed using primer3 programme. The efficiency of SCAR primer to distinguish male and female plants was tested by PCR amplification of DNA from five male and females. The SCAR primers amplified around 300 bp single band in five females. SCAR primer was again checked with four occasional fruiting males and it amplified 300 bp band in one occasional fruiting male. Expression of SCAR primers was tested in ten seedlings and got amplified in four samples.

Leaf samples from identified male and female trees were used for the biochemical marker analysis. Polyacrylamide gel electrophoresis (PAGE) for isozyme assay was standardized with standard protein and nutmeg leaf protein. Acid phosphatase assay recorded four zones of activity and all were monomorphic. Glutamate oxaloacetate transaminase showed polymorphism and need protocol refinement.

Accuracy of SCAR primer SP1 to distinguish male, female and occasional fruiting male has to be done with more samples. Commercialization of the technique can be explored based on the accuracy and cost benefit analysis.