MOLECULAR CHARACTERIZATION OF MALE STERILITY IN RIDGE GOURD [*Luffa acutangula* (L.) Roxb.]

By

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(2012-11-110)

THESIS

Submitted in partial fulfillment of the requirement

for the degree of

Master of Science in Agriculture

(PLANT BIOTECHNOLOGY)

Faculty of Agriculture

Kerala Agricultural University, Thrissur

CENTRE FOR PLANT BIOTECHNOLOGY AND MOLECULAR BIOLOGY

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KERALA, INDIA

2014

DECLARATION

I hereby declare that the thesis entitled **"Molecular characterization of male sterility in ridge gourd *Luffa acutangula* (L.) Roxb."** 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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Dedicated to my loving mother and my ever supporting Guide

ACKNOWLEDGEMENT

I bow to the lotus feet of ***Almighty*** *whose grace had endowed me the inner strength and confidence and blessed me with a helping hand at each step during my long sojourn at Kerala.*

At this moment of accomplishment, my heart is overwhelmed with gratitude and I wish if these words could convey the subtle feelings.

I am ineffable in expressing my deep sense of gratitude to my Major Advisor ***Dr. Deepu Mathew*** *Assistant Professor, CPBMB, College of Horticulture, Vellanikkara, for his inspiring guidance, critical comments, constant supervision, support and encouragement throughout the course of my research work. I am really grateful for the keen interest taken by him in the prompt correction of the manuscript.*

My sincere thanks are due to, ***Dr. P. A. Valsala,*** *Professor and Head, CPBMB, College of Horticulture, Vellanikkara, member of my advisory committee for the valuable guidance, timely suggestions and help rendered in the conduct of the experiment.*

I also owe my deep sense of gratitude and sincere thanks to ***Dr. P. A. Nazeem,*** *Professor and coordinator (DIC), CPBMB, College of Horticulture, Vellanikkara, member of my advisory committee for the her timely help, critical scrutiny of the manuscript and valuable guidance for the successful completion of my research work.*

I am extremely thankful to ***Dr. P. S. Abida,*** *Associate Professor, CPBMB, College of Horticulture, Vellanikkara, member of my advisory committee for her support, suggestions and guidance throughout the study.*

I take this opportunity to express my indebtedness with veneration and devotion to ***Dr. T. Pradeepkumar,*** *Associate Professor, Department of Olericulture, College of Horticulture, Vellanikkara,, member of my advisory committee for his constant support, suggestions, valuable guidance and timely help rendered in the conduct of the experiment.*

Words fall short as I place on record my indebtedness to ***Dr. Shylaja*** *of CPBMB for her untiring support and valuable guidance during my course of study.*

My heartfelt gratitude cannot be captured in words for the unflinching support, constant encouragement, warm concern, patience and valuable advice of my friends ***Vaishali,***

Renuka, Shriram, Sachin, Aoudumbar, Yogesh, Datta, Yashwant, Purushottam and Dheemanth, Vilas whose prayers, love and affection rendered me a successful path which propped up my career all along. I wish to express my sincere gratitude to my senior friends Parimal, Pujaita, Manibala, Subhashini, Shiny, Ravindra, Sharda & Ajay for their whole hearted support.

My duty is incomplete if I forget my junior friends Vinutha, Shruti, Deepthi, Sreelaxmi, Pallavi, Irfana, Asha, Devilaxmi, Dolagobinda, Naresh, Ramesh, Kallilu, Manikesh & Nabarun whose helping hands, love and affection fetched a remarkable place in my days in Kerala

The award of DBT fellowship is greatly acknowledged.

Above all, I am forever beholden to my all family members for their unfathomable love, boundless affection, personal sacrifice, incessant inspiration and constant prayers, which supported me to stay at tough tracks.

Sonwane Shital Marotirao

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ABBREVIATIONS

%	Percentage
>	Greater than
µg	Microgram
A	Ampere
AFLP	Amplified Fragment Length Polymorphism
AP-PCR	Arbitrarily Primed Polymerase Chain Reaction
bp	Base pair
cc	cubic centimetre
cm	Centimetre
CPBMB	Centre for Plant Biotechnology and Molecular Biology
CTAB	CetylTrimethyl Ammonium Bromide
DAF	DNA amplification fingerprinting
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
dNTPs	Deoxyribo Nucleoside Triphosphate
EDTA	Ethylene Diamine Tetra Acetic acid
EST	Expressed sequence tags
g	Gram
GD	Genetic Distance
ha	Hectare
ISSR	Inter Simple Sequence Repeat
kb	Kilo basepairs
L	Litre
M	Molar
MAS	Marker Assisted Selection
mg	Milligram
ml	Millilitre
mM	Milli mole

NBPGR	National Bureau of Plant Genetic Resources
ng	Nanogram
QTL	Quantitative trait loci
°C	Degree Celsius
OD	Optical Density
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
P^H	Hydrogen ion concentration
PIC	Polymorphic Information Content
pM	Pico molar
PVP	Poly vinyl pyrolidone
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RILs	Recombinant Inbred Lines
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
SCAR	Sequence Characterized Amplified Region
SRAP	Sequence Related Amplified Polymorphism
SSR	Simple Sequence Repeats
STMS	Sequence Tagged Microsatellite Sites
STR	Short Tandem Repeat
STS	Sequence Tagged Sites
TAE	Tris Acetate EDTA
TE	Tris EDTA
U	Unit
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
UV	Ultra violet
V	Volts
β	Beta
µl	Microlitre

Introduction

I. INTRODUCTION

The development of strategies to improve crop plants by the production of hybrid varieties is a major goal in plant breeding. Hybrids could enhance performance in different environments compared with the parental lines especially in terms of higher yield and increased resistance to diseases. Availability of cost effective mechanism and method to produce large scale hybrid seeds, utilizing the selected parental lines is one of the important factors which ultimately determine the commercial viability of hybrid varieties.

Manual emasculation increases cost of production, so various alternate genetic mechanisms such as male sterility, self incompatibility, gynoecious lines, sex regulators and chemical hybridizing agents are being used based on relative importance in hybrid development. Among these emasculation tools, male sterility is most commonly used for hybrid production. The practical utilization of heterosis in crop plants has been greatly facilitated during the past 15 years by the use of male sterility for low-cost, large-scale emasculation of the seed parents of hybrids. The method is now being used in large scale commercial production of hybrid onions, sugar beets, chillies, field corn, grain sorghum, and petunia (Duvick, 1965).

The first documentation of male sterility was done by Joseph Gottlieb Kolreuter (1763), who observed anther abortion within species and specific hybrids. Based on its inheritance and origin, male sterility can be classified as Cytoplasmic male sterility (CMS) which is maternally inherited, Genetic male sterility (GMS) which is nuclear inherited, Cytoplasmic genetic male sterility which is controlled by extra nuclear genes (CGMS) and Non genetic (NGMS) which is chemically induced using application of specific chemical like gametocides or chemical hybridizing agents.

Cytoplasmic male sterility (CMS) is a maternally inherited condition in which a plant is unable to produce functional pollens. Cytoplasmic male sterility has now been identified in over 150 plant species.

It is often associated with chimeric mitochondrial open reading frames, transcripts originated from these altered open reading frames are translated into unique proteins that appear to interfere with mitochondrial function and pollen development. Nuclear restorer (*Rf* or *Fr*) genes function to suppress the deleterious effects of CMS-associated mitochondrial abnormalities by diverse mechanisms. There are several well-characterized CMS systems, for which the mitochondrial sequences thought to be responsible (Schnable and Wise, 1998).

Ridge gourd [*Luffa acutangula* (L.) Roxb.] is also known as 'angular loofah', 'chinese okra' or 'fluted loofah'. It is an important vegetable rich in dietary fibre and minerals in the tropical and subtropical countries, especially in Asia (Jansen *et al.*, 1993) as well as Africa (Neuwinger, 1994).

Ridge gourd is characterized by diverse sex forms *viz.*, monoecious, androecious, gynoecious, gynomonoecious, andromonoecious and hermaphrodite (Choudhary and Thakur, 1966). Among all the forms, predominant is monoecious. The female flowers are solitary whereas male flowers are in recemes. Principally two genes are involoved in production of various sex forms (Richaria, 1948).

Regional Agricultural Research Station (RARS), Pilicode, under the Kerala Agricultural University have developed an improved ridge gourd (*Luffa acutangula*) variety with high yield potential. It has been released for commercial cultivation in the name "Haritham". This variety yields medium sized fruits, which are cylindrical, tapering sharply towards the base and distinguishable with prominent ridges.

An offtype plant was detected in a population of ridge gourd which was characterized by the production of rudimentary male flowers in racemes (Pradeepkumar *et al.*, 2007). It is maintained through tissue culture at the Department of Olericulture, Kerala Agricultural University (Pradeepkumar *et al.*, 2010). The knowledge about inheritance of fertility restoration of male sterility in CMS system is of vital importance in improving or transferring fertility restoring genes and formulating restorer line breeding.

Extensive studies by Pradeepkumar *et al.* (2012) showed that MS system in ridge gourd is under the influence of dominant fertility restorer genes *viz.*, *Rf1* and *Rf2*. This was the first report of cytoplasmically controlled male sterility (CMS) in cucurbit where two dominant male fertility restorer nuclear genes with complementary gene action governing the restoration of male fertility.

Molecular markers are identified as effective tools to detect the genes of interest (Reddy, 2002). SSRs (Simple Sequence Repeats) and ISSRs (Inter Simple Sequence Repeats) are proven useful in differentiating the male sterility and fertility in *Brassica juncea* (Pathania *et al.*, 2003), pearl millet (Kumar *et al.*, 2006; Yadav *et al.*, 2007), sunflower (Chen *et al.*, 2006), wheat (Li *et al.*, 2005), marigold (He *et al.*, 2009).

The prerequisite in any breeding programme is to ensure that the gene of interest is present in the plant material under use. Identifying a marker linked to the gene responsible for male sterility will enable the rapid identification of sterility at an earlier phase itself.

Present study was carried out with the objective of developing reliable SSR and ISSR markers to identify the genes responsible for male sterility in ridge gourd. Male sterile line derived from Haritham and male fertile lines Haritham, Deepthi, Arka Sumeet were used. Identified marker have great importance in order to use it for marker assisted selection and hybrid seed production.

Review of Literature

2. REVIEW OF LITERATURE

2.1 General Background

Ridge gourd *(Luffa acutangula* (L.) Roxb.) with the vernacular names angled loofah, ribbed gourd, chinese okra etc. is an important vegetable throughout Asia and Africa. It belongs to the family Cucurbitaceae ($2n = 26$) with the typical characters such as monoecy, annual, climbing or trailing growth and acutely 5-angled hairy stem with tendrils up to 6-feet. Leaves are alternate, simple, no stipules, and broadly ovate to kidney-shaped blade.

Ridge gourd is characterized by diverse sex forms *viz.*, monoecious, androecious, gynoecious, gynomonoecious, andromonoecious and hermaphrodite types (Choudhary and Thakur, 1966), among all predominant form is monoecious. Principally two genes are involved in the production of various sex forms in *Luffa* (Richaria, 1948). Male flowers are in racemes with 15–35 cm long peduncle, flowers are unisexual, regular and pentamerous with 5–9 cm in diameter, pale yellow free petals with 3 free stamens inserted on the receptacle tube. The female flowers are solitary on 2–15 cm long pedicels, with inferior, densely pubescent, longitudinally ridged ovary and stigma is 3-lobed. Fruits are club-shaped, dry and fibrous capsules with 15–50 cm × 5–10 cm size, acutely 10-ribbed, brownish, dehiscent by an apical operculum and many-seeded. Seeds broadly elliptical in outline, compressed up to 1.5 cm long, smooth and dull black in colour.

2.1.1 Origin and geographic distribution

The genus *Luffa* has essentially old world origin in subtropical Asia particularly India (Kalloo, 1993). *Luffa* comprises 7 species, 4 of which are native to the old world tropics and 3 are somewhat distantly related species indigenous to South America. *Luffa acutangula* L., cultivars grown as vegetables have larger fruits and are less bitter than the wild types. In West Africa, local cultivars are used as vegetables, whereas in East Africa, commercial growers use improved cultivars imported from Asian countries for the Asian customers (Neuwinger,

1994). In southern and eastern Asia also it is a widely cultivated vegetable (Jansen *et al.*, 1993).

2.2. Molecular markers

Genetic markers are the biological features that are determined by allelic forms of genes or genetic loci and can be transmitted from one generation to another, and thus they can be used as an experimental probes or tags to keep track of an individual, a tissue, a cell, a nucleus, a chromosome or a gene. Genetic markers used in genetics and plant breeding can be classified into two categories: classical markers and DNA markers (Xu, 2010).

Classical markers include morphological markers, cytological markers and biochemical markers. DNA markers have developed into many systems based on different polymorphism-detecting techniques or methods which includes southern blotting – nuclear acid hybridization, PCR based methods such as RFLP, AFLP, RAPD, SSR, ISSR, SNP etc. (Collard *et al.*, 2005).

2.2.1. DNA markers

DNA marker is defined as a fragment of DNA that reveal mutations/ variations, which can be used to detect polymorphism between different genotypes or alleles of a gene for a particular sequence of DNA in a population or gene pool. Such fragments are associated with a certain location within the genome and may be detected by means of certain molecular technology. In short, DNA marker is a small region of DNA sequence showing polymorphism (base deletion, insertion and substitution) between different individuals. There are two basic methods to detect the polymorphism: Southern blotting which is a nuclear acid hybridization (Southern, 1975), and PCR, a polymerase chain reaction (Mullis, 1990).

DNA markers are also called molecular markers in many cases and play a major role in molecular breeding. PCR is a simple, fast, specific and relatively

low cost technique. The main advantage of this technique over other techniques is its inherent simplistic analysis (a single reaction can contain all reagents) and the ability to conduct PCR test with extremely, small quantities of DNA (Welsch *et al.*, 1991).

2.3 SSR markers

SSRs, also called as microsatellites, short tandem repeats (STRs) or sequence-tagged microsatellite sites (STMS), are PCR-based markers. They are tandem repeats of short nucleotide motifs (2-6 bp/nucleotides long). Di-, tri- and tetra-nucleotide repeats, e.g. $(GT)_n$, $(AAT)_n$ and $(GATA)_n$. They are widely distributed throughout the genomes of plants and animals. The copy number of these repeats varies among individuals and is a source of polymorphism, because the DNA sequences flanking microsatellite regions are usually conserved, primers specific for these regions are designed for use in the PCR reaction. SSR markers are characterized by their hyper-variability, reproducibility, co-dominant nature, locus-specificity, and random genome-wide distribution in most cases (Kashi *et al.*, 1997).

One of the most important attributes of microsatellite loci is their high level of allelic variation, thus making them valuable genetic markers. The unique sequences bordering the SSR motifs provide templates for specific primers to amplify the SSR alleles via PCR. SSR loci are individually amplified by PCR using pair of oligonucleotide primers specific to unique DNA sequences flanking the SSR sequence. The PCR-amplified products can be separated in high-resolution electrophoresis systems (e.g. AGE and PAGE) and the bands can be visually recorded by fluorescent labelling or silver-staining.

The advantages of SSR markers include that they can be readily analyzed by PCR and easily detected by PAGE or AGE. SSR markers can be multiplexed, have high throughput genotyping and can be automated. SSR assays require only very small DNA samples (~100 ng per individual) and low start-up costs for manual assay methods. However, SSR technique requires nucleotide information

for primer design, labour-intensive marker development process and high start-up costs for automated detections. Since 1990s, SSR markers have been extensively used in constructing genetic linkage maps, QTL mapping, marker-assisted selection and germplasm analysis in plants. In many species, plenty of breeder-friendly SSR markers have been developed and are available for breeders (Song *et al.*, 2010).

2.4 SCAR markers

SCAR (Sequence Characterized Amplified Region) is PCR-based monolocus codominant marker, it is fast, reliable, less sensitive to reaction conditions and easy to conduct in any laboratory. It can be carried out using unknown genomic DNA from any developmental stage and body part. Consequently, SCAR markers, once developed, offer a practical method for screening numerous samples, accurately at one time thus, adding to the cost efficiency of the experiment (McDermott *et al.*, 1994).

2.5 ISSR markers

Inter simple sequence repeat (ISSR) technique is a PCR based method, which involves amplification of DNA segment present at an amplifiable distance in between two identical microsatellite repeat regions, oriented in opposite direction. The technique uses microsatellites, usually 16–25 bp long, as primers in a single primer PCR reaction, targeting multiple genomic loci to amplify mainly the inter- SSR sequences of different sizes.

The microsatellite repeats used as primers can be di-nucleotide, tri-nucleotide, tetranucleotide or penta-nucleotide. The primers used can be either unanchored (Gupta *et al.*, 1994; Meyer *et al.*, 1993; Wu *et al.*, 1994) or more usually anchored at 3' or 5' end with 1 to 4 degenerate bases extended into the flanking sequences (Zietkiewicz *et al.*, 1994).

ISSRs have high reproducibility, possibly due to the use of longer primers (16–25 mers) as compared to RAPD primers (10- mers) which permits the subsequent use of high annealing temperature (45–60 °C) leading to higher stringency. ISSRs segregate mostly as dominant markers, following the simple Mendelian inheritance (Gupta *et al.*, 1994; Tsumura *et al.*, 1996; Ratnaparkhe *et al.*, 1998; Wang *et al.*, 1998). However, they have also been shown to segregate as co-dominant markers in some cases thus enabling distinction between homozygotes and heterozygotes (Wu *et al.*, 1994; Akagi *et al.*, 1996; Wang *et al.*, 1998; Sankar and Moore, 2001).

2.6 Male Sterility

Male sterility is defined as an absence or disfunction of pollen grains in a plant or incapability of plants to produce or release functional pollen grains (Kaul, 1988).

The use of male sterility in hybrid seed production has a great importance as it eliminates the need of mechanical emasculation. Naturally occurring genetically male sterile plants in hermaphrodite species generally maintain fully normal female functions. The phenotypic manifestations of male sterility are very diverse from the complete absence of male organs, the failure to develop normal sporogenous tissues (no meiosis), the abortion of pollen at any step of its development, the absence of stamens dehiscence or the inability of mature pollen to germinate on compatible stigma. Although there exists multiple causes for pollen abortion, cytoplasmic male sterility, which is maternally inherited is most common in nature (Budar and Pelletier, 2001).

2.6.1 Mechanism of cytoplasmic male sterility

Cytoplasmic male sterility is a natural trait. It is a form of male sterility induced by the complementary action of nuclear and mitochondrial genes. Specific mutations in mitochondrial DNA (maternally inherited) are responsible for developing a disfunction in the respiratory metabolism which results in severe

deleterious phenotypes including stunting, striping and female sterility, in addition to male sterility (Chase, 2007). While in some instances, CMS phenotypes result from homeotic changes i.e. male reproductive organs are converted to petals, or to female reproductive organs, in other instances, they result from the degeneration of stamens or specific stamen components such as the anther or the tapetal cells, which line the anther and support pollen development within the anther (Cilier *et al.*, 2004).

This results in an abnormal development program of male gamete production (Budar *et al.*, 2003). Therefore, no pollen or non-viable pollen is produced by the plant. However, female fertility is not affected by CMS used in the breeding process, and male-sterile plants can set seeds if viable pollen is provided.

CMS was first discovered in onion and exploited for the production of hybrids (Jones and Clarke, 1943) since then CMS trait has been observed in over 150 plant species (Laser and Lersten, 1972) and has been characterized in many crop species, including maize, onion, petunia, rice, sorghum, melon, watermelon, ridge gourd, chilli, cabbage, sunflower, wheat and pigeon pea (Kuck and Wrick, 1995). Apart from their agronomic importance in hybrid seed production, CMS systems serve as an excellent model for studying nuclear-mitochondrial gene interactions because fertility restoration relies on nuclear genes that suppress cytoplasmic disfunctions.

2.6.2 Molecular mechanisms of male sterility in different crops

Detailed molecular analyses of a number of CMS systems in various plant species have revealed that male sterility is associated with the expression of novel, chimeric open reading frames (ORFs) encoded by mitochondrial genome. The chimeric ORFs differ among the CMS systems, but often carry recognizable segments of essential mitochondrial gene coding or flanking sequences (Hanson and Bentolila, 2004). ORFs encode novel proteins, which interfere in the expression of normal gene. A common feature shared by several of the CMS-

associated genes is the fact that they encode for proteins containing large hydrophilic domains (Schnable and Wise, 1998).

2.6.2.1 Maize

For the first time, Rhoades (1933) described CMS in Maize. Three major sources of cytoplasm for male sterility have been identified in maize which are T (Texas), C (Charrua) and S (USDA). These sources are differentiated by the genetics of fertility restoration and other molecular and biochemical features (Duvick, 1965; Laughnan and Laughnan, 1983).

2.6.2.2 Sorghum

The phenomenon of pollen sterility was first reported independently by Ayyangar and Ponnaiya (1937) in India and Stephens (1937) in America. In sorghum various male sterile cytoplasms have been identified based on mt-DNA differences (Xu *et al.*, 1995). Only two among those, *9E* and *A3* are known to be associated with unusual ORFs. CMS *9E* cytoplasm shows alteration in mitochondrial gene *coxI* (Bailey *et al.*, 1986). This altered *coxI* has been implicated in causing male sterility (Dixon and Leaver, 1982).

A chimeric gene *orf107* predicts a protein carrying N- terminal region related to the N terminal of *atp9* (Tang *et al.*, 1999). Sorghum *orf107* consists of sequences identical to *atp9* of sorghum (23 residues) and a stretch of 28 codons identical to that of the CMS-associated rice *orf79* (Iwabuchi *et al.*, 1993; Akagi *et al.*, 1994).

2.6.2.3. Rice

The first report of cytoplasmic male sterility in rice was documented in by Weeraratne *et al.* (1954).

There are three CMS/RF systems, which are named CMS-BT, CMS-WA, and CMS-HLD. In BT-type CMS, the cytoplasm derived from the rice line,

Chinsurah BoroII causes male sterility when combined with the nucleus from the rice line Taichung 65 that carries no restorer gene (Shinjyo, 1969). Wild-Abortive CMS (WA-CMS) system is derived from the common wild species *Oryza rufipogon* Griff, which is applied most often for hybrid rice production (Lin and Yuan, 1980). The CMS-HL line of rice was developed by the repeated backcrossing of a red-awned wild rice (*Oryza rufipogon*) from Hainan Island (Liu *et al.*, 2004).

IR58025A is the most widely used Cytoplasmic Male Sterile line (CMS) in hybrid rice production and contains WA type cytoplasm.

2.6.2.4 Wheat

Cytoplasmic male sterility in wheat was discovered by Kihara (1951) and Fukasawa (1953). It was adapted for use with bread wheats by Wilson and Ross (1962).

In wheat, several types of CMS such as T (*Triticum timopheevii*), K (*Aegilops kotschyi*), V (*Aegilops ventricosa*), and D2 (*Aegilops crassa*) have been identified (Murai, 2002; Chen, 2003). A chimeric open reading frame, *orf256*, is associated with the CMS in *T. aestivum* carrying the *T. timopheevi* cytoplasm (Rathburn and Hedgcoth, 1991). The *orf256* consisted of sequences duplicated from N terminal coding sequences of *coxI* and sequences of unknown origin. A 7 kD protein encoded by *orf256* was present in the inner mitochondrial membrane of CMS line but is absent in either parental or restorer lines (Song and Hedgcloth, 1994; Hedgcoth *et al.*, 2002).

2.6.2.5 Common Bean

CMS in common bean, derived from the cytoplasm of the line G08063 has several unusual features and is associated with the unique 3.7 kb sequence known as *Phaseolus vulgaris* sterility sequence (*pvs*) (Johns *et al.*, 1992).

There are two restorers for the CMS, one leads to stoichiometric shift but not the other. The *pvs* region includes two open reading frames *orf239* and *orf98*, that were predicted to encode two polypeptides of 26.7 kD and 109 kD, respectively (Abad *et al.*, 1995). Only the protein product of *orf239* was found to accumulate in the reproductive tissues of CMS plants (Abad *et al.*, 1995; Sarria *et al.*, 1998). The role of *orf239* in causing male sterility in common bean was confirmed when tobacco plants expressing the gene from a nuclear transgene were found to be male sterile (He *et al.*, 1996).

2.6.2.6 Sunflower

In sunflower more than 30 CMS sources are known. Interspecific cross of *Helianthus petiolaris* and *Helianthus annuus* (Leclerq, 1983) gave male sterile lines designated as PET-1.

A 522 bp open reading frame (*orf522*) is created by 5 kb insertion in the downstream of the *atpA* gene and is co-transcribed with *atpA* gene in the CMS line (Kohler *et al.*, 1991; Laver *et al.*, 1991). This ORF encodes a 16 kD protein that accumulates in both sterile and restored PET-1 seedlings (Horn *et al.*, 1994; Moneger *et al.*, 1994).

2.6.2.7. Nicotiana

The CMS lines of tobacco had an alloplasmic or a novel transcript pattern indicating the role of nucleus in creating expression differences (Hakansson and Glimelius, 1991). A CMS derived from the cybrid involving *N. undulata* and *N. bigelovii* had a chimeric *atpA*. Analysis of *atpA* showed that loss of 3' part of *atp* α was correlated with restoration of fertility (Bergman *et al.*, 2000). Hakansson *et al.* (1988) found variant mitochondrial DNA and protein patterns in CMS lines of Nicotiana carrying *N. repanda, N. suaveolens* or *N. debney* cytoplasm.

2.6.2.8 Petunia

The CMS locus in Petunia was identified through the analysis of somatic hybrids between fertile and CMS lines (Boeshore *et al.*, 1983). The mitochondrial RFLP analysis of somatic hybrids containing recombinant mtDNA revealed a chimeric ORF. Petunia CMS was associated with a fused *pcf* that co-segregated with male sterility. The *pcf* is composed of portions of *atp9* and *coxII* coding regions and an unidentified reading frame termed *urfS* (Young and Hanson, 1987).

2.6.2.9 Pigeon pea

The first report on male-sterility in pigeon pea was published by Deshmukh (1959). Dundas *et al.* (1982) reported a male-sterile mutant within a photo-insensitive pigeon pea breeding line. Wanjari *et al.* (1999) recorded the first dominant gene in an inter-specific progeny that controlled male-sterility in pigeon pea.

2.6.2.10 Tomato

Male sterility in tomato was first described in 1915 (Crane, 1915). For the first time male sterility was used in tomato hybrid seed production by Rick (1945).

Structural male sterile mutants in tomatoes result from aberrations in genes that affect the structure of the stamens. In addition to the stamen phenotype, abnormalities in the carpel, petals and sepals are also common (Gorman and McCormick, 1997). Functional male sterile mutants produce viable pollens but are sterile for mechanical reasons. They either have indehiscent anthers, or have dehiscent anthers with morphological abnormalities that prevent pollen from reaching the stigma. Relatively few mutants displaying this type of sterility have been described in *Lycopersicon*, and three of them have been considered as useful

in breeding: positional sterile (*ps*), (Larson and Paur, 1948); positional sterile-2, (*ps2*), (Tronickova, 1962) and the exerted mutants, (Rick and Robinson, 1951).

2.6.2.11 Chilli

Male sterility in pepper was first documented by Martin and Grawford (1951) and somewhat later by Peterson (1958) has been utilized worldwide for the production of F_1 hybrids.

Experiments by Deng *et al.* (2012) had shown that the *orf456-2* had one nucleotide deleted in codon 150 and consists of a 507-bp single-coding exon which encodes 168 amino acid compared with the original sequence data of *orf456*. RT-PCR results showed that the *Orf507* was transcribed in buds and leaves at all developmental stages of CMS-9704A and a hybrid F1, while no band was detected in the maintainer line.

2.6.2.12 Cole crops

Genic male sterility has been reported in most of the cole vegetables (Cole, 1957; Nieuwhof, 1961; Borchers, 1966; Sampson, 1966a; Dickson, 1970). In Brussels sprout (Johnson, 1958; Nieuwhof, 1968b), cabbage (Nieuwhof, 1961), broccoli (Dickson, 1970) and cauliflower (Nieuwhof, 1961) recessive gene (ms) controlling male sterility has been reported. However, in broccoli (Duneman and Grunewaldt, 1987), cabbage (Fang *et al.*, 1997) and cauliflower (Crips and Tapsel, 1993) dominant genes controlling male sterility have been reported.

2.7 Male sterility in cucurbits

2.7.1 Watermelon

Male sterility in watermelon was first reported by Watts (1962) who found a male sterile mutant in the X2 generation of 'Sugar Baby', irradiated with gamma rays. The mutant was described as glabrous male sterile (*gms*) due to the associated lack of hairs on the plant foliage. It was further reported by that the

gms gene not only disrupts the male reproductive function, but also reduces female reproduction (Watts, 1967) and hence the commercial application of the *gms* gene was limited (Zang *et al.*, 1994).

Two spontaneous male sterile mutants were found in a self-pollinated population of a commercial cultivar 'Nongmei 100' in China in 1983. The Chinese male sterile mutation had inherited as a single recessive nuclear gene and assigned the gene symbol, *ms* or *ms-1*, *ms-1* produced plants with small, shrunken anthers and aborted pollen (Zang and Wang, 1990). A third type of male sterile mutant appeared simultaneously with dwarfism, and in the fourth, the dwarf gene was different from the three known dwarf genes which was named male sterile dwarf (*ms-dw*) by Huang *et al.* (1998).

2.7.2 Cucumber

Male sterility (MS) is of practical importance in cucumber (*Cucumis sativus* L.) breeding because it can facilitate F_1 hybrid seed production without hand pollination. Five MS forms are known: 1) gynoecious (*G*) flowering, 2) an apetalous sterile mutant (*ap*), 3) a pleiotropic pollen-aborted mutant (*ms-1*), 4) an aborted male flower type (*ms-2*) and 5) a closed-flower type.

The *G* character is under the control of several loci. Usually, *G* is considered to be a form of sex expression rather than MS because it is one of several sex segregates (Lower and Edwards, 1986; Pierce and Wehner, 1990). In *ap*, the corolla lacks staminate and pistillate flowers, and anthers become sepal-like (Grimbly, 1980). The recessive pleiotropic gene *ms-1* determines MS in which failure of staminate flower anthesis and pollen sterility (PS) varies from 30 to 90 per cent (Shifriss, 1954). Grimbly (1980) and Hutchins (1936) each reported that *ms-1* conditioned sterility in which staminate flowers are devoid of pollen, and fertility of pistillate flowers is decreased. In rare instances of *ms-2*, when the flowers matured to anthesis, only rudimentary anthers that contained no pollen were present but usually, male flower aborts (Barnes, 1960; Miller and Quisenberry, 1978; Whelan, 1974).

Except for *G*, these forms of MS have not been used in hybrid cucumber seed production because their inheritance is determined by nuclear genes and because they are associated with undesirable traits such as missing corolla, malformed ovary, and closed female flowers (Grimbly, 1980; Hutchins, 1936). In cucumber, gene for gynoecy has been characterized using AFLP markers (Lou *et al.*, 2005) and the sequences are available at public domain NCBI (Lou and Chen, 2004).

2.7.3 Muskmelon

GMS was reported in melon by Bohn and Whitaker (1949) and exploited by Foster (1968). Nandpuri *et al.* (1982) were the pioneer for its commercial utilization in India.

Five single recessive genes for male sterility including *ms-1* to *ms-5* have been identified in melon. Each of these have unique phenotype (Bohn and Principe, 1964; Bohn and Whitaker, 1949; Lecouviour *et al.*, 1990; McCreight and Elmstrom, 1984; Pitrat, 1991, 2002). The *ms*-1 and *ms*-2 are difficult to detect whereas *ms-3* gene is easily detectable with naked eye (McCreight 1984). The *ms-4* and *ms-5* are easily detectable due to male flower abortion at the bud stage (Lecuviour *et al.*, 1990). No allelism was found between different male sterile genes (Bohn and Principe, 1964; Lecuviour *et al.*, 1990; McCreight and Elmstrom 1984).

These results were confirmed by Pitrat (1991) who reported that these genes are located on five different linkage groups (LGs) of the classical melon map.

2.8 Molecular markers for characterizing male sterility

2.8.1 Maize

Molecular markers like RFLPs and PCR-based markers that are designed upon the unique characteristics of chimeric mtDNA regions can be used for

distinguishing the main maize CMS types, much more rapidly than by the traditional test-crossing procedure (Nakajima *et al.*, 1999; Sato, 1998).

Multiplex PCR assay was shown to be a quick and a reliable method and combining three primer pairs in a single reaction makes it convenient for analysis of a huge number of samples (Liu *et al.*, 2002.). Identification of sterile cytoplasm in maize was done using specific mtDNA primers by Dragena *et al.* (2006). This assay revealed 398, 440 and 799 bp specific DNA fragments identifying C, T and S cytoplasm respectively.

2.8.2 Rice

Molecular characterization of different cytoplasmic male sterile lines using mitochondrial DNA specific markers in rice was done by Khera *et al.* (2012). Dagang, (2006) studied thermosensitive genic male sterility by mapping male sterile gene tms5with EST and SSR markers. *Rf3* locus in rice was mapped using SSR and CAPS markers by Alavi (2009).

2.8.3 Wheat

Cao *et al.* (2009) reported the alternate allele at *Xwmc617* of 228bp for male sterile line T5-S5-109 and mapped the marker *Xwmc617* to the distal position of chromosome arm 4DS, just 1cM away from the male sterile *Ms2* gene concurrent with the *Rht-d1c* gene responsible for dwarfism. Validation of male sterile, fertility restorer and hybrid lines in wheat (*Triticum aestivum* L.) with linked SSR markers studied by Prakash (2012).

The SSR marker *Xgwm413* was identified to be closely linked to the male sterile gene and was found to be linked to yellow rust resistance genes in earlier reports (Peng, 1999; Ma *et al.*, 2001).

2.8.4 Sunflower

RFLP analysis revealed that PET-1 cytoplasm differs from normal fertile cytoplasm within a 17 kb region of mitochondrial genome. The rearranged region includes a 12 kb inversion and a 5 kb insertion flanked by 261 bp inverted repeats (Horn, 1994). Chen *et al.* (2006) done molecular mapping of a nuclear male-sterility gene in sunflower (*Helianthus annuus* L.) using TRAP and SSR markers.

2.8.5 Safflower

Development of SCAR markers linked to male sterility and very high linoleic acid content in safflower was done by Hamdan *et al.* (2008). In which they have developed molecular markers for the closely linked genes *Li*, controlling very high linoleic acid content and male sterility.

2.8.6 Tomato

Staniaszek *et al.* (2010) created CAPS marker designated as C4-301000 which is linked to *ps-2* gene responsible f functional in tomato and it can be useful for selection of male sterile tomato plants. This marker was developed based on a conserved ortholog set II (*COSII*). sequence C2_At3g20020 located on tomato chromosome 4.

2.8.7 Chilli

Bartoszewski *et al.* (2012) done mapping of the *ms8* male sterility gene in sweet pepper (*Capsicum annuum* L.) on the chromosome P4 using PCR-based markers useful for breeding programmes In this study, F2 population resulting from a cross between the sweet pepper male sterile line 320 and the male fertile variety Elf was used to identify DNA markers linked to the *ms8* locus.

With the use of RAPD–BSA technique, seven markers linked to the *ms8* locus were found. Four of them were converted into SCAR markers. In addition, two COSII/CAPS markers linked to the *ms8* locus were identified. Comparative

mapping with reference pepper maps indicated that the ms8 locus is located on the lower arm of the pepper chromosome P4.

In coloured sweet pepper CAPS marker linked to ms gene of unknown origin was identified (Lee *et al.*, 2010). In chili pepper a codominant SCAR marker linked to the genic male sterility gene *ms1* and three AFLP markers linked to the *ms3* gene were found (Lee *et al.* 2010). Study by Ji *et al.* (2014) was aimed to develop a new CMS-specific SCAR, for reliable identification of S-cytoplasm in pepper, while the new and three previous molecular markers were used to determine the cytoplasm types of pepper lines. Based on mitochondrial genome sequence related amplified polymorphism (SRAP) analysis of the CMS lines and the maintainer lines, SCAR was developed from a 10-bp deletion at the SRAP primer binding site in the CMS line (130 bp) compared with that in the maintainer line (140 bp) which indicated that factors other than *orf507* may exist for the regulation of male sterility in pepper.

2.8.8 Cole crops

Various molecular markers that are linked to the restorer genes for Ogu, Pol and Tour CMS in Brassica have been identified (Hansen *et al.* 1997; Jean *et al.*, 1997; Janeja *et al.*, 2003). Miao *et al.* (2000) identified four sequence-tagged site (STS) markers that are tightly linked to a recessive male-sterile gene in Chinese cabbage. Zhang *et al.* (2008) identified a random amplified polymorphic DNA (RAPD) marker that is tightly linked to a dominant male sterile gene and converted it into a sequence characterized amplified region(SCAR) marker.

Wang *et al.* (2005); Ke *et al.* (2004); Yi *et al.* (2006); Hong *et al.* (2006); Lei *et al.* (2007) and Huang *et al.* (2007) identified a set of molecular markers that are linked to the male sterile gene in *Brassica napus*. Wang *et al.* (2005) identified 12 amplified fragment length polymorphism (AFLP) markers, a SCAR marker and an extended random primer amplified region (ERPAR) marker that are closely linked to a dominant male-sterile gene in *Brassica oleracea*, and mapped the gene to linkage group 09, corresponding to chromosome 3 of *B. oleracea*.

2.9 Male sterility in ridge gourd

Male sterility was first reported in ridge gourd in India by Deshpande *et al.* (1979) which has not been cogitated for crop improvement programmes.

An offtype plant was detected in a population of ridge gourd which was characterized by the production of rudimentary male flowers in racemes. The anthers of the suspected male sterile line were compared with fertile plants Haritham, which showed a marked difference with respect to the appearance of anther lobes, the microspores of the suspected male sterile line were shrunken, small and sterile. Nodal cuttings of the identified male sterile line were maintained under *in vitro* conditions with the standardized protocol for the *in vitro* multiplication of male sterile mutant in ridge gourd (Pradeepkumar *et al.*, 2007). This CMS line in ridge gourd can be extensively exploited in commercially hybrid seed production (Pradeepkumar *et al.*, 2010). Inheritance of male sterility and presence of dominant fertility restorer gene in ridge gourd was studied by Pradeepkumar *et al.* (2010).

Male sterile (MS) ridge gourd mutant regenerated through *in vitro* culture were crossed with five pollen parents. Analysis of F3 families validated that two dominant fertility restorer genes (*Rf1* and *Rf2*) either in homozygous dominant or heterozygous dominant condition restores the male fertility in presence of sterile cytoplasm. All three way crosses *viz.*, (MS×Deepthi) × ArkaSumeet, (MS×IC-92685)× Arka Sumeet, (MS×IC-92671)× Arka Sumeet and (MS×CO.2)×Arka Sumeet regained fertility indicating the presence of dominant fertility restorer genes in Arka Sumeet (Pradeepkumar *et al.*, 2012). This was the first report of cytoplasmically controlled male sterility (CMS) in cucurbit where two dominant male fertility restorer nuclear genes with complementary gene action governing the restoration of male fertility.

2.10 Molecular marker analysis in *Luffa*

Molecular studies in ridge gourd are limited. The ISSR conditions in the genus *Luffa* were optimized by Ren *et al.* (2008) and Chen *et al.* (2011). Sikdar *et al.* (2010) used isozyme, RAPD and ISSR markers to estimate the genetic relation among the members of the cucurbitaceae family, including *Luffa*. Liu *et al.* (2010) used SSR and SRAP markers to estimate the genetic distances among the different *Luffa* accessions.

2. 11 Male sterile plants by genetic engineering

Recently it has been demonstrated in tobacco and rape (*B. napus*) that male sterile plants can be produced by introducing chimeric ribonuclease gene. In these transgenic plants the chimeric RNase gene is expressed within the anther. Selective destruction of the tapetal cell layer, preventing pollen formation resulted in the male sterility (Mariani *et al.*, 1990). This was achieved by crossing the male sterile plants with transgenic male fertile plants carrying chimeric tapetal cell specific RNase inhibitor gene. F_1 progeny expressed genes, RNase and RNase inhibitor, and were found to be restored to pollen fertility by suppression of cytotoxic RNase activity in the anther by formation of cell specific RNase-RNase inhibitor complexes (Mariani *et al.*, 1992).

Transgenic tobacco (*Nicotiana tabaccum*) plants, generated by genetic engineering methods expressing the *rolC* gene of the T-DNA of *Agrobacterium rhizogenes* are shown to be male sterile (Schmulling *et al.*, 1988). Over expression of the *rolC* gene has also been shown to cause male sterility in other transgenic plants (Fladung, 1990). The male sterility induced by *rolC* gene is of nuclear type and shows dominant Mendelian inheritance. Recently, restoration of fertility in *rolC* induced male sterility in transgenic tobacco plants by introduction of *rolC*-antisense constructs has been demonstrated (Schmulling *et al.*, 1993).

2.12 Conclusion

Molecular markers are identified as effective tools to detect the gene of interest (Reddy, 2002). The DNA markers are sequences showing polymorphism between two individuals or Species. SSRs and ISSRs are proven useful in differentiating the male sterility and fertility in pearl millet (Kumar *et al.*, 2006; Yadav *et al.*, 2007), sunflower (Chen *et al.*, 2006), wheat (Li *et al.*, 2005).

Marker-trait association identification will play an important role in plant marker assisted selection/quantitative trait loci (MAS/QTL) breeding programs, especially in plants that no other genetic information such as linkage map and quantitative trait loci are available (Ruan, 2010). In the present study polymorphism between male sterile and male fertile line was identified using SSR, SCAR and ISSR to formulate molecular marker linked with male sterility in ridge gourd. This will help hybrid seed production and reduce selection cycle time.

Materials and methods

3. MATERIALS AND METHODS

The study on "Molecular characterization of male sterility in ridge gourd *Luffa acutangula* (L.) Roxb." was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, KAU during the period 2012-2014. The materials used and methodologies adopted are discussed in this chapter.

3.1 Materials

3.1.1 Plant Materials

Haritham (male sterile) line identified at KAU was used and three male fertile lines Haritham (fertile), Deepthi (fertile) and Arka Sumeet (fertile) were also used. Seeds of Haritham (fertile) and Deepthi (fertile) were collected from Kerala Agricultural University and Arka Sumeet (fertile) from Indian Institute of Horticulture Research, Bangalore. The leaf samples of Haritham (male sterile line) were obtained from Department of Olericulture, College of Horticulture, Kerala Agricultural University.

Plants for three male fertile lines were maintained at CPBMB, College of Horticulture and flower features were recorded. Data regarding Haritham (male sterile) was received from Department of Olericututre, Kerala Agricutural Uniersity, where it was maintained.

3.1.2. Laboratory chemicals and glass wares

The chemicals used in the study were obtained from Merck India Ltd. and HIMEDIA. The *Taq* DNA *polymerase*, dNTPs, Taq buffer and molecular markers (λDNA/*Hind*III+*Eco*RI double digest) (100bp) were obtained from Genei Ltd. Bangalore. The plastic wares were obtained from Tarsons India Ltd. SSR, SCAR and ISSR primers, were synthesized and supplied by Sigma Aldrich Chemicals Pvt. Ltd.

3.1.3. Equipments

Centrifugation was done in high speed refrigerated centrifuge (KUBOTA 6500). NanoDrop^{R} ND-1000 spectrophotometer was used for the estimation of quality and quantity of DNA. The DNA amplification was carried out in Thermal cycler from Agilent Technologies and Applied Biosystems. Agarose gel electrophoresis was done in horizontal gel electrophoresis from BIORAD, USA. Polyacrylamide gel electrophoresis was done in PAGE unit from BIORAD. Gel documentation was done in GelDoc unit (BIORAD).

3.2. Methods

Molecular analysis of the three fertile ridge gourd lines Haritham (fertile), Deepthi (fertile), and Arka Sumeet (fertile) and Haritham (male sterile) was carried out with three different marker systems- SSR (Simple Sequence Repeats), SCAR (Sequence Characterized Ampilfied Regions), ISSR (Inter Simple Sequence Repeats).

3.2.1 Total Genomic DNA extraction

Total Genomic DNA extraction was done by using CTAB method developed by Rogers and Bendich (1994).

Reagents

I. CTAB buffer (2X)

II. TE buffer

III. 1 per cent PVP

IV. Chloroform: isoamyl alcohol (24:1) v/v

V. Isopropanol

VI. Ethanol 70% and 100%

VII. Sterile distilled water obtained from Millipore unit

3.2.1.1 Procedure for the extraction of total genomic DNA

- One gram of cleaned leaf tissue was ground in a pre-chilled mortar and pestle in the presence of liquid nitrogen, 50 µl of β-mercaptoethanol and a pinch of PVP, until a smooth paste was obtained.
- 4 ml of extraction buffer (2 per cent CTAB) was added.
- The homogenized samples were transferred into an autoclaved 50 ml oak ridge tube and 3ml of pre-warmed extraction buffer was added (total 7 ml).
- Mixed well and incubated the mixture at 65°C for 45 min with occasional mixing by gentle inversions.
- Equal volume (7 ml) of chloroform: isoamyl alcohol (24:1) was added and the contents were mixed by inversions to emulsify.
- The contents were centrifuged at 10,000 rpm for 15 min at 4°C. After centrifugation, the contents got separated into three distinct phases. Aqueous top most layer containing DNA and RNA, interphase with fine particles and proteins and lower layer having chloroform, pigments and cell debris.
- The top aqueous layer was transferred to a clean centrifuge tube and equal volume of chloroform: isoamyl alcohol (24:1) was again added and the contents were mixed by inversion.
- Second centrifugation was done at 10,000 rpm for 10 min at 4°C.
- The aqueous phase was transferred into a clean centrifuge tube and 0.6 volume of chilled isopropanol was added and mixed by quick gentle inversion till the DNA got precipitated.
- Complete precipitation was obtained by keeping at -20°C for one hour.
- Contents were centrifuged at 8,000 rpm for 5 min at 4°C and the supernatant was gently poured off.

- DNA pellet was washed with 70% ethanol.
- Spun for 1 min at 8,000 rpm and the ethanol was decanted.
- The pellet was air dried, dissolved in 50 µl sterilized water and stored at -20°C.
- The samples were loaded on 0.8 per cent agarose gel and the quantity and quality of DNA was confirmed.

3.2.1.2. Assessing the quality and quantity of DNA using spectrophotometer

The purity of DNA was further checked using NanoDrop ND-1000 spectrophometer. Nucleic acid shows absorption maxima at 260nm whereas proteins show peak absorbance at 280nm. Absorbance has been recorded at both wavelength and the purity was indicated by the ratio OD_{260}/OD_{280}. The values between 1.8 and 2.0 indicate that the DNA is pure and free from proteins. The quantity of DNA in the pure sample was calculated using the relation 1 OD_{260} equivalent to 50ng double stranded DNA/µl sample.

1OD at 260 nm = 50 ng DNA/µl

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

Procedure for Nanodrop:

- The Nanodrop spectrophotometer was connected to the computer and the operating software ND-100 was opened.
- Option Nucleic acid was selected.
- With the sampling arm open, 1µl distilled water was pipetted on to the lower measurement pedestal.
- The sampling arm was closed and a spectral measurement was initiated using the operating software on the computer. The sample column has

been automatically drawn between the upper and lower measurement pedestals and the spectral measurement was made.

- The reading was set to zero with blank.
- 1µl sample was pipetted onto measurement pedestal and selected 'Measure'.
- When the measurement was completed, the sampling arm was opened and the sample was wiped from both upper and lower pedestals, using a soft laboratory wipe. Simple wiping was done to prevent sample carryover in successive measurements, for the samples varying by more than 1000 fold in concentration.

3.2.1.3 Purification of DNA

The DNA which had RNA as a contaminant, was purified by RNAse treatment and subsequently precipitated.

Reagents

- Chloroform: isoamyl alcohol (24:1)
- Chilled isopropanol
- 70 % ethanol
- TE buffer
- 1:9 diluted RNAse solution (1µl of RNAse (Sigma, USA) was mixed with 9 µl sterile distilled water and the solution was stored at -20^{0}C).

Procedure

- 250 µl of sterile distilled water was added to 50 µl DNA sample, to make up the volume up to 300 µl. To this, 6 µl RNAse solution was added and the contents were incubated at 37^{0}C in dry bath for 1 hr with occasional mixing.

- After the incubation equal volume of chloroform: isoamyl alcohol (24: 1) mixture was added.
- The contents were gently mixed and subsequently centrifuged at 10,000 rpm for 10 min at 4^0C.
- The aqueous phase was transferred into a fresh centrifuge tube and equal volume of chloroform: isoamyl alcohol (24: 1) was added, the contents were gently mixed and subsequently centrifuged at 10,000 rpm for 10 min at 4^0C.
- The aqueous phase was transferred into a clean centrifuge tube and 0.6 volume of chilled isopropanol was added. Quick but gentle mixing was done by inversions till the DNA got precipitated.
- Sample was kept at -20°C for one hour for complete precipitation.
- After incubation, centrifuged at 8,000 rpm for 5 min at 4^0C.
- DNA pellet was washed with 70 % ethanol and centrifuged for 1 min at 8,000 rpm and the ethanol was decanted.
- The pellet was air dried, dissolved in 50 µl sterilized water and stored at -20°C.
- The samples were loaded on 0.8 per cent agarose and the quantity and quality of DNA was verified.

3.2.1.4. Electrophoresis of DNA

Reagents and Equipments

1. Agarose

   0.8 per cent (for genomic DNA)

2. 50X TAE buffer (pH 8.0)

3. 6X Gel loading dye

4. Ethidium bromide

5. Electrophoresis unit, power pack, gel casting tray and combs

Procedure for agarose gel electrophoresis

- The gel tray was prepared by sealing the ends with tape. Comb was placed in gel tray about 1 inch from one end of the tray and positioned the comb vertically such that the teeth are about 1 to 2 mm above the surface of the tray.

- Prepared 0.8 per cent agarose (0.8 g in 100 ml) in a conical flask with 100 µl 1X TAE buffer. It was heated for 60 seconds to dissolve the agarose.

- The solution was allowed to cool to 45 ^{0}C and 4 µl ethidium bromide was added immediately. After dissolving ethidium bromide the warm solution was poured into the tray to a depth of about 5 mm.

- The gel was allowed to solidify for about 30 to 45 min at room temperature.

- To run the gel, the comb and the tape used for sealing were gently removed, the gel was placed in electrophoresis chamber along with the tray. Electrophoresis buffer (the same buffer used to prepare the agarose) was poured to the chamber to submerge the gel (just until wells are covered).

- To the prepared samples for electrophoresis, added 1 µl of 6x gel loading dye for every 5µl of DNA solution. Mixed well and 6 µl DNA per well was loaded. A suitable molecular weight marker (λDNA *Eco*RI/ *Hind*III double digest) was also loaded in a well.

- DNA samples were electrophoresed at 80 V until dye has migrated two third the length of the gel.

- Intact DNA appeared as orange fluorescent bands. Degraded DNA appeared as a smear because of a large number of fragments, which differed in one or two bases only.

3.2.1.5 Gel Documentation

Gel documentation was done with BioRad Gel Documentation System, using Quantity One software. Quantity One is a software package for imaging, analyzing and data basing of electrophoresed gels. The software acquired images of gels displayed on computer screen.

Equipments

1. UV transilluminator (BIORAD)

2. Gel documentation and analysis system Gel DOC-XR ™

Procedure for Gel documentation

- Positioning the Gel: The Gel Doc XR camera was set at Live/Focus mode. While focussing the position of the gel was carefully kept within the frame.
- Selection of Illumination Mode: Selected the type and scale of GelDoc XR data, using the Image Mode option buttons.
- Acquiring the Image: Auto Expose was clicked. After the automatic exposure time has been reached, the exposure was fine tuned by entering slightly different times (in seconds) in the Exposure Time field. Alternatively, Manual Expose was also clicked to enter an exposure time directly. Once the image was acquired click on 'Freeze' was clicked.
- Optimizing the Display: The Display controls were useful for quickly adjusting the appearance of image for output to a video printer.
- Analyzing the Image: The Analysis step of the Gel Doc XR acquisition window allowsed the addition of annotations and analyzing the newly acquired image.
- Selection of Output: The image was video printed and saved to a file.

3.3. Molecular Marker analysis

Three different types of markers were used for the study which included SSR (Simple Sequence Repeats), SCAR (Sequence Characterized Amplfied Regions) and ISSR (Inter Simple Sequences Repeats).

DNA amplification conditions

The PCR conditions required for effective amplifications in SSR, SCAR and ISSR analysis included appropriate proportion of the component of the reaction mixture. The reaction mixture included template DNA, Tàg buffer A or B, $MgCl_2$, *Taq* DNA *polymerase*, dNTPs and primers. The aliquot of this master mix were dispensed into 0.5ml or 0.2ml PCR tubes. The PCR was carried out in an Agilent Technologies thermal cycler. An important factor, which affected amplification rate was the temperature profile of thermal cycles. The thermocycler was programmed for desired duration and temperature.

3.3.1. SSR and SCAR analysis

The good quality genomic DNA (40ng/µl) isolated from ridge gourd leaf samples were subjected to SSR and SCAR assay, as per the procedure. SSR and SCAR primers supplied by Sigma Aldrich, USA with good resolving power were used for amplification of DNA. SSR and SCAR primers for the assay were selected after an initial screening.

3.3.1.1 SSR and SCAR PCR reaction mixture

a) Genomic DNA (40ng/µl) - 2.0 µl

b) 10X Taq assay buffer A - 2.0 µl

c) dNTP mix (2.5mm) - 1.5 µl

d) *Taq* DNA *Polymerase* (3U/m) - 0.4 µl

e) Forward Primer (0.1µM/ml) - 0.75µl

f) Reverse Primer (0.1µM/ml) - 0.75µl

g) Autoclaved Distilled Water	-	12.6µl
Total volume	-	20.0µl

3.3.1.2 The PCR programme

94^0C for 4 minutes-		Initial denaturation	
94^0C for 45 seconds	-	Denaturation	35 cycles
43^0C to 55^0C for 1 minutes	-	Primer annealing	
72^0C for 2 minutes	-	Primer extension	
72^0C for 8 minutes	-	Final extension	

4^0C for infinity to hold the sample

3.3.1.3 SSR and SCAR primers used for the study

3.3.1.3.1 Selection of SSR primers

Due to lack of molecular work in ridge gourd SSR primers from related species like watermelon, cucumber, muskmelon were selected.

Watermelon primers

36 SSRs were developed from BAC library constructed by Jarret *et al.* (1996) Joober *et al.* (2006) and reported SSR primers for water melon. Among which 6 primers were selected based on the high PIC value as watermelon belongs to the same family *i.e.* Cucurbitaceae (Table 3.1).

Cucumber primer

In cucumber, Identification of SSR marker linked to gynoecious loci in cucumber was done by Shengjun *et al.* (2013) (Table 3.2).

Muskmelon primers

SSR primers suggested by Diaz *et al.* (2011) and designed by Syngenta for analysis of muskmelon accessions having high PIC value were also used in the study (Table 3.3).

3.3.1.3.2 Selection of SCAR primers

Quon *et al.* (2007) developed SCAR marker using AFLP for locus controlling gynoecy reported primers (Table 3.4). Transcriptome profile analysis of floral sex determination in cucumber was done by Tao *et al.* (2010). Using RT-PCR, for the confirmation of the gene *CsACS1* 1 G three SCAR primers were designed, which were also used in the present study (Table 3.5).

Universal mitochondrial primers

Khera *et al.* (2012) reported universal mitochondrial SCAR primers (Table 3.6). As cytoplasmic male sterility is associated with mitochondrial disfunction universal mitochondrial primers were also used in the study.

Table 3.1 List of watermelon primers with highest PIC values (Jarret *et al.*, 1996; Joobeur *et al.*, 2006)

Sl. No	Primer	Nucleotide Sequence	Annealing temperature (°C)
1	MCPI- 14F	5'TCAAATCCAACCAAATATTGC3	56.1
	MCPI- 14R	5'GAGAAGGAAACATCACCAACG3'	58.4
2	MCPI- 33F	5'CGTCATTTGAGAGCATTGGA3'	58.8
	MCPI- 33R	5'TCCAATTTTGTTTAGTGACATAGAGT3'	60.7
3	C.I.1- 20F	5'CGCGCGTGAGGACCCTATA3'	63.3
	C.I.1- 20R	5'AGCAATTGATTGAGGCGGTTCT3'	62.7
4	C.I.1- 21F	5'ACCCTTTCGCTGCTGTTATTCA3'	59.6
	C.I.1- 21R	5'TGTCCCACCCAACATTTCATT3'	61
5	C.I.1- 61F	5'TTCTGCTCAGTTTCTTCCTAAT3'	53.6
	C.I.1- 61R	5'CATCCTCAAAAAAAAGGCTAAG3'	55.6
6	C.I.1- 140F	5'CTTTTTCTTCTGATTTGAACTGG3'	55.6
	C.I.1- 140R	5'ACTGTTTATCCCGACTTCACTA3'	54.1

Table 3.2 List of SSR primers reported in cucumber for gynoecy (Shengjun *et al.*, 2013)

Sl. No.	Primer	Nucleotide sequence	Annealing temperature (°C)
1	CSWCT25F	5'AAAGAAATTAAGTCAATCAAACCG3'	56.1
	CSWCT25R	5'CCCACCAATAGTAAAATTATACAT3'	51.7
2	SSR18956F	5'CGTATGTACGACAAAATGTGAACAG3'	59.1
	SSR18956R	5'TCGAAACCTCAATACTTCTACCAA3'	57.5

Table 3.3 List SSR primers for muskmelon with high PIC value (Diaz *et al.*, 2011)

Sl. No.	Primer	Nucleotide sequence	Annealing temperature (°C)
1	DE1805 F	5'GATTTGTTTTGGCTGGCCA 3'	59.9
	DE1805 R	5'GGAGACGACAGATGAGGAC 3'	54.8
2	DM0325 F	5'AGCTGAGTTTTGGTTGTTG 3'	52.9
	DM0325 R	5'AAGGAATCCCAAGAGAATG 3'	53.1
3	DE1507 F	5'CGATCGTTGCTAGGAGAAC 3'	55
	DE1507 R	5'TTGAGCACATCTCTTTCCC 3'	50.4
4	DE0144 F	5'TTAGCCAACAACAGTTTCC 3'	52.8
	DE0144 R	5'GAGAAACTTGATACAAAACTCG 3'	52.1
5	DM0168 F	5'GTGTAGGCCATGAAAATG 3'	51.3
	DM0168 R	5'TTCCTTCTCTTCCTTCATC 3'	50.8
6	DM1296 F	5'AACGCTCCAACAAAAACTC 3'	54.4
	DM1296 R	5'CACTTTAACTTGCAGCTGTG 3'	53.7
7	DM0933 F	5'AGTCCACTCCTCCAATAAAAC 3'	53.6
	DM0933 R	5'GGCCTTTAAATTGCCATAAC 3'	54.9
8	DM01320 F	5'TCGAAGTCCAGGAAGAGAG 3'	54.7
	DM01320 R	5'TAAAGAATGTGGAGAGCGG 3'	55.4

Table 3.4 List of SCAR primers developed for cucumber for gynoecy (Quon *et al.*, 2007)

Sl. No	Primer	Nucleotide sequence	Annealing temperature (°C)
1	SCARSA200a F	5'TGCAAAATGTCCTATGACTGGT3'	58.2
	SCARSA200a R	5'CACTATTTTGATCGATAATTATAC ATG3'	53.5

Table 3.5 List of SCAR primers for *CsACS* 1G gene reported by Tao *et al.* (2010) for flowering in cucumber

Sl. No.	Primer	Nucleotide sequence	Annealing temperature (°C)
1	CsACS 1G F1	5'TTTAACTACTTCGGACGGGCATAG3'	59.9
2	CsACS 1G F2	5'TACCTGCTCTGGTCGGAGACACT3'	63.3
3	CsACS 1G F3	5' GGGTCTTTTTTCCCATCTTCTC 3'	58.7
	CsACS 1G R	5'AGGTGTTCAGCAAACATAGGGTG 3'	61

Table 3.6 List of universal mitochondrial SCAR primers reported by Khera *et al.* (2012)

Sl. No.	Primer	Nucleotide sequence	Annealing Temperature (°C)
1	Nad4ex1 F	5' CAGTGGGTTGGTCTGGTATG 3'	54.3
	Nad4ex1 R	5' TCATATGGGCTACTGAGGAG 3'	51.3
2	Nad5D F	5' ATAAGTCAACTTCAAAGTGGA 3'	50.4
	Nad5D R	5' CATTGCAAAGGCATAATGAT 3'	52.4
3	Nad1 F	5'GCATTACGATCTGCAGCTCA 3'	55.6
	Nad1 R	5'GGAGCTCGATTAGTTTCTGC 3'	53.1
4	Nad4ex2 F	5'TGTTTCCCGAAGCGACACTT 3'	52
	Nad4ex2R	5'AACCAGTCCATGACTTAACA 3'	50.8
5	Rps14 F	5'CACGGGTCGCCCTCGTTCCG 3'	50.3
	Rps14 R	5'GTGTGGAGGATATAGGTTGT 3'	54

3.3.1.4 Agarose electrophoresis

The amplified products were electrophoresed using 2 per cent agarose with ethidium bromide staining. Gel run was done with 50X TAE and sizing was done using a marker (100-bp DNA ladder). The profile was visualized under UV (312 nm) transilluminator and documented for further analysis.

3.3.1.5 Composition and procedure for Native PAGE electrophoresis

The compostion of the PAGE gel is given below

% of Gel	30% Acrylamide (ml)	Distilled water (ml)	5X TBE (ml)	10% APS (µl)	TEMED (µl)
10	4.0	5.6	2.4	200	10

a. Procedure

1. The glass plates and spacers were thoroughly cleaned. The plates were rinsed with deionized water and ethanol and they were set aside to dry.
2. The glass plates were assembled with spacers in gel caster.
3. Ten per cent polyacrylamide gel solution was prepared according to the composition.
4. Sufficient care was taken to wear gloves and to work quickly after addition of TEMED to complete the gel before the acrylamide polymerizes.
5. The appropriate comb was immediately and carefully inserted into the gel, to avoid air bubbles getting trapped under the teeth.
6. The acrylamide was allowed to polymerize for 30-60 min at room temperature.

7. After polymerization is complete, gel was removed from gel caster, carefully cleaned to remove spilled gel from the back of the white plates.

8. Gel was inserted into gel electrophoresis unit and after adding running buffer, the combs were pulled carefully from the polymerized gel.

9. The DNA samples were mixed with the appropriate amount of gel loading buffer and the mixtures were loaded into the wells using a micropipette equipped with a drawn-out plastic tip.

10. The electrodes were connected to a power pack and the power was turned on to begin the electrophoresis run at 75 V. When electrophoresis was carried out at a higher voltage, differential heating in the center of the gel was observed bowing of the DNA bands and even melting of the strands of small DNA fragments.

11. The gel was run until the marker dyes have migrated to the desired distance. The electric power was turned off and the leads were disconnected.

12. The glass plates were detached to remove the gel which was subsequently stained with silver stain.

b. Silver staining

Fixer- Ethanol 250 ml and Acetic acid 12.5 ml

Stain- Silver nitrate 5 gm

Developer- Sodium hydroxide 37.5 gm and Formaldehyde 12.5 ml

Procedure

1. The gel was removed from plates and put in the staining tray.

2. Fixer was added and put the tray on the shaker for 5 min.

3. After 5 min the fixer was removed and stain was added and kept for 5 more min.

4. Gel was washed with distilled water to remove the stain
5. Developer was poured slowly and as soon as bands started to get visualize developer was removed.
6. Distilled water was added to remove the traces of developer and gel was observed on the white board and documented.

3.3.2. ISSR analysis

The good quality genomic DNA (35ng/µl) isolated from ridge gourd leaf samples was subjected to ISSR analysis using ISSR primers with good resolving power. The ISSR primers for assay were selected after an initial screening. List of ISSR used for screening are provided in Table 3.7

Table 3.7 List of ISSR primers screened for the study

Sl. No	Primer	Nucleotide Sequence
1	UBC 811	5'GAGAGAGAGAGAGAGAC3'
2	UBC 813	5'CTCTCTCTCTCTCTCTT3'
3	UBC 814	5'CTCTCTCTCTCTCTCTA3'
4	UBC 815	5'CTCTCTCTCTCTCTCTG3'
5	UBC 834	5'AGAGAGAGAGAGAGAGYT3'
6	UBC 835	5'AGAGAGAGAGAGAGAGYC3'
7	UBC 836	5'AGAGAGAGAGAGAGAGYA3'
8	UBC 840	5'GAGAGAGAGAGAGAGAYT3'
9	UBC 844	5'CTCTCTCTCTCTCTCTRC3'
10	UBC 890	5'VHVGTGTGTGTGTGTGT3'
11	UBC 866	5'CTCCTCCTCCTCCTCCTC3'
12	UBC 807	5'AGAGAGAGAGAGAGAGT3'
13	UBC 843	5'CTCTCTCTCTCTCTCTRA3'
14	UBC S2	5'CTCTCTCTCGTGTGTGTG3'
15	UBC 820	5'GTGTGTGTGTGTGTGTC3'
16	UBC 854	5'TCTCTCTCTCTCTCTCRG3'
17	UBC 845	5'CTCTCTCTCTCTCTCTRG3'
18	UBC 817	5'CACACACACACACACAA3'
19	UBC 826	5'ACACACACACACACACC3'
20	UBC 818	5'CACACACACACACACAG3'
21	Oligo ISSR 04	5'ACACACACACACACACC3'
22	ISSR 05	5'CTCTCTCTCTCTCTTG3'
23	ISSR 06	5'GAGAGAGAGAGAGAGAC3'
24	ISSR 7	5'CTCTCTCTCTCTCTTG3'

25	ISSR 08	5'GAGAGAGAGAGAGAGAT3'
26	ISSR 09	5'CTCTCTCTCTCTCTCTCG3'
27	ISSR 10	5'ACACACACACACACACG3'
28	ISSR 15	5'TCCTCCTCCTCCTCC3'
29	SPS 03	5'GACAGACAGACAGACA3'
30	SPS 08	5'GGAGGAGGAGGA3'
31	UBC 868	5'GAAGAAGAAGAAGAAGA3'
32	UBC 895	5'- AGAGTTGGTAGCTCTTGATC-3'
33	UBC 899	5'-CATGGTGTTGGTCATTGTTCCA -3'
34	UBC 880	5'- GGAGAGGAGAGGAGA-3'
35	UBC 892	5' TAGATCTGATATCTGAATTCCC-3'
36	UBC900	5'- ACTTCCCCACAGGTTAACACA-3'
37	UBC880	5'-GGAGAGGAGAGGAGA-3'
38	ISSR SY 88	5'CCACCACCACCA3'
39	UBC873	5'-GACAGACAGACAGACA-3'
40	ISSR5	5'GACACGACACGACACGACAC 3'
41	UBC834	5'AGAGAGAGAGAGAGAGYT3'
42	ISSR6	5'GGAGGAGGAGGA3'
43	UBC855	5'-ACACACACACACACACYT-3'
44	UBC841	5'-GAGAGAGAGAGAGAGAYC-3'
45	ISSR SY 83	5'CTGACTGACTGACTGA3'
46	C.Renetria 6	5'GTGTGTGTGTGTCC3'
47	ISSR SY 81	5'GTTGTTGTTGTTGTT3'
48	Sigma	5'-GAAGTGGGGAAGTGGG-3'
49	ISSR 2	5' ATTATTATTATTATTCAT3'

3.3.2.1 PCR reaction mixture

PCR amplification was performed in a thermal cycler in 20 µl reaction mixture and the composition of the reaction mixture consists of

a) Genomic DNA (35 ng/µl) - 2.0 µl
b) 10X Taq buffer B - 2.0 µl
c) $MgCl_2$ - 2.0µl
c) dNTP mix (10mM each) - 1.5 µl
d) Taq DNA polymerase (1U - 0.4 µl
e) Primer (10 pM) - 1.5 µl
f) Autoclaved distilled water - 10.6 µl

Total volume - 20.0 µl

3.3.2.2. The PCR programme

The PCR programme set for amplifying the DNA samples is as follow

94^0C for 4 min	- Initial denaturation	
94^0C for 45 seconds	- Denaturation	35 cycles
43^0C to 55^0C for 1 min	- Primer annealing	
72^0C for 2 min	- Primer extension	
72^0C for 8 min	- Final extension	
4^0C for infinity to hold the sample		

3.3.2.3 Gel electrophoresis and visualisation of PCR products

The amplified products were run on 2 per cent agarose gel using 1X TAE buffer stained with ethidium bromide. The profile was visualized under UV (312 nm) transilluminator and documented using gel documentation system Gel DOC-XR™ Imaging system UVP (USA). The documented ISSR profiles were carefully examined for polymorphism in banding pattern among the varieties. The size of polymorphic bands was recorded in comparison with the selected marker.

3.4 Scoring of bands and data analysis

Scoring of bands on agarose was done with the QuantityOne software (BioRad) in the Gel Doc imagination system, using 100 bp ladder as molecular weight size marker. Only distinct and well resolved fragments were scored, Jaccards coefficient of similarity was measured and a dendrogram based on similarity coefficient was generated using Unweighted pair Group Method with Arithmetic means (UPGMA). The resulting data was analysed using the software package NTSYS (Rohlf, 2005).

The unique band which is present in the male sterile line and absent in all the male fertile lines or the unique band which is absent in the male sterile line and present in all the male fertile lines was considered as a polymorphic band linked with male sterility in ridge gourd.

3.4 Elution of marker from PAGE and sequencing

Polymorphic bands were eluted from PAGE as well as agarose gels. For PAGE bands, elution was done using crush and soak method (Maxam and Gilbert, 1977).

1. Polymorphic bands were cut using a sharp razor blade.
2. The gel slice was placed on a glass plate and chopped into fine pieces with a razor blade and transferred the pieces to a small test tube and 1 volume of Taq buffer A was added.
3. The tube was incubated at 37°C overnight.
4. The sample was centrifuged at 10,000 rpm for 10 min. The supernatant was recovered carefully to avoid transfer of acrylamide fragments.
5. The supernatant was used for doing PCR to confirm that the eluted DNA contains only single genomic region.6. PCR products were directly sequenced on NGS through outsourcing.

Results

4. RESULTS

The study on "Molecular characterization of male sterility in ridge gourd. *Luffa acutangula* (L.) Roxb" with the objective to identify molecular markers linked with the male sterility in ridge gourd was carried out using SSR and ISSR markers.

The results of various aspects of the experiment are presented in this chapter.

4.1 Morphological observations

There were no observed differences with respect to vegetative characters in the Haritham (male sterile) line as compared with the other fertile lines, but there were differences in flower characters.

In Haritham (male sterile) rudimentary male flower buds were produced in racemes which failed to open. Anthers were poorly developed which produced shrunken, non viable pollen grains. In male fertile, Haritham (fertile), Deepthi (fertile) and Arka Sumeet (fertile) male flower buds were well developed, flowers were opened having well filled anthers with viable pollen grains (Plate 4.1 and 4.2). Details of flower observations in male fertile and Haritham (male sterile) line are provided in Table 4.1.

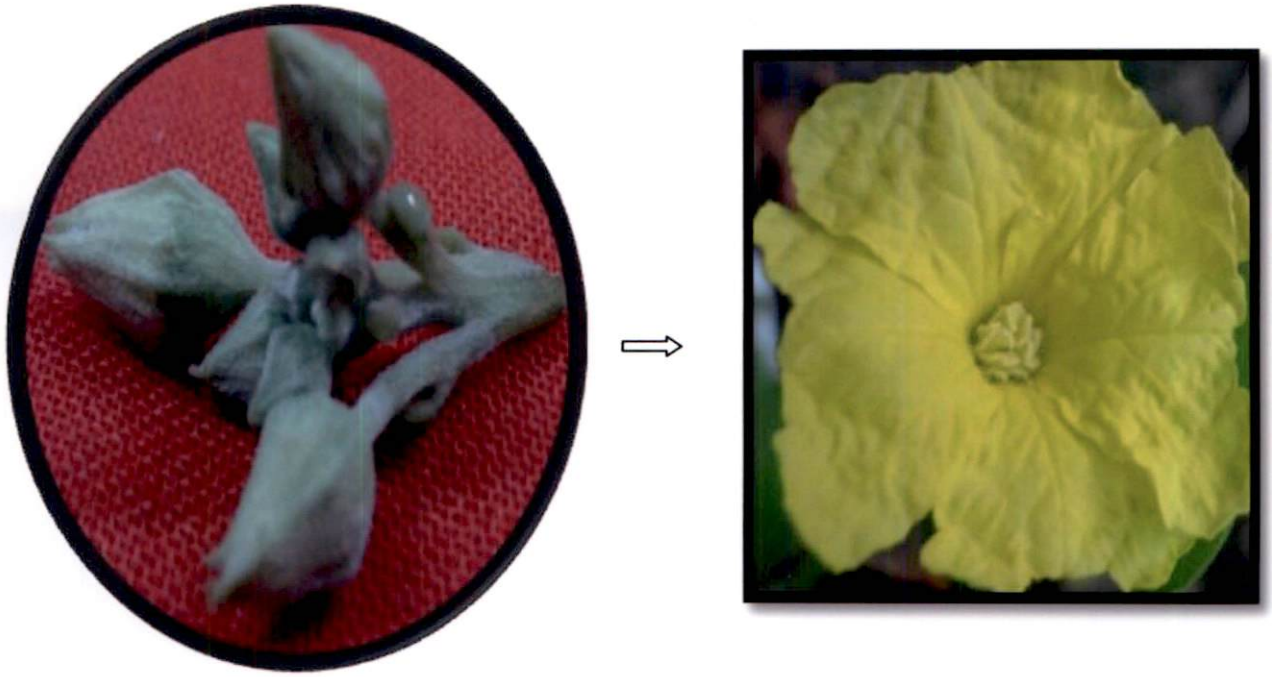
4.2 Total genomic DNA extraction

4.2.1 Source of DNA

Emerging pale green leaves of three male fertile lines *viz.*, Haritham (fertile), Deepthi (fertile), Arka Sumeet (fertile) and Haritham (male sterile) line were used to extract total genomic DNA using CTAB method reported by Rogers and Bendich (1994).

Male fertile plants **Haritham (male sterile)**

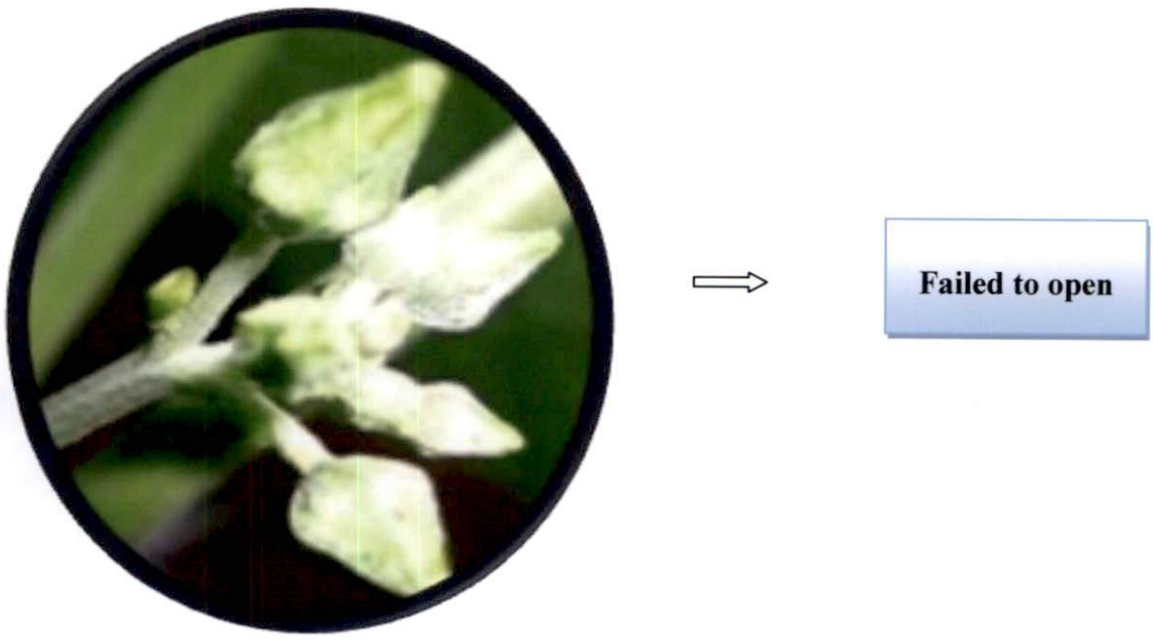
Plate 4.1- Male fertile plants and Haritham (male sterile) line



A

Flower bud

Opened flower



B

Flower bud

Plate 4.2- Comparison between male fertile and male sterile flowers A- Haritham (fertile), B- Haritham (male sterile)

C

Flower bud

Opened flower

D

Flower bud

Opened flower

Plate 4.2- Comparison between male fertile and male sterile flowers C- Deepthi (fertile), D- Arka Sumeet (fertile)

Table 4.1 Comparison between male fertile and male sterile flowers

Sl. No.	Variety/ Line	Flower buds	Flowers	Anthers	Pollen grains
1	Haritham (fertile)	Well developed	Opened	Well filled	Mature, viable
2	Deepthi (fertile)	Well developed	Opened	Well filled	Mature, viable
3	Arka Sumeet (fertile)	Well developed	Opened	Well filled	Mature, viable
4	Haritham (male sterile)	Rudimentary	Failed to open	Poorly developed	Shrunken, non- viable

Table 4.2 Quality and quantity of isolated total genomic DNA using NanoDrop spectrophotometer

Sl. No	Variety/line	UV absorbance at 260 nm (A_{260})	UV Absorbance at 280 nm (A_{280})	$A_{260}/_{280}$	Quantity (ng/µl)
1	Haritham (fertile)	4.956	2.588	1.82	247.81
2	Deepthi (fertile)	9.661	5.102	1.89	483.05
3	Arka Sumeet (fertile)	15.918	8.584	1.85	795.92
4	Haritham (male sterile)	9.584	4.914	1.87	478.99

4.2.2 Quality and quantity of DNA

The quality and quantity of DNA isolated was determined by agarose gel electrophoresis and NanoDrop ND-1000 spectrophotometer (Table 4.2). Intact, clear bands were observed on agarose gel which indicated good quality DNA without degradation (Plate 4.3).

Spectrophotometer method had shown that the ratio of UV absorbance ranged between 1.8 to 1.89 which indicated the purity of DNA *i.e.* the DNA is pure and free from proteins and RNA. The quantity of DNA in the pure sample was calculated using the relation 1 OD_{260} equivalent to 50µg double stranded DNA/ml sample. Therefore OD_{260} X 50 gave the quantity of DNA in ng/µl. RNA contamination was removed by RNAse treatment.

4.3 Molecular marker analysis

SSR, SCAR and ISSR marker systems were used in this study. The protocols were standardised. The PCR conditions required for effective amplification of DNA in SSR, SCAR, ISSR analysis includes appropriate proportions of the components of the reaction mixture. The reaction mixture included template DNA, Taq buffer A or B, $MgCl_2$, *Taq* DNA *polymerase,* dNTPs and primers. Another important factor, which affected the amplification rate was the temperature profile of thermal cycles.

The thermal cycler was programmed for desired number of cycles and temperatures of denaturation, annealing and polymerization steps. PCR products were analysed on 10 per cent native PAGE for SSR and SCAR and 2 per cent agarose gel for ISSR.

4.3.1 SSR and SCAR analysis

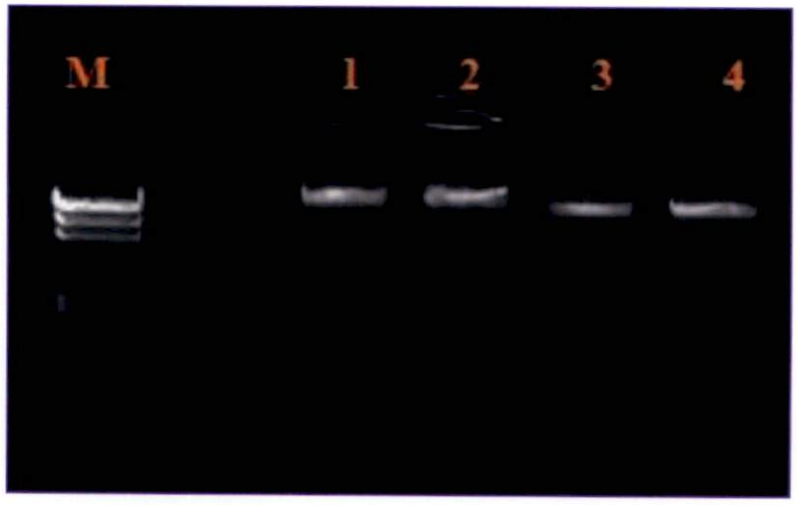
Microsatellites, also known as simple sequence repeats (SSRs) are repeating sequences of 2-5 base pairs of DNA. Due to lack of molecular research in ridge gourd, SSR and SCAR primers reported in related crops, watermelon, muskmelon and cucumber were used. Certain studies have revealed that male

M: Molecular weight marker λ DNA (*Eco*RI/ *Hind* III digest)

1-Haritham (fertile) 2-Deepthi (fertile) 3-Arka Sumeet (fertile)

4- Haritham (male sterile)

A. Before RNase treatment



M: Molecular weight marker λ DNA (*Eco*RI/ *Hind* III digest)

1-Haritham (fertile) 2-Deepthi (fertile) 3-Arka Sumeet (fertile)

4- Haritham (male sterile)

B. After RNase treatment

Plate 4.3- Isolation of total genomic DNA from tender leaves of ridge gourd (Rogers and Bendich method)

sterility is associated with the expression of novel, chimeric open reading frames (*orfs*) encoded by mitochondrial genome (Schnable and Wise 1998; Hanson and Bentolila, 2004) and hence universal mitochondrial SCAR primers were also used for the study.

Intially 2 per cent agarose gel was used for separating SSR and SCAR fragments but as primers are very much crop specific, most of the primers showed no amplification hence to resolve even small fragments, native PAGE was used.

Polyacrylamide gel electrophoresis (PAGE) offered high resolution of low-molecular-weight nucleic acids. In particular, small DNA fragments (20-1000 base pairs) that are poorly resolved by ordinary agarose gels are easily separated on polyacrylamide gels.

4.3.1.1 Details of the results obtained with SSR primers

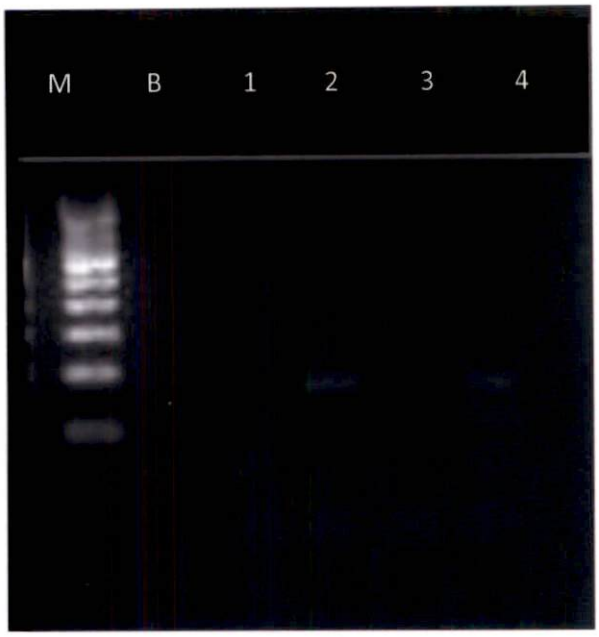
4.3.1.1.1 DE 0325

Total genomic DNA of four ridge gourd lines, Haritham (fertile), Deepthi (fertile), Arka Sumeet (fertile) and Haritham (male sterile) line were amplified with DE0325. DE0325 primer gave 8 bands in Haritham (fertile), 12 with Deepthi (fertile), 14 in Arka Sumeet (fertile), 8 in Haritham (male sterile), on PAGE. Among all the lines, 6 polymorphic bands were observed. Polymorphism in relation to male sterility was absent. Amplification pattern is as shown in Plate 4.4.1.a.

4.3.1.1.2 DE1805

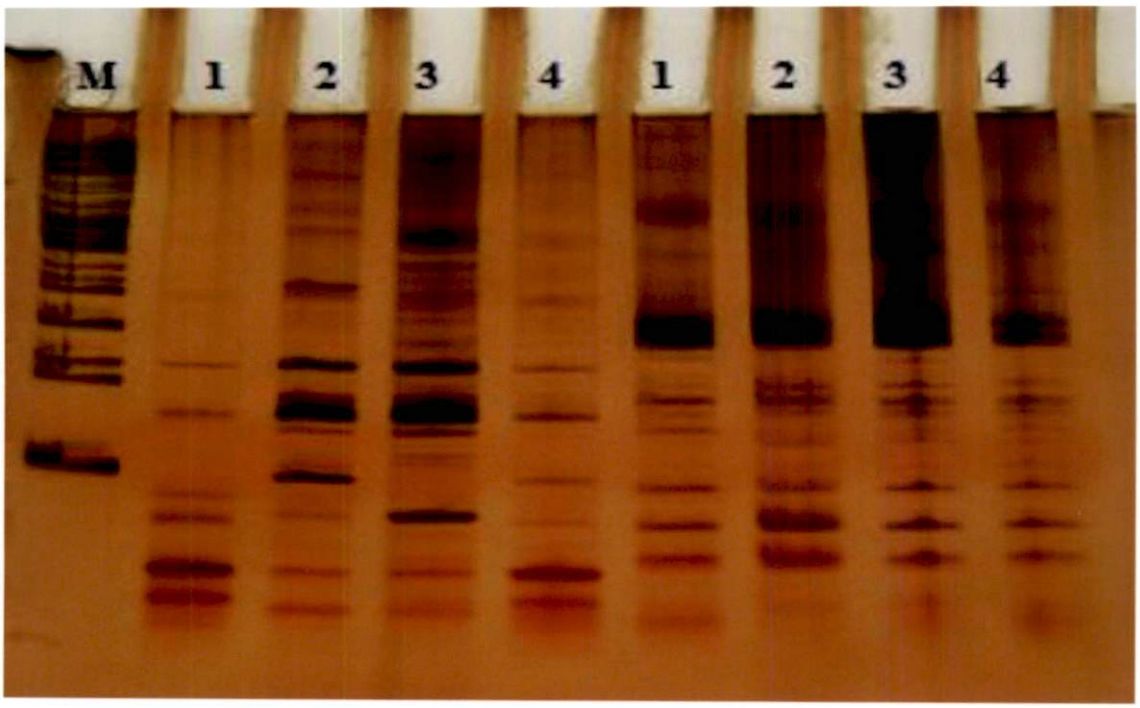
Total genomic DNA of four ridge gourd lines was amplified with DE1805. Eleven monomorphic bands were observed with the primer in all the lines. As all amplified bands were monomorphic no polymorphism for male sterility was observed. Amplification pattern is shown in Plate 4.4.1.b.

Agarose profile



a. DE0325 **b. DE1805**

PAGE profile



a. DE0325 **b. DE1805**

M- 100 bp ladder (100bp-3kb), B- Blank 1- Haritham (fertile), 2- Deepthi (fertile), 3- Arka Sumeet (fertile), 4- Haritham (male sterile)

Plate 4.4.1- The amplification pattern of DNA of three male fertile and one male sterile line of ridge gourd using SSR primers DE0325 and DE1805

4.3.1.1.3 CI1-21

Total genomic DNA of four ridge gourd lines, Haritham (fertile), Deepthi (fertile), Arka Sumeet (fertile) and Haritham (male sterile) were amplified with CI1-21. The amplification gave 2 bands in Haritham (fertile), 7 bands in Deepthi (fertile), 5 in Arka Sumeet (fertile) and 2 in Haritham (male sterile). The bands in male fertile and male sterile lines were compared and it was found that 1 band is present in Haritham (male sterile) line which is absent in all fertile lines. The size of the band was 125bp. Amplification pattern is as shown in Plate 4.4.2.a.

4.3.1.1.4 DE0144

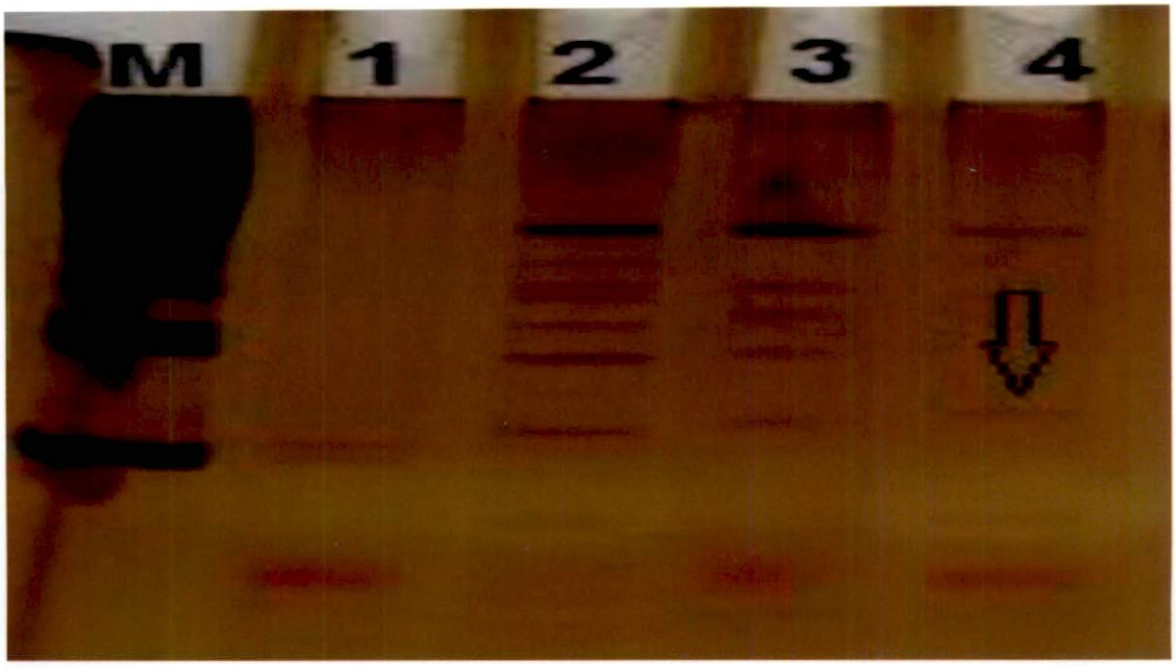
Total genomic DNA of all the four ridge gourd lines were amplified with DE0144. On amplification, it gave 1 band in Arka Sumeet (fertile) and 1 in Haritham (male sterile) line, at different size. The band in Haritham (male sterile) line was absent in all fertile lines. The size of the band was 50bp. Amplification pattern is as shown in Plate 4.4.2.b.

4.3.1.1.5 CsWCT 25

Total genomic DNA of four ridge gourd lines, Haritham (fertile), Deepthi (fertile), Arka Sumeet (fertile) and Haritham (male sterile) were amplified with CsWCT25. It gave 1 band in Haritham (fertile), 6 bands in Deepthi (fertile), 9 bands in Arka Sumeet (fertile) and 4 bands in Haritham (male sterile). A band of 350bp was observed in Haritham (Male sterile) line which is polymorphic for male sterility as it is present only in male streilie line. Amplification pattern is as shown in Plate 4.4.3.a

4.3.1.1.6 SSR18956

Total genomic DNA of four ridge gourd lines, Haritham (fertile), Deepthi (fertile), Arka Sumeet (fertile) and Haritham (male sterile) were amplified with SSR18956. On amplification, SSR18956 gave 2 bands in Haritham (fertile), 4 in Deepthi (fertile), 6 in Arka Sumeet (fertile) and 2 in Haritham (male sterile). Polymorphism has been observed between four lines but there was no

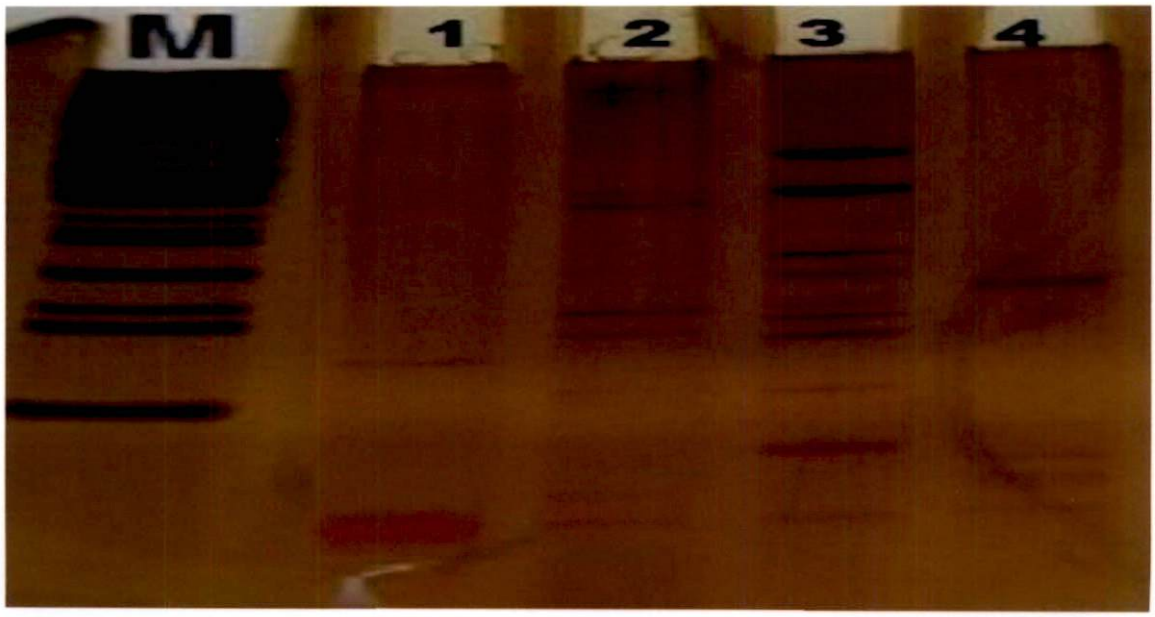


a.CI1-21

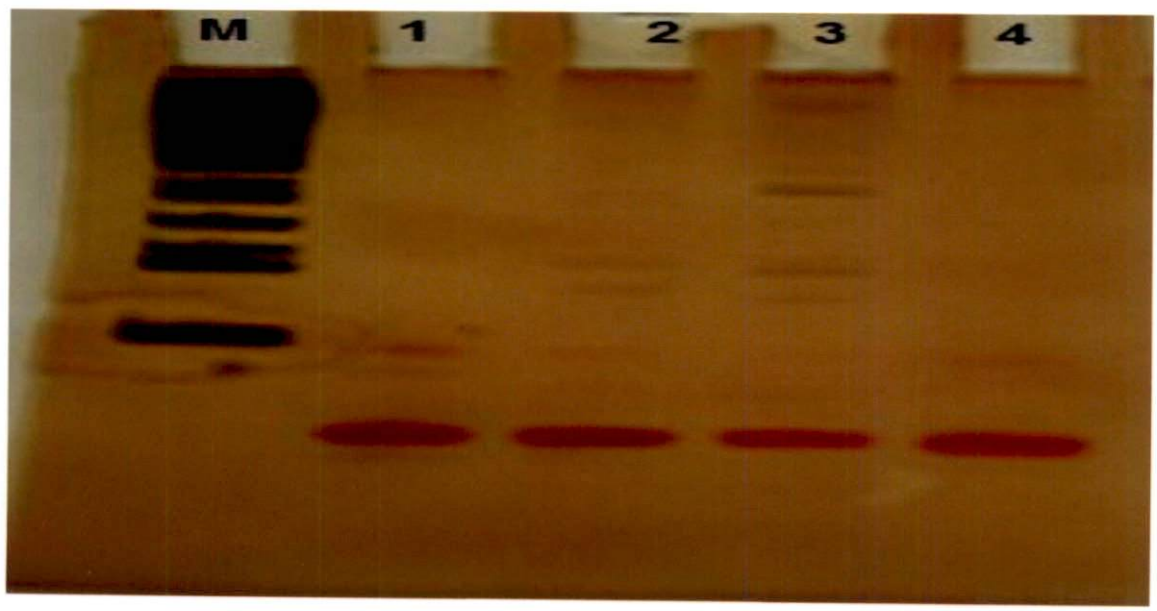
b- DE0144

M- 100 bp ladder (100bp-3kb), 1- Haritham (fertile), 2- Deepthi (fertile), 3- Arka Sumeet (fertile), 4- Haritham (male sterile)

Plate 4.4.2- The amplification pattern of DNA of three male fertile and one male sterile line of ridge gourd using SSR primers CI1-21 and DE0144



a.CsWCT25



b. SSR18956

M- 100 bp ladder (100bp-3kb), 1- Haritham (fertile), 2- Deepthi (fertile), 3- Arka Sumeet (fertile), 4- Haritham (male sterile)

Plate 4.4.3- The amplification pattern of DNA of three male fertile and one male sterile line of ridge gourd using SSR primers CsWCT25 and SSR18956

polymorphism for male sterility. Amplification pattern is as shown in Plate 4.4.3.b.

4.3.1.1.7 DM01320

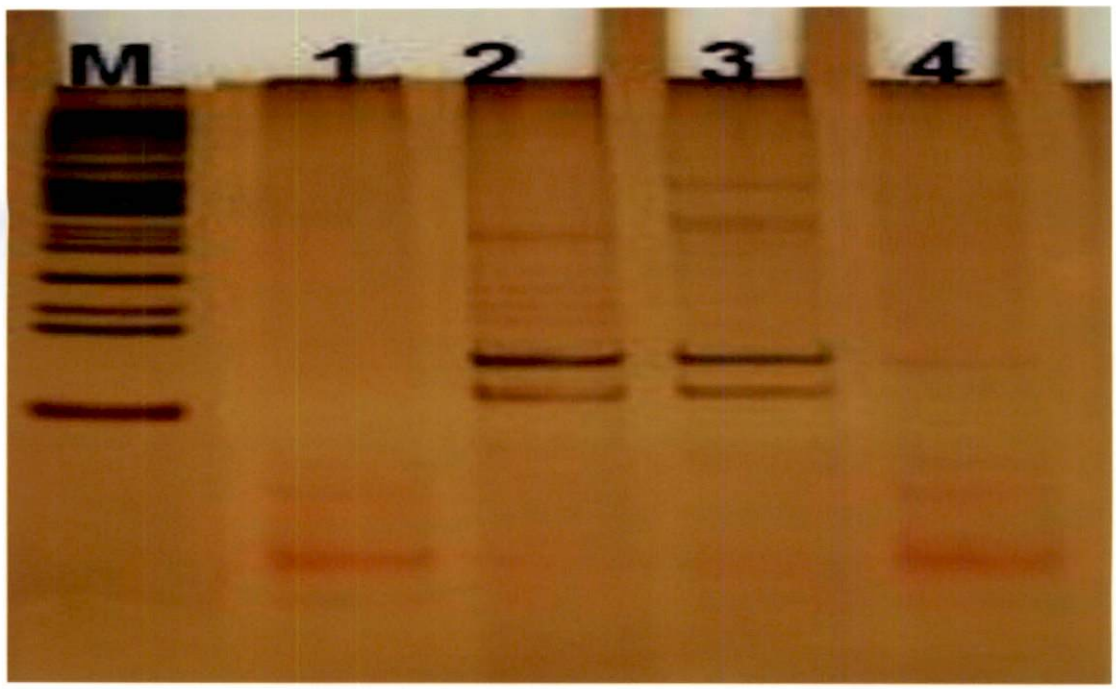
Total genomic DNA of four ridge gourd lines, Haritham (fertile), Deepthi (fertile), Arka Sumeet (fertile) and Haritham (male sterile) line was amplified with DM01320. Fifteen bands were observed in Haritham (fertile), Deepthi (fertile), Arka Sumeet (fertile) and Haritham (male sterile). No polymorphic band for male sterility was observed. Amplification pattern is as shown in Plate 4.4.4.a.

4.3.1.1.8 DM0168

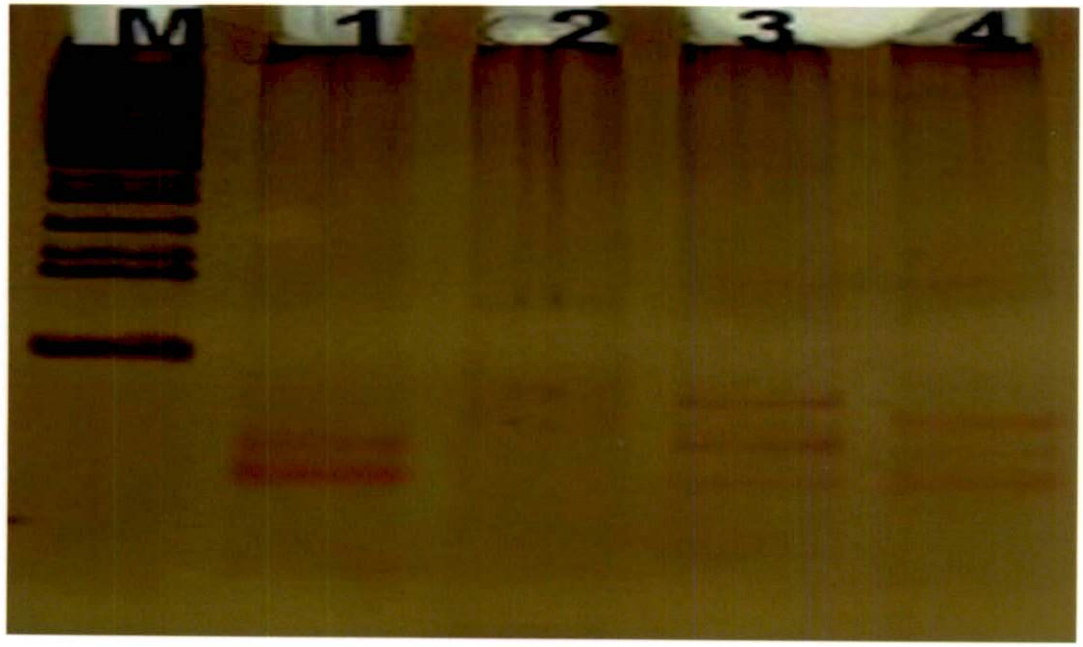
Total genomic DNA of four ridge gourd lines, Haritham (fertile), Deepthi (fertile), Arka Sumeet (fertile) and Haritham (male sterile) were amplified with DM0168 which gave 1 band in Haritham (fertile), 6 bands in Deepthi (fertile), 5 bands in Arka Sumeet (fertile) and 2 bands in Haritham (male sterile) line. Though polymorphic bands were observed between four lines, polymorphism for male sterility was absent. Amplification pattern is as shown in Plate 4.4.4.b.

4.3.1.1.9 DM1296

Total genomic DNA of four ridge gourd lines, Haritham (fertile), Deepthi (fertile), Arka Sumeet (fertile) and Haritham (male sterile) were amplified with the primer DM1296. Three bands in Haritham (fertile), 6 in Deepthi (fertile), 5 in Arka Sumeet (fertile) and 2 in Haritham (male sterile) were observed but no polymorphism for male sterility was detected. Amplification pattern is as shown in Plate 4.4.5.a



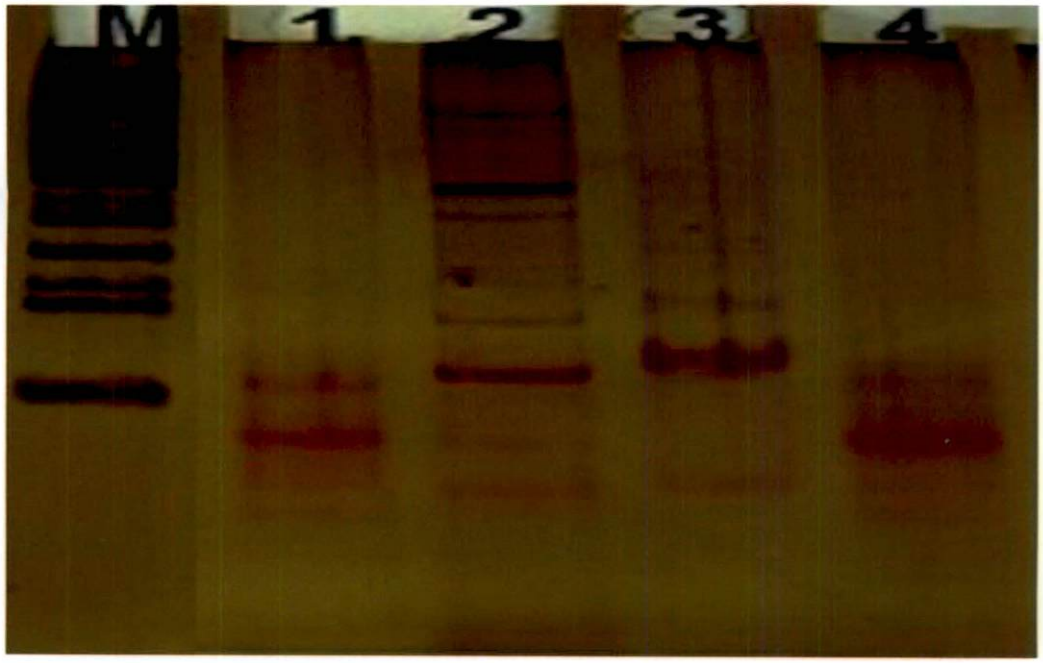
a- DM1320



b- DM 168

M- 100 bp ladder (100bp-3kb), 1- Haritham (fertile), 2- Deepthi (fertile), 3- Arka Sumeet (fertile), 4- Haritham (male sterile)

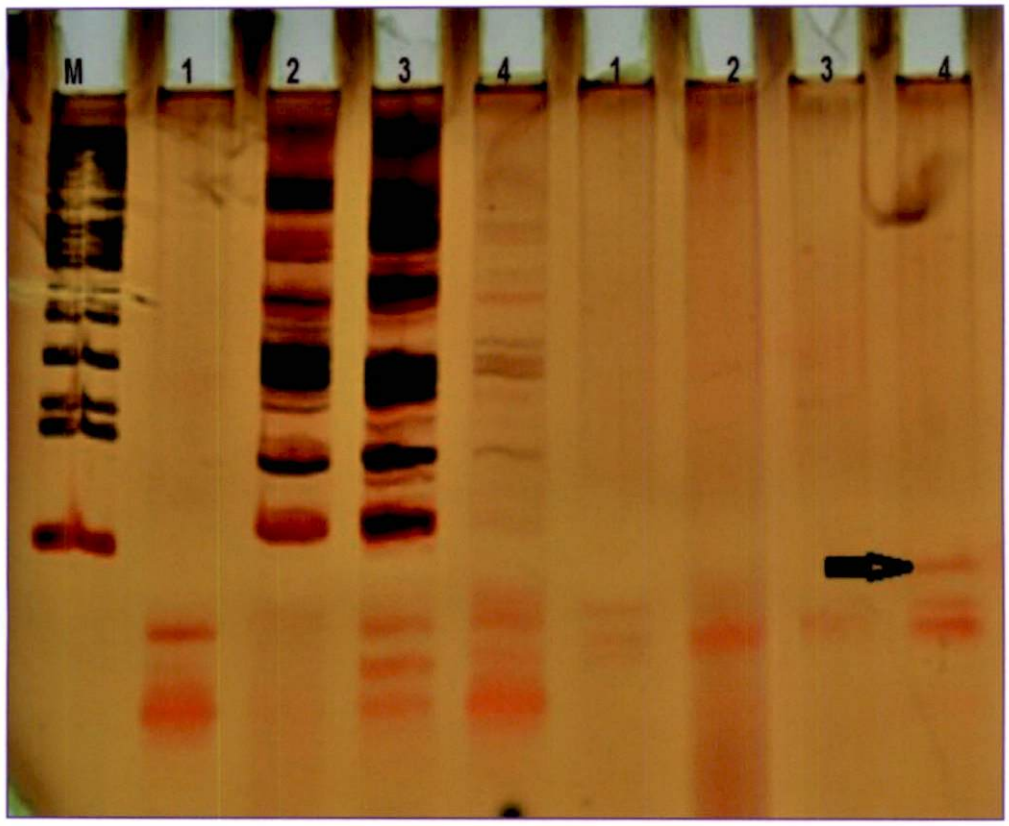
Plate 4.4.4- The amplification pattern of DNA of three male fertile and one male sterile line of ridge gourd using SSR primers DM1320 and DM168



a- DM1296

M- 100 bp ladder (100bp-3kb), 1- Haritham (fertile), 2- Deepthi (fertile), 3- Arka Sumeet (fertile), 4- Haritham (male sterile)

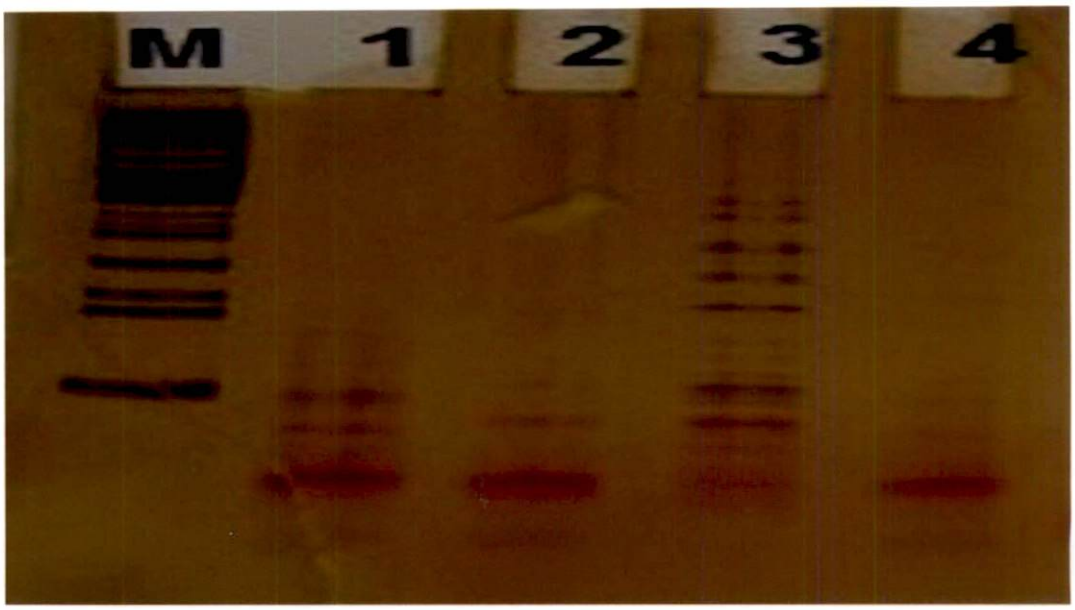
Plate 4.4.5 - The amplification pattern of DNA of three male fertile and one male sterile line of ridge gourd using SSR primer DM1296



a- Nad4ex2 **b- Rps14**

M- 100 bp ladder (100bp-3kb), 1- Haritham (fertile), 2- Deepthi (fertile), 3- Arka Sumeet (fertile), 4- Haritham (male sterile)

Plate 4.5.1- The amplification pattern of DNA of three male fertile and one male sterile line of ridge gourd using SCAR primers Nad4ex2 and Rps14



a. CsACS 1G F1

b. CsACSG F3

M- 100 bp ladder (100bp-3kb) B- Blank 1- Haritham (fertile), 2- Deepthi (fertile), 3- Arka Sumeet (fertile), 4- Haritham (male sterile)

Plate 4.5.2- The amplification pattern of DNA of three male fertile and one male sterile line of ridge gourd using SCAR primers CsACS 1G F1 and CsACS 1G F3

4.3.1.2 Details of the results obtained with SCAR primers

4.3.1.2.1 Nad4ex2

Nad4ex2 was amplified with total genomic DNA of all the four ridge gourd lines. Three bands in Haritham (fertile), 13 in Deepthi (fertile), 15 in Arka Sumeet (fertile) and 9 in Haritham (male sterile) were observed. In spite of polymorphic bands observed between these lines, polymorphism for male sterility was absent. Amplification pattern is as shown in Plate 4.5.1.a.

4.3.1.2.2 Rps14

Total genomic DNA of four ridge gourd lines was amplified with Rps14, a mitochondrial primer. Two bands were observed in Haritham (fertile), 1 each in Deepthi (fertile) and Arka Sumeet (fertile) and 3 in Haritham (male sterile). Out of 3 bands, 1 band at 80bp size was observed only in Haritham (male sterile) line. Amplification pattern is shown in Plate 4.5.1.b.

4.3.1.2.3 CsACS1G F1

Total genomic DNA of four ridge gourd lines, Haritham (fertile), Deepthi (fertile), Arka Sumeet (fertile) and Haritham (male sterile) line was amplified with CsACS 1G F1. Three bands were observed in Haritham (fertile), 2 in Deepthi (fertile), 12 in Arka summet (fertile) and 2 in Haritham (male sterile). Polymorphism for male sterility was not observed when all the male fertile lines were compared with Haritham (male sterile) line. Amplification pattern is shown in Plate 4.5.2.a.

4.3.1.2.4 CsACS1G F3

Total genomic DNA of four ridge gourd lines, Haritham (fertile), Deepthi (fertile), Arka Sumeet (fertile) and Haritham (male sterile) line was amplified with CsACS1G F3. Five bands in Haritham (fertile), 11 in Deepthi (fertile), 15 in Arka Sumeet (fertile) and 8 bands were observed in Haritham (male sterile) line were

observed. No polymorphism for male sterility was observed. Amplification pattern is as shown in Plate 4.5.2.b.

4.3.1.2.5 SCARSA200a

Total genomic DNA of four ridge gourd lines, Haritham (fertile), Deepthi (fertile), Arka Sumeet (fertile) and Haritham (male sterile) line was amplified with SCAR SA200a. It produced 4 bands in Haritham (fertile), 2 bands in Deepthi (fertile), 5 bands in Arka Sumeet (fertile) and only 1 in Haritham (male sterile). Observed polymorphism was further checked for male sterility but no polymorphism was found. Amplification pattern is as shown in Plate 4.5.3.a.

4.3.2 ISSR assay

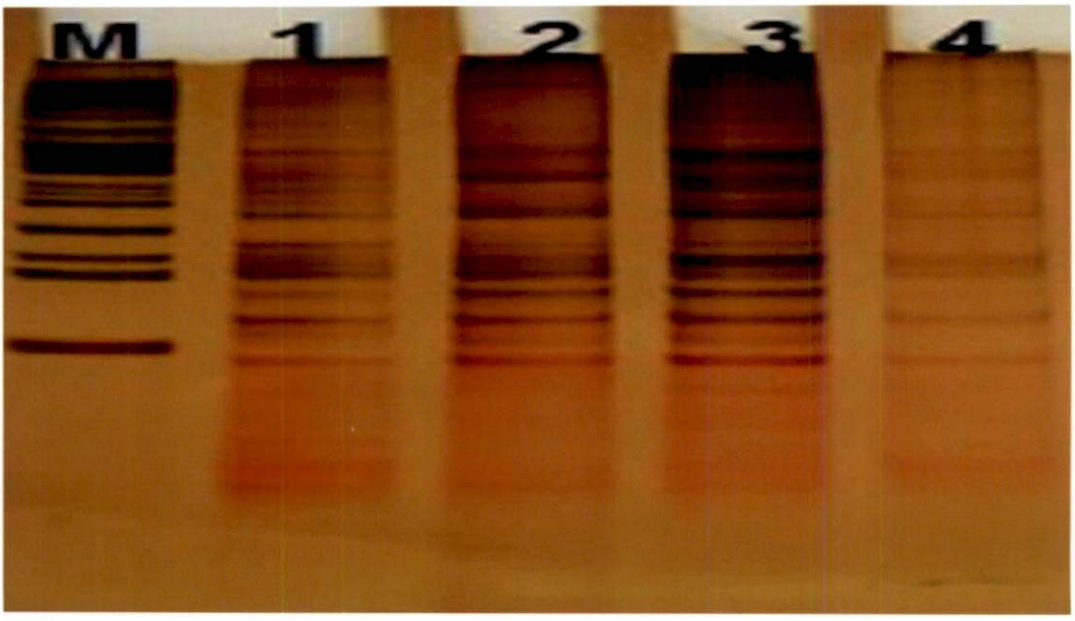
4.3.2.1 Standardization of PCR conditions

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

4.3.2.2. Screening of primers for ISSR analysis

Forty nine primers belonging to series UBC, ISSR, C. Reneteria were tested for amplification of genomic DNA. Based on the amplification pattern, 16 out of 49 primers were selected for ISSR assay of four ridge gourd accessions

Selected primers are provided in Table 4.3.



a. SCARSA200a

M- 100 bp ladder (100bp-3kb) B- Blank 1- Haritham (fertile), 2- Deepthi (fertile), 3- Arka Sumeet (fertile), 4- Haritham (male sterile)

Plate 4.5.3- The amplification pattern of DNA of three male fertile and one male sterile line of ridge gourd using SCAR primer SCARSA200a

Table 4.3 List of ISSR primers selected for molecular characterization

Sl. No.	Primer	Nucleotide Sequence	Annealing Temperature (^{0}C)
1	ISSR 15	5'TCCTCCTCCTCCTCC3'	47.4
2	UBC 880	5'-GGAGAGGAGAGGAGA-3'	44.1
3	ISSR SY 88	5'CCACCACCACCA3'	40.5
4	UBC 873	5'-GACAGACAGACAGACA-3'	40.1
5	UBC 834	5'AGAGAGAGAGAGAGAGYT3'	41.8
6	ISSR 05	5'GGAGGAGGAGGA3'	41.5
7	Oligo ISSR 04	5'ACACACACACACACACC3'	42
8	UBC 855	5'-ACACACACACACACACYT-3'	41.9
9	UBC 841	5'-GAGAGAGAGAGAGAGAYC-3'	41
10	UBC 813	5'CTCTCTCTCTCTCTCTT3'	38.5
11	C.Renetria 6	5'GTGTGTGTGTGTCC3'	41.2
12	ISSR 7	5'CTCTCTCTCTCTCTTG3'	47.6
13	ISSR SY 81	5'GTTGTTGTTGTTGTT3'	56.2
14	Sigma	5'-GAAGTGGGGAAGTGGG-3'	53.9
15	ISSR 2	5' ATTATTATTATTATTCAT3'	57.5
16	UBC 815	5'CTCTCTCTCTCTCTCTG3'	39.9

4.3.2.3.1 Sigma

Total genomic DNA of four ridge gourd lines, Haritham (fertile), Deepthi (fertile), Arka Sumeet (fertile) and Haritham (male sterile) line was amplified with sigma. Total 15 bands were observed with 3 polymorphic bands. No polymorphism was found when all male fertile lines were compared with Haritham (male sterile) line for male sterility. Amplification pattern is as shown in Plate 4.6.1.a.

4.3.2.3.2 ISSR 2

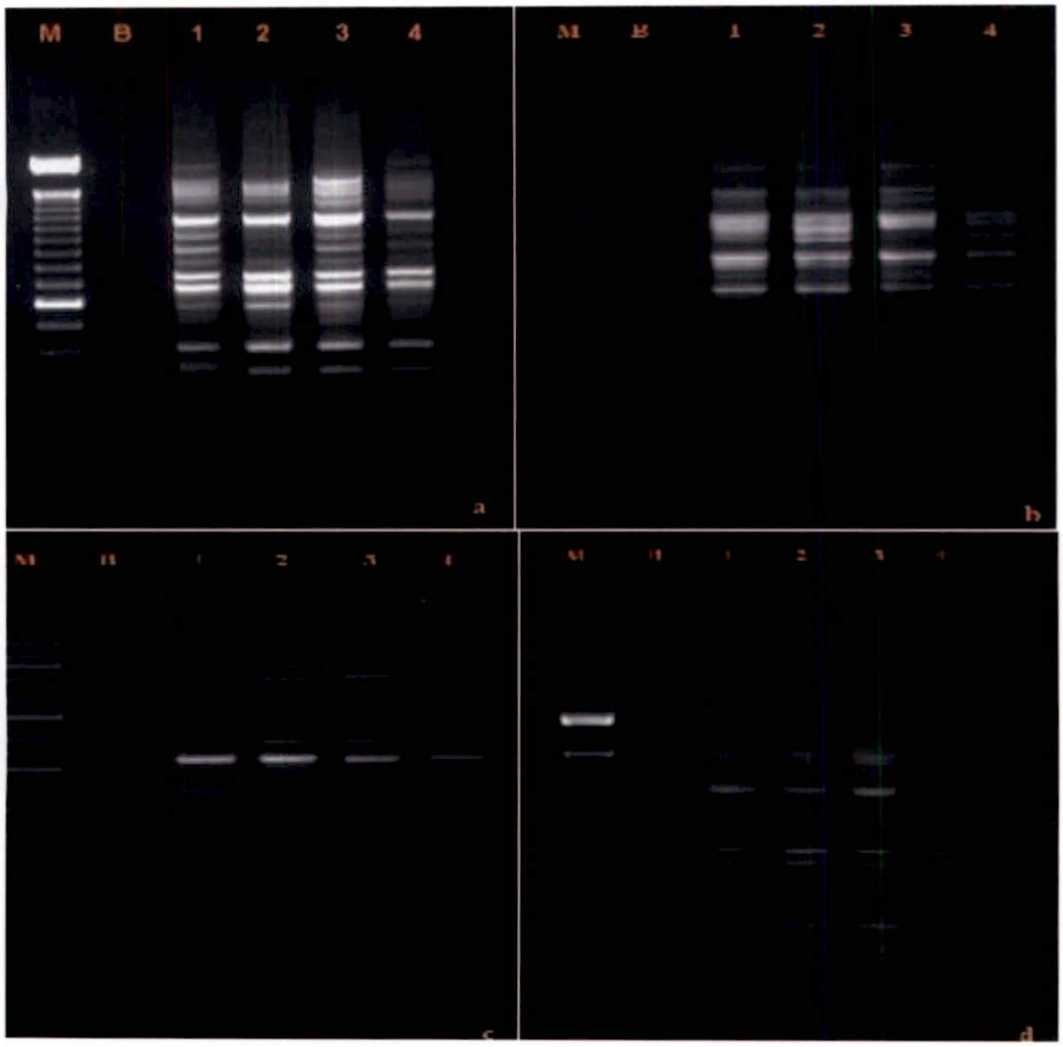
Total genomic DNA of four ridge gourd lines, Haritham (fertile), Deepthi (fertile), Arka Sumeet (fertile) and Haritham (male sterile) line was amplified with ISSR 2. Total 8 bands were observed, out of 8 bands, 2 were polymorphic. The observed polymorphism was not related with male sterility. Amplification pattern is as shown in Plate 4.6.1.b.

4.3.2.3.3 ISSR 05

Total genomic DNA of four ridge gourd lines, Haritham (fertile), Deepthi (fertile), Arka Sumeet (fertile) and Haritham (male sterile) line was amplified with ISSR05 and total 11 bands were obtained, including 8 polymorhic bands. No polymorphism for male sterility was observed between Haritham (male sterile) and male fertile lines. Amplification pattern is as shown in Plate 4.6.1.c.

4.3.2.3.4 UBC 873

Total genomic DNA of four ridge gourd lines, Haritham (fertile), Deepthi (fertile), Arka Sumeet (fertile) and Haritham (male sterile) line was amplified with UBC 873. 16 bands were observed including 4 polymorphic bands and 12 monomorphic bands. Polymorphism for male sterility was not found. Amplification pattern is as shown in Plate 4.6.1.d.



M- 100 bp ladder (100bp-3kb), B- Blank 1- Haritham (fertile), 2- Deepthi (fertile), 3- Arka Sumeet (fertile), 4- Haritham (male sterile)

a- Sigma, b- ISSR2, c- ISSR05, d- UBC873

Plate 4.6.1- The amplification pattern of DNA of three male fertile and one male sterile line of ridge gourd using ISSR primer Sigma, ISSR2, ISSR05 and UBC873

4.3.2.3.5 UBC834

Total genomic DNA of four ridge gourd lines, Haritham (fertile), Deepthi (fertile), Arka Sumeet (fertile) and Haritham (male sterile) line was amplified with UBC834. Six polymorphic bands were observed with total 14 bands. All the fertile lines were compared with Haritham (male sterile) line but no polymorphism for male sterility was detected. Amplification pattern is as shown in Plate 4.6.2.e.

4.3.2.3.6 ISSR 7

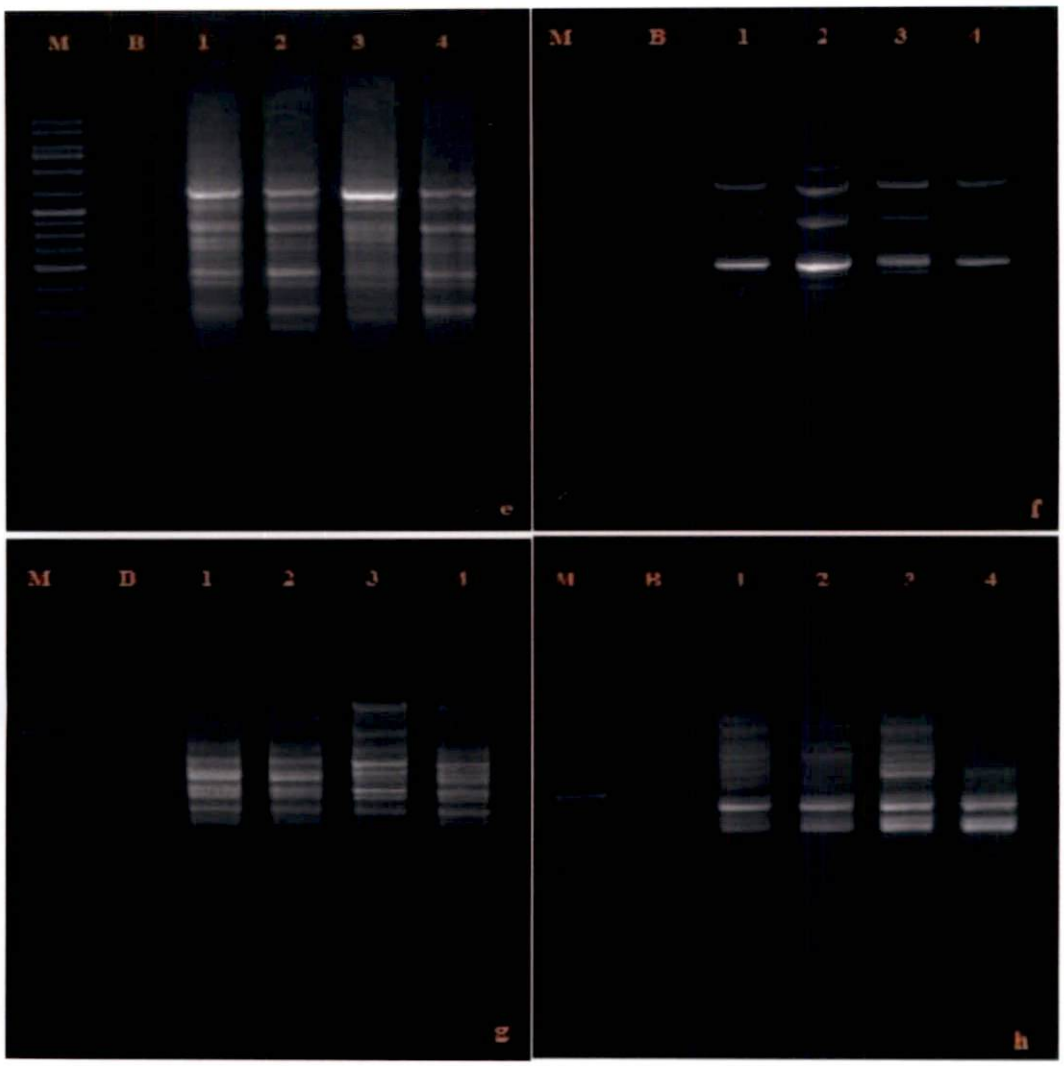
Total genomic DNA of four ridge gourd lines, Haritham (fertile), Deepthi (fertile), Arka Sumeet (fertile) and Haritham (male sterile) line was amplified with ISSR7. It has given 7 bands with 2 polymorphic bands. No polymorphism was found when all male fertile lines were compared with Haritham (male sterile) line for male sterility. Amplification pattern is as shown in Plate 4.6.2.f

4.3.2.3.7 OligoISSR04

Total genomic DNA of four ridge gourd lines, Haritham (fertile), Deepthi (fertile), Arka Sumeet (fertile) and Haritham (male sterile) line was amplified with Oligo ISSR 04. It gave 11 bands with only 1 polymorphic band. No polymorphism for male sterility was detected. Amplification pattern is as shown in Plate 4.6.2.g.

4.3.2.3.8 UBC 815

Total genomic DNA of four ridge gourd lines, Haritham (fertile), Deepthi (fertile), Arka Sumeet (fertile) and Haritham (male sterile) line was amplified with UBC815. Two polymorphic and 7 monomorphic bands were observed, no polymorphism for male sterility was found. Amplification pattern is as shown in Plate 4.6.2.h



M- 100 bp ladder (100bp-3kb) B- Blank 1- Haritham (fertile), 2- Deepthi (fertile), 3- Arka Sumeet (fertile), 4- Haritham (male sterile)

e- UBC834, f- ISSR7, h- Oligo ISSR 04, i- UBC 815

Plate 4.6.2- The amplification pattern of DNA of three male fertile and one male sterile line of ridge gourd using ISSR primers UBC834, ISSR7, Oligo ISSR04 and UBC815

4.3.2.3.9 UBC 841

Total genomic DNA of four ridge gourd lines, Haritham (fertile), Deepthi (fertile), Arka Sumeet (fertile) and Haritham (male sterile) line was amplified with UBC 841. It gave total 8 bands in each fertile line but in Haritham (male sterile) line it gave 7 bands. 1 band of 1185 bp size was found absent in Haritham (male sterile) line and it was present in all the fertile lines. The band can be considered as polymorphic band for male sterility, which can be used to differentiate Haritham (male sterile) and male fertile lines. Amplification pattern is as shown in Plate 4.6.3.i.

4.3.2.3.10 C. Renetria6

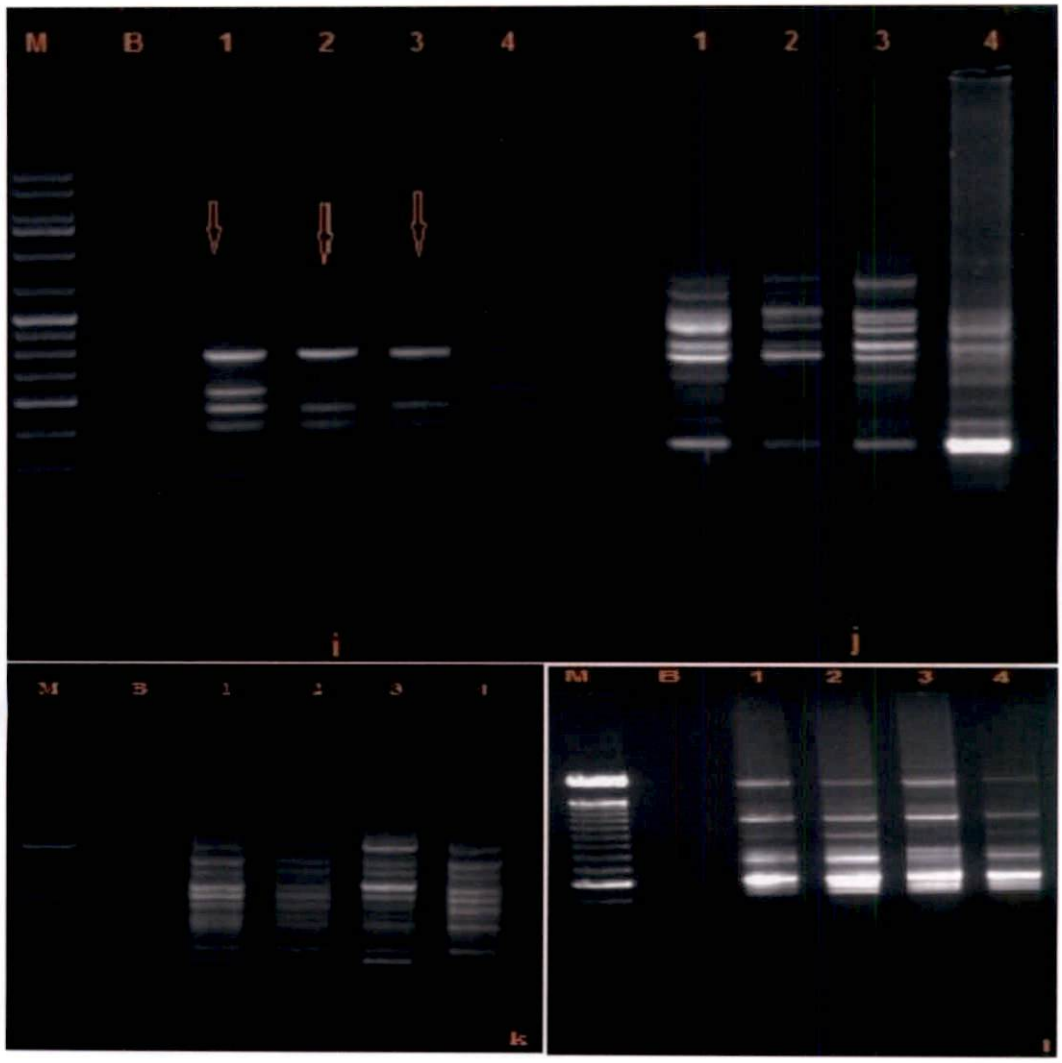
Total genomic DNA of four ridge gourd lines, Haritham (fertile), Deepthi (fertile), Arka Sumeet (fertile) and Haritham (male sterile) line was amplified with C. Reneteria6 gave 6 polymorphic bands and 8 monomorphic bands. No polymorphism for male sterility was detected. Amplification pattern is as shown in Plate 4.6.3.j.

4.3.2.3.11 UBC 855

Total genomic DNA of four ridge gourd lines, Haritham (fertile), Deepthi (fertile), Arka Sumeet (fertile) and Haritham (male sterile) line was amplified with UBC855. It gave 11 bands with 1 polymorphic and 10 monomorphic bands. All the fertile lines were compared with Haritham (male sterile) line to check polymorphism for male sterility but it was found to be absent. Amplification pattern is as shown in Plate 4.6.3.k.

4.3.2.3.12 ISSR SY 81

Total genomic DNA of four ridge gourd lines, Haritham (fertile), Deepthi (fertile), Arka Sumeet (fertile) and Haritham (male sterile) line was amplified with ISSR SY 81. It gave 12 bands, 6 polymorphic and 6 monomorphic bands. This polymorphism was further checked for male sterility but no polymorphism was found. Amplification pattern is as shown in Plate 4.6.3.l.



M- 100 bp ladder (100bp-3kb) B- Blank 1- Haritham (fertile), 2- Deepthi (fertile), 3- Arka Sumeet (fertile), 4- Haritham (male sterile)

i- UBC841, j- C.Reneteria 6, k- UBC855, l- ISSR SY81

Plate 4.6.3- The amplification pattern of DNA of three male fertile and one male sterile line of ridge gourd using ISSR primers UBC841, C. Reneteria 6, UBC855 and ISSR SY81

4.3.2.3.13 ISSR SY 88

Total genomic DNA of four ridge gourd lines, Haritham (fertile), Deepthi (fertile), Arka Sumeet (fertile) and Haritham (male sterile) line was amplified with ISSR SY 88. 3 polymorphic and 2 monomorphic bands were observed. No polymorphic band was observed when compared for male sterility. Amplification pattern is as shown in Plate 4.6.4.m.

4.3.2.3.14 UBC813

Total genomic DNA of four ridge gourd lines, Haritham (fertile), Deepthi (fertile), Arka Sumeet (fertile) and Haritham (male sterile) line was amplified with UBC813. 10 bands were obtained, Out of 10 bands, 5 bands were polymorphic. Inspite of 5 polymorphic bands, polymorphism for male sterility was not obtained. Amplification pattern is as shown in Plate 4.6.4.n.

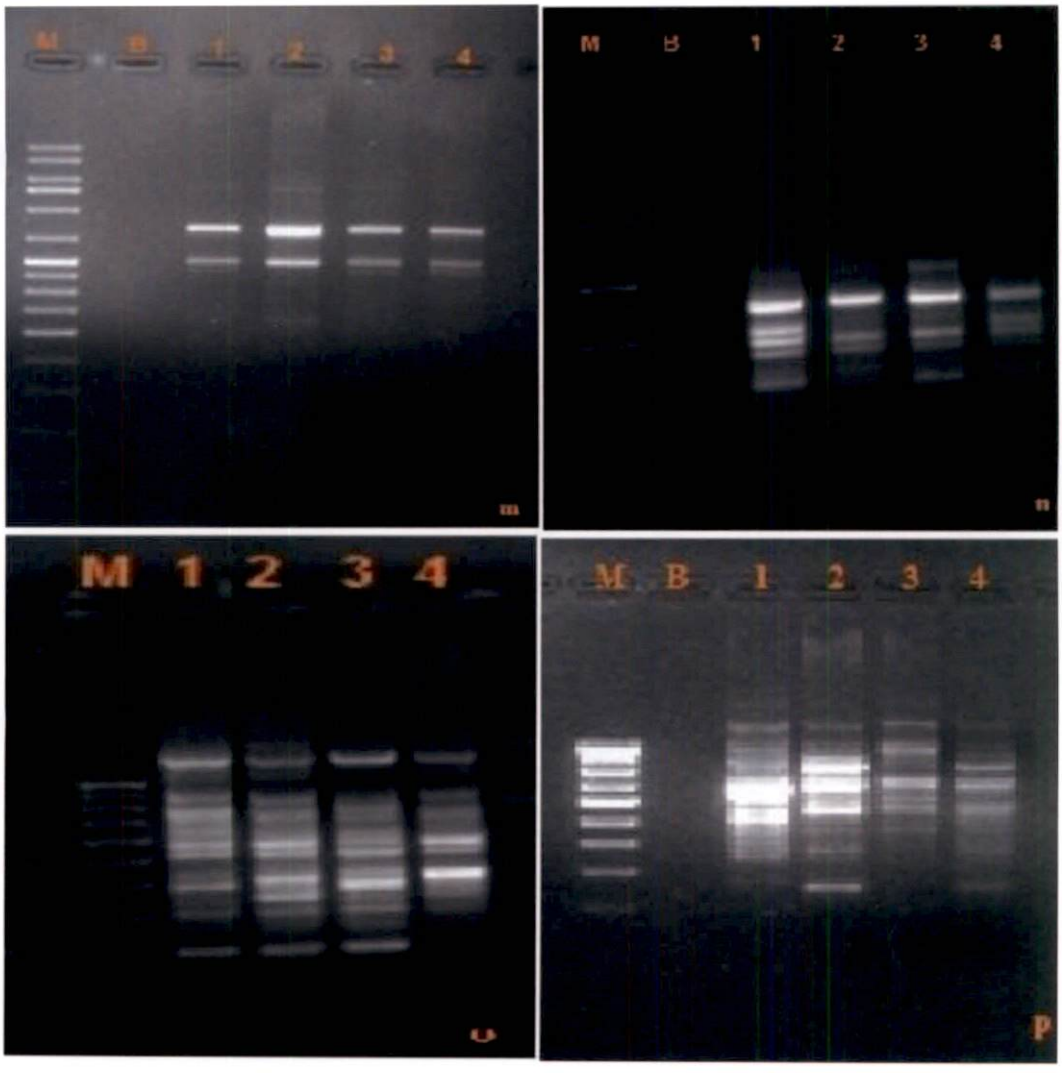
4.3.2.3.15 ISSR 15

Total genomic DNA of four ridge gourd lines, Haritham (fertile), Deepthi (fertile), Arka Sumeet (fertile) and Haritham (male sterile) line was amplified with ISSR 15. Total 11 bands were observed with 7 polymorphic and 4 monomorphic bands. Polymorphism was further compared with Haritham (male sterile) line in order to check polymorphism for male sterility but no polymorphism was found. Amplification pattern is as shown in Plate 4.6.4.n.

4.3.2.3.16 UBC880

Total genomic DNA of four ridge gourd lines, Haritham (fertile), Deepthi (fertile), Arka Sumeet (fertile) and Haritham (male sterile) line was amplified with UBC880. Total 7 bands were observed out of which 2 bands were polymorphic. No polymorphism detected for male sterility. Amplification pattern is as shown in Plate 4.6.4.o.

Details of amplification with the 16 primers for ISSR assay in ridge gourd are given in Table 4.4.



M- 100 bp ladder (100bp-3kb) B- Blank 1- Haritham (fertile), 2- Deepthi (fertile), 3- Arka Sumeet (fertile), 4- Haritham (male sterile)

m- ISSR SY 88, n- UBC813, o- ISSR15, p- UBC 880

Plate 4.6.4- The amplification pattern of DNA of three male fertile and one male sterile line of ridge gourd using ISSR primers ISSR SY88, UBC813, ISSR15 and UBC880

Table 4.4 Details of amplification with the 16 primers for ISSR assay in ridge gourd

Sl. No.	Primer	Total no of amplicons	No of polymorphic amplicons	No of monomorphic amplicons
1	Sigma	15	3	12
2	ISSR2	8	2	6
3	ISSR05	11	8	3
4	UBC873	16	4	12
5	UBC834	14	6	8
6	ISSR7	7	2	5
7	Oligo ISSR 04	11	1	10
8	UBC815	9	2	7
9	UBC841	8	1	7
10	C.Renetria 6	14	6	8
11	UBC855	11	1	10
12	ISSR SY 81	12	6	6
13	ISSR SY 88	5	3	2
14	UBC813	10	5	5
15	ISSR15	11	7	4
16	UBC880	7	2	5

4.4 Genetic relationship among ridge gourd lines using ISSR data

Genetic similarity coefficient for the varieties ranged from 0.51 to 0.84 Dendrogram was generated using NTSYS software based on the data generated through ISSR. It was divided into two clusters, Haritham (fertile), Deepthi (fertile) and Haritham (male sterile) lines from Kerala Agricultural University in one cluster and Arka Sumeet (fertile) from Indian Institute of Horticulture Research, Bangalore in one cluster. Haritham (fertile) and Haritham (male sterile) showed 82 per cent similarity (Fig. 4.1).

4.5 Sequencing

Three SSR, 1 ISSR and 1 SCAR markers were eluted and sent for sequencing. The direct sequencing on NGS platform was not successful for the markers CI1-21 (SSR), DE0144 (SSR) and Rps14 (SCAR), UBC841 (ISSR). The SSR maker CsWCT25 that registered a total size of 350 bp has yielded a sequence of 225 bp (Fig. 4.2) and from this SCAR primers were designed using Primer3 software (Koressaar, 2007), with an expected product size of 216 bp (Table 4.5). The BLASTn analysis of the sequence has not shown any homology, may be because the SSR marker amplifies only the non-coding repeat region and ridge gourd is a minor crop with only very few molecular works done on it.

4.6 Summary

The summary of the primitive results obtained using SSR, SCAR and ISSR marker is given in Table 4.6. In SSR assay 16 primers were screened, 9 SSR primers were selected. SSR primer, CI1-21, DE0144, CsWCT25 gave polymorphic band linked with male sterility at 125, 50, 350 bp size respectively.

9 SCAR primers were screened and 5 are selected. Out of 5, Rps14 gave polymorphic band of 80bp size.

In ISSR, 16 primers were analysed to find polymorphism between Male fertile lines and Haritham (male sterile) line. Out of 16 only, UBC841 gave

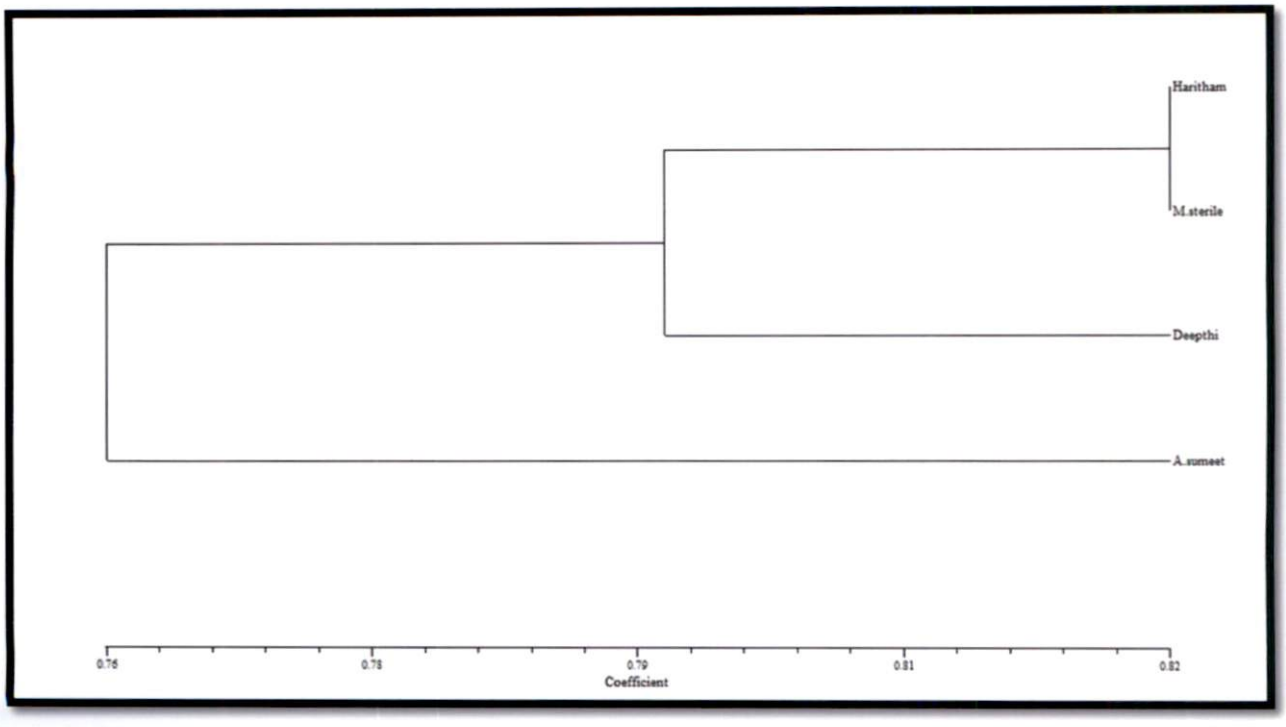


Fig. 4.1- Genetic relationship among ridge gourd lines using ISSR data

TCAATCAAACCGTCCCACCAATAGTAAAATTATACATCATGCTAAGTTTGGGG
ACAAAATTGATTTTTCCCTTAACTCAAGAATTTTCCGGTGAGTTTTGTGAATCA
GAAGCTTCAAGCTCACATTTGATGACATCTTTCTGTTTTGAGAACTCGTTACTT
TTTCAAGTGTTAAGCCTTGAAATGCCAGTTATAAATAGGGCGGTTTGATTGACT
TAATTTCTTT

Fig. 4.2- Sequence obtained with CsWCT25 (Total length 225 bp)

polymorphism for male sterility. Band of size 1185bp was missing Haritham (Male sterile) line whereas it was present in all fertile lines.

Among all polymorphic bands sequencing of CsWCT25 was successful. These identified markers could be characterized and further utilized used for early identification of male sterility and use for hybrid seed production.

Table 4.5 SCAR primer designed using the sequence obtained with CsWCT25

Sl. No	Primer	Nucleotide sequence	Annealing temperature (°C)
1	Forward	5' CAATCAAACCGTCCCACCAA 3'	53.68
	Reverse	5' TAAGTCAATCAAACCGCCC 3'	52.13

Table 4.6 Specific bands for male sterility generated using SSR, ISSR, and SCAR primers

Sl. No.	Primer	Type of marker	Reported in	Size of polymorphic band for male sterility (bp)
1	CI1-21	SSR	Watermelon	125
2	DE0144	SSR	Muskmelon	50
3	CsWCT25	SSR	Cucumber	350
4	Rps14	SCAR	Universal mitochondrial	80
5	UBC 841	ISSR	Universal	1185


Discussion

5. DISCUSSION

Among the agricultural crops, vegetables are most extensively utilized for the exploitation of heterosis through hybrid varieties. Heterosis can lead to higher yields and are less susceptible to diseases and environmental stresses than conventional varieties. In the production of hybrid seeds, therefore, efforts are made to use purely female - or more precisely "Male sterile" – parent plants for easiness in hybridization by skipping the emasculation. Public sector in most of the developed nations, has successfully utilized male sterility system in several vegetable crops to produce hybrid seeds at commercial scale. Similarly, private sector is also involved in the development of male sterile based hybrids of the important temperate and tropical vegetables (Kalloo *et al.*, 1998).

In traditional hybridization, pollen is transferred to the female organs manually, consequently, cost of hybrid seeds is comparatively higher, which is one of the major constraints in achieving more rapid adoption of vegetable hybrid technology.

Identification of male sterile female parent will simplify the process, simply by growing the female and male parents side by side, hybrid seeds could be harvested from the male sterile plants.

5.1 Male sterility

Male sterility refers to either absence of pollen grain or if present it is non-functional. The phenomenon of male sterility has always been of long term interest for the researchers of various disciplines of applied, strategic and basic sciences. Male sterility can be classified as Cytoplasmic male sterility (CMS) which is maternally inherited, Genetic male sterility (GMS) which is nuclear inherited, Non genetic (NGMS), chemically induced male sterility due to application of specific chemical (gametocides or chemical hybridizing agents).

Cytoplasmic male sterility is most widely used among all male sterilities because it is more convenient for the production of hybrid seeds, their mode of maintenance and restoration of fertility. Furthermore, CMS which have been successfully selected in natural populations are less prone to be associated with characters which are not convenient for seed production.

Male sterilities have been identified in different crops such as, maize (Rhoades,1933), sorghum (Ayyangar *et al.* (1937) (India); Stephens (1937) (America)), rice (Weeraratne *et al.*, 1954), wheat (Kihara, 1951; Fukasawa, 1953), common bean (Johns *et al.*, 1992), sunflower (Leclerq, 1983), tobacco (Hakansson and Glimelius, 1991), petunia (Boeshore *et al.*, 1983), pigeon pea (Deshmukh, 1959), tomato (Crane, 1915), chilli (Martin and Grawford, 1951), cole crops (Nieuwhof, 1961; Borchers, 1966; Sampson, 1966; Dickson, 1970), watermelon (Watts 1962) and muskmelon (Bohn and Whitaker (1949).

5.2 Male sterility in ridge gourd

Ridge gourd is characterized by diverse sex forms *viz.*, monoecious, androecious, gynoecious, gynomonoecious, andromonoecious and hermaphrodite types (Choudhary and Thakur, 1965), among which monoecious is mostly found with pistillate (female) flowers borne in axil of flowers and staminate (male) flowers in raceme Principally two genes are involved in production of various sex forms (Richaria, 1948).

In a population of ridge gourd, a plant having shrunken, small and sterile microspore was found, nodal cuttings of the male sterile line were maintained under *in vitro* conditions with the standardized protocol (Pradeepkumar *et al.*, 2007; 2010). Male sterile (MS) ridge gourd mutant regenerated through *in vitro* culture when crossed with pollen parents showed that two dominant male fertility restorer nuclear genes having complementary gene action governs the restoration of male fertility (Pradeepkumar *et al.*, 2012).

A molecular marker (identified as genetic marker) is a fragment of DNA that is associated with a certain location within the genome. Molecular markers are now widely used to track loci and genome regions in several crop-breeding programmes, as molecular markers tightly linked with a large number of agronomic and disease resistance traits are available in major crop species (Phillips and Vasil, 2001; Jain *et al.*, 2002; Gupta and Varshney, 2004).

Hence, the present study was undertaken with the objective to identify molecular marker linked with male sterility in ridge gourd in comparison with three male fertile lines Haritham, Deepthi and Arka Sumeet and Haritham (Male sterile). Isolated total genomic DNA was used for SSR, SCAR and ISSR analysis and polymorphism for male sterility was checked.

5.3 Morphological observations

There were no observed differences with respect to vegetative characters in the Haritham (male sterile) line as compared with the other fertile lines, difference was only with respect to flower characters which relates to male sterility. In Haritham (male sterile) rudimentary male buds were produced in racemes which failed to open, anthers were undeveloped and produced shrunken, non viable pollen grains whereas in male fertile lines anthers were well filled with viable pollen grains.

5.4 Total genomic DNA isolation

Isolation of good quality DNA is one of the important prerequisites in a reliable molecular biology work. Tender, green leaves were used for the total genomic DNA using CTAB method suggested by Rogers and Bendich (1994).

The major problems occurred during DNA isolation are the contaminants such as polysaccharides and polyphenols co-precipitate with DNA, making it unsuitable for molecular analysis and degradation of DNA due to endonucleases.

To overcome these hurdles, β-mercaptoethanol and PVP (poly vinyl pyrolidone) was added at the time of grinding the leaf samples. PVP and β-mercaptoethanol were added to the DNA isolation buffer as they were previously reported to be effective in removing polyphenols from leaf tissues (Dawson and Magee, 1995; Clark, 1997; Zidani *et al.*, 2005). According to Maliyakal (1992), PVP forms complex hydrogen bonds with poly-phenolic compounds which can be separated from DNA by centrifugation. Furthermore, PVP tend to reduce the oxidation of polyphenols (Howland *et al.*, 1991). Further, chloroform: isoamyl alcohol treatment was given three times instead of two times, in order to minimise the chances of protein contamination.

Isolated DNA was highly contaminated with RNA, this RNA contamination was removed with the 1 per cent RNAse A treatment. Use of RNase A has been reported by several workers (Raval *et al.*, 1998; Gallego and Martinez, 1996).

5.5 Determination of the quality and quantity of DNA

The quality and quantity of isolated DNA was determined by Nanodrop ND-1000 spectrophotometer and agarose gel electrophoresis. In the spectrophotometeric method, the ratio of optical density at 260 and 280 nm was used to test the quality. The absorbance ratio was calculated as OD at 260/280, for the various samples. The ratios between 1.8 and 2.0 were considered to be of high quality. All the samples under the study recorded a ratio between1.82 to 1.89. The quantity of DNA was also good and is ranged from 247.81 to 795.92 ng/µl.

Agarose gel electrophoresis had shown intact, clear band in genomic DNA isolated from all four ridge gourd samples which indicated good quality DNA without degradation.

5.6 Molecular marker analysis

A molecular marker is a fragment of DNA that is associated with a certain location within the genome. In the present study, three molecular marker systems, SSR, SCAR and ISSR were used.

SSRs (Simple Sequence Repeats) and ISSRs (Inter Simple Sequence Repeats) are proven useful in differentiating the male sterility and fertility in *Brassica juncea* (Pathania *et al.*, 2003), pearl millet (Kumar *et al.*, 2006; Yadav *et al.*, 2007), wheat (Li *et al.*, 2005), marigold (He *et al.*, 2009).

The protocols like reaction mixture and thermal profile for different marker systems such as SSR, SCAR and ISSR were standardized with the bulk DNA from the three fertile lines *viz.*, Haritham (fertile), Deepthi (fertile), Arka Sumeet (fertile). The protocols were standardized by altering the quantities of DNA, $MgCl_2$, primer and dNTP mix and the optimum quantity of these components was found to be 2µl, 2µl, 1.5µl and 1.5µl respectively A total of seventy four primers suitable to the three markers were screened with the standardized protocols.

5.6.1 SSR and SCAR analysis

Simple sequence repeats (SSRs) or Microsatellites are becoming increasingly attractive markers in molecular breeding (Morgante and Olivieri, 1993; Powell *et al.*, 1996).

Due to lack of molecular work, in ridge gourd SSR primers from related species like watermelon, cucumber, muskmelon was selected. SSR primers for water melon reported by Joober *et al.* (2006) and Jarret *et al.* (1996) were used. Cucumbar primers based on the study by Shengjun *et al.* (2013); Quon *et al.* (2007) and Tao *et al.* (2010) for gynocey in cucumber primers were selected. Primers reported by Diaz *et al.* (2011) and synthesized by syngenta reported for muskmelon having high PIC value were selected.

As cytoplasmic male sterility is associated with mitochondrial disfunction universal mitochondria primers were also used. Identification of sterile cytoplasm in maize was done using specific mtDNA primers by Dragena *et al.* (2006). This assay reveals 398, 440 and 799 bp specific DNA fragments identifying C, T and S cytoplasm, respectively. Molecular characterization of different cytoplasmic male sterile lines using mitochondrial DNA specific markers in rice was done by Khera *et al.* (2012). Hence for the present study, universal mitochondrial primers reported by Khera *et al.* (2012) were used.

In this study 2 per cent agarose gel was used for separating SSR fragments. Most of the primers showed no amplification at 2 per cent agarose gel electrophoresis and to resolve hence in order to resolve small fragements 10 per cent native PAGE was used. Polyacrylamide gel electrophoresis (PAGE) offers high resolution of low-molecular-weight nucleic acids. In particular, small DNA fragments (25-750 bp) that are poorly resolved by ordinary agarose gels are easily separated on polyacrylamide gels and even small difference can be quantified (Jack and Pollard, 1998). Silver staining was used for staining the gel. Silver staining is a highly sensitive method for the visualization of nucleic acid and protein bands after electrophoretic separation on polyacrylamide gels. Nucleic acids and proteins binds with silver ions, which can be reduced to insoluble silver metal granules. Sufficient silver deposition is visible as a dark brown band on the gel (Chevallet *et al.*, 2006).

Among 16 SSR and 9 SCAR primers, 9 SSR primers and 5 SCAR primers were selected for the final assay. DNA of three male fertile and one Haritham (male sterile) line was amplified using these 14 primers with standardized PCR conditions.

Among 6 watermelon primers, CI1-21 on amplification gave polymorphic band relative to male sterility at 125bp size. Cucumber primer CsWCT25 gave 350 bp polymorphic band for male sterility on amplification with genomic DNA

of four ridge gourd lines. DE0144 when amplified with Haritham (male sterile) and fertile line gave 50 bp polymorphic band for male sterility.

Universal SCAR mitochondrial primers Rps14 gave 80 bp polymorphic band for male sterility. S14 protein is coded by Rps14. Rps14 was identified to be involved in cytoplasmic male sterility in petunia (Budar *et al.*, 2001). Polymorphic band found using Rps14 may lead to male sterility in ridge gourd used in the present study, in order to confirm further characterization is needed. These polymorphic bands can be used to differentiate male fertile lines from male sterile.

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

SSR markers can be efficiently used for identification of markers linked with male sterility. Feng *et al.* (2009) identified two SSR markers, cnu_m273 and cnu_m295 linked to the *Ms* gene, at the genetic distances of 4.95 cM and 7.92 cM respectively using 250 SSR primers. Dagang, (2006) studied thermosensitive genic male sterility by mapping male sterile gene *tms5* with EST and SSR markers. *Rf3* locus in rice was mapped using SSR and CAPS markers by Alavi (2009). Validation of male sterile, fertility restorer and hybrid lines in wheat (*Triticum aestivum* L.) with linked SSR markers was studied by Prakash, (2012). Molecular mapping of a nuclear male- sterility gene in sunflower was done using TRAP and SSR markers by Chen *et al.* (2006).

5.6.2 ISSR analysis

The technique uses microsatellites as primers in a single primer PCR reaction targeting multiple genomic loci to amplify mainly inter simple sequence repeats of different sizes. ISSRs exhibit the specificity of microsatellite markers, but need no sequence information for primer synthesis enjoying the advantage of random markers (Joshi *et al.*, 2000).

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

For the identification of molecular marker linked with male sterility 49 primers were screened out of which 16 primers were selected for the final assay. All the selected ISSR primers were amplified with Haritham (fertile), Deepthi (fertile), Arka sumeet (fertile) and Haritham (male sterile) line. The PCR product was subjected to 2 per cent agarose gel electrophoresis in order to get well separated bands. Polymorphic bands were observed among Haritham (fertile), Deepthi (fertile), Arka Sumeet (fertile), Haritham (male sterile) line (Table 4.4) but when the polymorphism was compared between all the male fertile lines with Haritham (male sterile) line to check presence or absence of unique band in Haritham (male sterile) line it was found only in UBC 841.

UBC 841 gave polymorphic band for male sterility. In UBC 841 band having 1185bp size was absent in Haritham (male sterile) line whereas it was present in all male fertile line *i.e.* it was polymorphic for male sterility. The experiment was repeated in order to confirm the absence of this particular band, which was found to be absent in each experiment.

Using ISSR marker, SCAR marker linked to a recessive male sterile gene was developed in marigold by He, (2008). JinBing *et al.* (2005) identified ISSR marker for cytoplasmic male sterility in sweet pepper. Different cytoplasmic male sterile lines, restorer lines and hybrid of pearl millet were characterized by (Kumar *et al.*, 2005) using ISSR markers. The amplification pattern observed in ISSR and SSR analysis was scored and analysed for relatedness/ variability among the genotypes. The computer package NTSYS-PC (Rohlf, 2005) was used for cluster analysis. The dendrogram and the similarity matrix obtained by UPGMA cluster analysis of the ISSR and SSR markers are given in Fig- 4.1.

5.7 Sequence analysis of the identified markers

Polymorphic bands obtained with CI1-21, DE0144, CsWCT25, Rps14, UBC 841 were eluted and sent for sequencing in order to analyse the genes which are involved in the male sterility. On sequencing, polymorphic band of 350 bp obtained with CsWCT25 has yielded a sequence of 225 bp. CsWCT25 was the primer reported for gynoecious character in cucumber by Shengjun *et al.* (2013). In order to confirm the sequence obtained with CsWCT25 forward and reverse primers were designed using Primer3 software (Koressaar and Remm, 2007).

Most CMS associated genes are chimeric mitochondrial sequences including *atp, Nad, Rps, Cox* (Schnable and Wise, 1998). Alterations in mitochondrial genes will lead to male sterility. In plant mitochondria C changes to U to create new start/stop codons (UAA, UAG or UGA) by RNA editing *i.e.* tissue-specific editing only can result male sterility. In case of sorghum *atp6* editing leads to reduced fertile plants and progenies that have been restored to fertility have an increase in editing of *atp6* (Howad and Kempken, 1997). *Rps* is the ribosomal protein found in mitochondria. Hence the band obtained with Rps14 has a wide importance in order to confirm the mitochondrial genes involved in cytoplasmic male sterility of ridge gourd.

5.8 Conclusion

One of the important applications of molecular markers is Marker Assisted Selection. In Marker Assisted Selection, based on the presence of linked marker with the gene, presence of gene can be confirmed. Marker-trait association identification will play an important role in plant marker assisted selection or quantitative trait loci (MAS/ QTL) breeding programs, especially in plants that no other genetic information such as linkage map and quantitative trait loci are available (Ruan, 2010). This strategies utilize phenotypic and genotypic information to increase gain from selection and reduce selection cycle time.

Among the marker systems studied, SSR was found to be better than ISSR and SCAR to distinguish between Haritham (male sterile) and male fertile line. The four polymorphic bands identified with SSR and ISSR and one in ISSR marker for male sterility, are of great value for use in the Marker Assisted Breeding. Sequencing of the identified polymorphic bands, to validate the marker linked with male sterility and to identify genes involved in male sterility, early identification of male sterility and in hybrid seed production.

Summary

6. SUMMARY

The study entitled "Molecular characterization of male sterility in ridge gourd *Luffa acutangula* (L.) Roxb." was carried out at Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Kerala Agricultural University, during 2012-2014. The objective of the study was to identify molecular marker linked with the recently identified male sterility in ridge gourd, using SSR and ISSR markers.

A male sterile line shall enable the easier hybrid seed production avoiding the labour intensive emasculation process and thus the development and maintenance of CMS/ GMS/ CGMS lines is almost important in all the crops.

For the study, Three male fertile lines, Haritham and Deepthi (Kerala Agricultural University), Arka Sumeet (Indian Institute of Horticulture Research, Bangalore) and Haritham (male sterile) (Department of Olericulture, College of Horticulture, Kerala Agricultural University) were used.

The salient findings of the study can be summarized as follows:

Morphological characters were recorded for all the four lines Anthers were undeveloped and produced shrunken, non viable pollen grains were observed in the Haritham (male sterile) whereas in male fertile lines anthers were well developed.

The protocol suggested by Rogers and Bendich (1994) was found ideal for isolation of the total genomic DNA from the ridge gourd lines. The male sterility reported in ridge gourd is cytoplasmic. Since the total genomic DNA contains cpDNA and mtDNA along with nuclear DNA, total genomic DNA was isolated. The RNA contamination was completely removed through RNase treatment. After RNAse treatment, the $A_{260/280}$ ratio of all the samples was within the accepted range of 1.8 to 2.0 and bands were intact on 0.8 per cent agarose gel elctrophoresis.

Three molecular marker systems namely, SSR, SCAR and ISSR were employed for characterisation of male sterility.

Since no *Luffa* specific SSRs are reported, SSRs reported for watermelon, cucumber, muskmelon were tried for their capability to amplify the DNA.

SCAR primers developed for gynoecy in cucumber were used and since male sterility reported is cytoplasmic, mitochondrial specific universal primers were also used.

All the four lines were analysed with SSR, SCAR and ISSR primers, polymorphism for male sterility was checked by comparing banding pattern of three male fertile lines with male sterile line.

A total of 16 SSR, 9 SCAR, 49 ISSR primers were screened for their ability to amplify the DNA fragments. Out of these 9 SSR, 5 SCAR and 16 ISSR primers were selected for studying the polymorphism between male fertile and male sterile lines.

Electrophoresis using 2 per cent agarose gel has failed to bring proper amplification, Hence 10 per cent native PAGE followed by silver staining was used to separate the SSR and SCAR bands. Using SSR molecular marker system among 9 primer sets, 3 SSR primers CI1-21, DE0144, CsWCT25 gave polymorphic bands for male sterility. Size of polymorphic band found were, 125bp, 50bp and 350 bp respectively. Universal mitochondrial SCAR primer, Rps14 gave polymorphic band of 80bp size.

Using ISSR molecular marker system, 16 primers were analysed to find polymorphism between male fertile lines and male sterile line. Out of 16, only UBC841 gave polymorphic band for male sterility at 1185bp which was missing in male sterile line and present in all male fertile lines. The results for the polymorphism were reported and were found to be highly reproducible.

Sequencing of the SSR marker CsWCT25, which is already reported to be tightly linked with gynoecy in cucumber, has yielded 225 bp. SCAR primers are also designed using this sequence. These markers will have wide application in marker assisted selection and hybrid seed production.

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Annexures

ANNEXURE I

List of laboratory equipments used for the study

Refrigerated centrifuge	:	Kubota, Japan
Horizontal electrophoresis System	:	Biorad, USA
SDS- PAGE electrophoresis System	:	Biorad, USA
Thermal cycler	:	Veriti Thermal Cycler (Applied Biosystem, USA)
Gel documentation system	:	Biorad, USA
Nanodrop® ND-1000 spectrophotometer USA	:	Nanodrop®Technologies Inc.

ANNEXURE II

Reagents required for DNA isolation

Reagents:

1. **2x CTAB extraction buffer (100 ml)**

CTAB	:	2g
(Cetyl trimethyl ammonium bromide)		
Tris HCl	:	1.21 g
EDTA	:	0.745 g
NaCl	:	8.18 g
PVP	:	1.0 g

Adjusted the pH to 8 and made up final volume up to 100 ml.

2. **CTAB (10 %, 100 ml)**

CTAB	:	10 g
NaCl	:	4.09 g

3. **Chloroform- Isoamyl alcohol (24:1 v/v)**

To chloroform (24 parts), isoamyl alcohol (1 part) was added and mixed properly.

4. **Chilled isopropanol**

Isopropanol was stored in refrigerator at 0°C and was used for the study.

5. **Ethanol (70 %)**

To the 70 parts of absolute ethanol (100 %), 30 parts of sterile distilled water was added to make 70 per cent ethanol.

6. **TE buffer (pH 8, 100 ml)**

Tris HCl (10 mM)	:	0.1576 g
EDTA (1 mM)	:	0.0372 g

The solution was prepared, autoclaved and stored at room temperature.

ANNEXURE III

Composition of Buffers and Dyes used for Gel electrophoresis

1. TAE Buffer 50X

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

2. Loading Dye (6X)

0.25% bromophenol blue

0.25% xylene cyanol

30% glycerol in water

3. Ethidium bromide

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

MOLECULAR CHARACTERIZATION OF MALE STERILITY IN RIDGE GOURD [*Luffa acutangula* (L.) Roxb.]

By

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(2012-11-110)

ABSTRACT OF THESIS

Submitted in partial fulfillment of the requirement

for the degree of

Master of Science in Agriculture

(PLANT BIOTECHNOLOGY)

Faculty of Agriculture
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2014

ABSTRACT

The development of hybrids with desirable heterosis is a major goal in plant breeding. In traditional hybridization, following the emasculation which is a labour intensive process, pollen is manually transferred to the female organs. The higher cost of the hybrid seeds, considerably owing to the labour costs for emasculation, is the major constraint in achieving more rapid adoption of vegetable hybrid technology. Use of male sterile parent is understood to reduce the cost of hybrid seed production by avoiding the manual emasculation. In this context, the recently reported male sterile line of ridge gourd from Kerala Agricultural University, is commercially very important.

A system to confirm the male sterility at an early phase of parent itself, is very important since any kind of fertility restoration at a later phase will fail the hybridization programme. A molecular marker associated with the male sterility will be enormously useful at this phase, to confirm the male sterility of the female parent used in the hybridization programme. The study entitled "Molecular characterization of male sterility in ridge gourd *Luffa acutangula* (L.) Roxb." was carried out at Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Kerala Agricultural University, with the objective to identify the molecular marker/s linked with the male sterility in the newly reported sterile line of ridge gourd from KAU, using SSR and ISSR marker systems.

Plant materials used in this study were three male fertile lines, Haritham, Deepthi, and Arka Sumeet and the Haritham (male sterile line of KAU). In the male sterile line, unopened rudimentary male buds with poorly developed anthers containing shrunken, non viable pollen grains were observed. In male fertile plants, the anthers were well developed with viable pollen grains.

Total genomic DNA was extracted from the young leaves, using CTAB method and SSR and ISSR marker systems were employed for characterizing the male sterility. SSR primers are highly genus specific and since genomic data on *Luffa* is lacking, suitability of SSR primers from the related genus such as *Citrullus* and *Cucumis* was attempted. Initial attempts on electrophoresis of SSR amplicons on 2 per cent agarose gel were successful for few primers only. Thus, to resolve the small fragments, 10 per cent native PAGE was subsequently used. In SSR assay 25 primers were screened, among which 14 were selected. Total genomic DNA of the male fertile and sterile lines were amplified with these SSR

primers. The primers CI1-21, DE0144 and CsWCT25 have yielded distinctly polymorphic bands associated with the male sterility with 125, 50, 350 bp sizes, respectively.

In ISSR assay, among the 49 primers screened initially, 16 were selected for the study. UBC841 has yielded a distinctly polymorphic band for male fertility at 1185bp. This marker was missing in the male sterile line. Dendrogram was generated based on the ISSR study in order to study genetic relationship among the ridge gourd lines. Haritham (fertile) and Haritham (male sterile) showed 82 per cent similarity. The universal mitochondrial primers were additionally screened in this study and among them; Rps14 gave polymorphic band linked with male sterility, at 80 bp size.

Direct sequencing with NGS platform was done of the identified polymorphic bands, it was failed with CI1-21, Rps14, DE0144 and UBC841. Only with CsWCT25 sequence of 225 bp was obtained. CsWCT25 is already reported to be tightly linked with gynoecy in cucumber, SCAR primers are also designed using this sequence. These markers will have wide application in marker assisted selection and hybrid seed production.