IDENTIFICATION OF MOLECULAR MARKER LINKED TO THE RESISTANCE FOR VASCULAR STREAK DIEBACK DISEASE IN COCOA (*Theobroma cacao* L.)

By

EKATPURE SACHIN CHANDRAKANT
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THESIS

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Faculty of Agriculture

Kerala Agricultural University, Thrissur

CENTRE FOR PLANT BIOTECHNOLOGY AND MOLECULAR BIOLOGY

COLLEGE OF HORTICULTURE

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DECLARATION

I hereby declare that the thesis entitled **"Identification of molecular marker linked to the resistance for vascular streak dieback disease in cocoa (*Theobroma cacao* L.)."** 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.

Vellanikkara
Date: 28/09/14

Ekatpure Sachin Chandrakant
(2012-11-103)

CERTIFICATE

Certified that the thesis entitled **"Identification of molecular marker linked to the resistance for vascular streak dieback disease in cocoa (*Theobroma cacao* L.)."** is a record of research work done independently by **Mr. Ekatpure Sachin Chandrakant** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, associateship, fellowship to him.

Vellanikkara
Date: 23.09.2014

Dr. Deepu Mathew
(Chairman, Advisory Committee)
Assistant Professor
Centre for Plant Biotechnology
and Molecular Biology
College of Horticulture
Vellanikkara, Thrissur.

CERTIFICATE

We, the undersigned members of the advisory committee of **Mr. Ekatpure Sachin Chandrakant** a candidate for the degree of **Master of Science in Agriculture** with major field in **Plant Biotechnology,** agree that the thesis entitled **"Identification of molecular marker linked to the resistance for vascular streak dieback disease in cocoa (*Theobroma cacao* L.)."** may be submitted by **Mr. Ekatpure Sachin Chandrakant** in partial fulfilment of the requirement for the degree.

Dr. Deepu Mathew
(Chairman, Advisory Committee)
Assistant Professor
CPBMB
College of Horticulture,
Vellanikkara, Thrissur.

Dr. P. A. Valsala
(Member, Advisory committee)
Professor and Head
CPBMB
College of Horticulture,
Vellanikkara, Thrissur.

Dr. P. A. Nazeem
(Member, Advisory committee)
Professor and Co-ordinator
DIC, CPBMB
College of Horticulture
Vellanikkara, Thrissur.

Dr. P. S. Abida
(Member, Advisory committee)
Associate Professor,
CPBMB
College of Horticulture
Vellanikkara, Thrissur.

Dr. J. S. Minimol
(Member, Advisory committee)
Assistant Professor
Plant Breeding and Genetics
Cocoa Research Centre
College of Horticulture
Vellanikkara, Thrissur.

Dr. Sainamol Kurian P.
(Member, Advisory committee)
Assistant Professor
(Plant Pathology), AICVIP
College of Horticulture
Vellanikkara, Thrissur.

External Examiner

Dr. Rekha K.
Senior Scientist
Rubber Research Institute of India
Kottayam

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Ekatpure Sachin Chandrakant

Dedicated to my beloved parents and Teacher

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ABBREVIATIONS

%	Percentage
@	At the rate
<	Less than
=	Equal to
>	Greater than
µg	Microgram
µl	Microlitre
AFLP	Amplified Fragment Length Polymorphism
ai	Active ingredient
AP-PCR	Arbitrarily Primed Polymerase Chain Reaction
APS	Ammonium per sulphate
BLAST	Basic Local Alignment Search Tool
bp	Base pair
cm	Centimetre
cM	Centi Morgan
CMS	Cytoplasmic Male Sterile
CTAB	Cetyl Trimethyl Ammonium Bromide
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
dNTPs	Deoxyribo Nucleoside Triphosphate
EDTA	Ethylene Diamine Tetra Acetic acid
EST	Expressed sequence tags
g	Gram
ha	Hectare
Kb	Kilo base pairs
L	Litre

M	Molar
MAS	Marker Assisted Selection
mg	Milligram
ml	Millilitre
mM	Milli mole
ng	Nanogram
°C	Degree Celsius
OD	Optical Density
PAGE	Polyacryl Amide Gel Electrophoresis
pH	Hydrogen ion concentration
PIC	Polymorphic Information Content
pM	Pico Mole
PVP	Poly vinyl pyrolidone
QTL	Quantitative Trait loci
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
SCAR	Sequence Characterized Amplified Region
SSCP	Single Stranded Conformational Polymorphism
TAE	Tris Acetate EDTA
TBE	Tris Borate EDTA
TE	Tris EDTA
U	Unit
UBC	University of British Columbia
UV	Ultra violet
V	Volts
VSD	Vascular streak dieback
WBD	Witches Broom Disease
β	Beta

Introduction

1. INTRODUCTION

The cacao tree, *Theobroma cacao* L. (2n = 20), is one of the important beverage crops belonging to the family *Malvaceae* (Alverson *et al.*, 1999). It is native of the rainforests of humid tropical regions of the northern parts of South America and the northern parts of Central America. Cocoa is mostly cultivated in many South American, African and East Asian countries where the agro climatic conditions are good for its growth. In 1960's commercial cultivation of cocoa was started in India. Current world production of cocoa is about 4 million tonnes but India contributes only 0.02 per cent. In Kerala, area under the cocoa is 12,480 ha. with the production of 6.14 metric tonnes and productivity 490 kg/ ha (India Stat, 2014).

Under the humid condition of Kerala, various diseases and pest act as a serious challenge to the commercial cocoa cultivation. Among the all diseases Vascular Streak Dieback (VSD) has now become the most devastating one. This disease was first reported from Papua New Guinea by Keane (1972) and Prior (1979). This disease was caused by a new genus and species of fungus which was given the name *Oncobasidium theobromae* (Talbot and Keane, 1971). Since then occurrence of this disease has been reported in many South East Asian countries.

In India, initial record of this disease was made by Abraham (1981) and Chandramohan and Kaveriappa (1982). Abraham and Ravi (1991), after a detailed survey, has observed that this disease was present in almost all the district of Kerala except Thrissur and Palakkad. Later it was observed that the disease is spreading at an alarming rate in all the cocoa gardens of Kerala including Thrissur and Palakkad districts (KAU, 1993; 1995). Global crop loss due to this disease was up to 40 per cent.

Generally, the pathogen *Oncobasidium theobromae* grows very slowly in culture medium and has not so far been reported to sporulate in axenic culture. Due to this difficulty, screening for resistance was done by exposing the test plant to natural inoculum in the field. Further, the pathogen has got a long incubation

period and according to Keane and Prior (1992), *Oncobasidium theobromae* is highly specialised near obligate parasite of cocoa and is the only known wind born, leaf penetrating basidomycets of vascular pathogen.

VSD can be easily identified by its characteristic symptoms such as yellowing of leaf with green islets, defoliation and enlargement of lenticels on the stem, production of the sporophores on the fallen leaf scars, vascular streaking and finally the death of the infected parts. The disease causes considerable damage to the grown up plants and is found to be lethal in seedlings (Talbot and Keane, 1971).

Cocoa is a perennial tree, it requires long breeding cycle. To reduce the length of the breeding cycle, early screening methods for major diseases using molecular markers have been developed (Nyasse *et al.*, 1995; Iwaro *et al.*, 1998). By employing molecular marker techniques, accelerated breeding programs have been initiated in several countries (Efron *et al.*, 2002). Now days, molecular markers play a significant role in enhancing the speed of breeding programs.

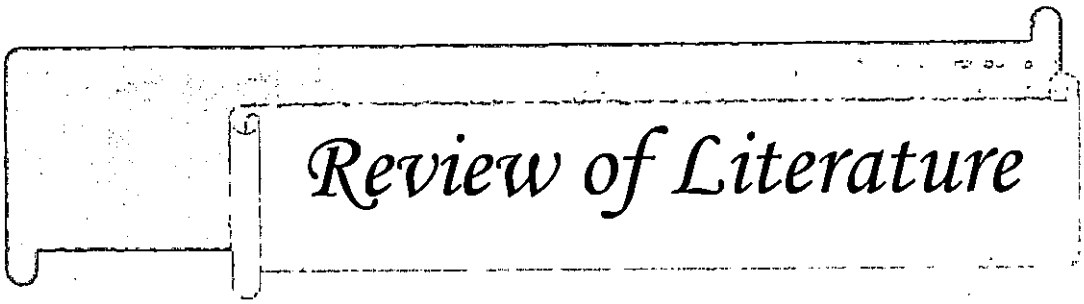
Using molecular markers on the specific regions of a chromosome near the target gene, which effectively flag to breeders whether specific traits have been inherited, early identification of the most desirable genotypes will be possible. The size of the population that needs to be evaluated can be reduced substantially and thus the efficiency of the breeding could be enhanced (Figueira, 2004).

As of now, there is no marker system for the identification of VSD resistance gene in cocoa. By considering the potential of molecular markers, an attempt to identify the SSR (Simple Sequence Repeats) and ISSR (Inter Simple Sequence Repeats) markers linked with the VSD resistance gene has been made. SSR are proved to be the best markers since they are highly polymorphic, uniformly distributed throughout the genome, co-dominant, reproducible and easy to interpret. As such, SSR markers have been used widely in various genetic studies of cacao, including genetic mapping and identification of quantitative trait loci (QTLs) (Queiroz *et al.*, 2003; Brown *et al.*, 2005). ISSR targets the highly

variable microsatellite regions of the nuclear genome providing a large number of polymorphic fragments (Gupta *et al.,* 1994). Therefore this technique has been used extensively to evaluate genetic diversity both within and between plant populations in angiosperms and gymnosperms (Osborn *et al.*, 2005). The high level reproducibility of ISSR markers is already well established in many crops and hence these markers offer the potential for direct usage in MAS (Bornet and Branchard, 2001; Reddy *et al.*, 2002).

Cocoa Research Centre, Kerala Agricultural University, India has developed numerous high yielding and VSD resistant hybrids and the performance of these hybrids is being evaluated for the past one and half decade. Among the parents used in the extensive crossing programmes, clones GVI 55, GVI 18.5 and GVI 126 were the best source of resistance with maximum recovery of good hybrids having VSD resistance (Minimol and Amma, 2013). More recently, five best performing hybrids S IV1.26, S IV2.29, S IV4.29, S IV6.18 and VSD I31.8 are identified to be released for commercial cultivation (Amma, 2013). For identification of resistant gene twelve genotypes of cocoa with three levels of response to the VSD disease *viz.* resistant, partially resistant and susceptible have been selected from Cocoa Research Centre, College of Horticulture, Kerala Agricultural University and employed in the study.

The objective of the study was to identify the reliable and reproducible molecular markers linked to the gene offering resistance to vascular streak dieback disease of cocoa (*Theobroma cacao* L.).



Review of Literature

2. REVIEW OF LITERATURE

2.1 Introduction

Theobroma cacao has originated from the tropical rainforest of South America and is one of the major cash crops for many tropical countries (Whitelock *et al.*, 2001). *Theobroma* literally means *food of the gods*, referring to the cultural and religious status of cocoa. Archaeological records have revealed the evidences of cocoa plantation in Central America dated to 2000-4000 years before Spanish contact (Bergman, 1969). These plantations extended from Mexico to Costa Rica and over the time cocoa cultivation spread to other locations in South America and the Caribbean (Wood and Lass, 1985).

Currently, cocoa is cultivated on over five million hectares of tropical lowlands worldwide (Kraus and Soberanis, 2001). *T. cacao* is often cultivated as one component of complex agro ecosystems, providing both economic and ecological benefits to the farmers and the producing countries (Wood and Lass, 1985)

2.2 Botany of cocoa

Theobroma cacao was the name given to the cocoa by Linnaeus in the first edition of his *Species Plantarum* published in 1753. Cocoa is a diploid fruit tree species (2n=2x=20) with a small genome (380 Mb), similar to rice genome (Figueira *et al.*, 1992). The natural habitat of *Theobroma* is the lower canopy of the evergreen rain forest; hence it is cultivated under shade canopies.

The flowers are borne on the trunk and branches, a habit referred to as cauliflory or truncate. The flowers are only produced on wood of a certain minimum physiological age, usually two or three years old under favourable growing conditions. Flowers are born on long pedicels and have five free sepals, five free

petals, ten stamens and ovary of five united carpels. The ten stamens which form the androecium of the flower are in two whorls, the outer whorl consists of five long staminodes, while the inner whorl has five fertile stamens. The stamens bear two anthers which lie in the pouch of the corresponding petal. The ovary has five parts containing many ovules arranged around a central axis. The flowers are generally pink with darker tissue in the staminodes and petals, but there is a considerable variation between cultivars in the size and colour of the flowers (Wood and Lass, 1985).

Cocoa trees produce large numbers of flowers at certain times of the year depending on local conditions and genotype. However, only 1-5 percent of the flowers are successfully pollinated to produce pods, although higher proportions have been recorded for the Amelonado type (Posnette and Entwistle, 1958). Pollination is effected by various small insects; the most important of these pollinating insects are midges belonging to several genera of the family *Ceratopogonidae* (Wood and Lass, 1985).

2.3 Pests and diseases of cocoa

Cocoa growing environment is often confining, shaded and humid, conditions strongly favourable for pathogen development (Fulton, 1989). Because cacao plants are grown in many non native tropical environments around the world, its yield is affected by a number of opportunistic pathogens. Diseases account for over 40 per cent of annual global cocoa losses (Entwistle *et al.* 1987; Bowers *et al.* 2001; Flood *et al.* 2004; Hebbar 2007). Fungal pathogens are the most devastating, the important being pod rot, also called black pod (caused by several species of *Phytophthora*), vascular streak dieback (*Oncobasidium theobromae),* frosty pod rot (*Moniliophthora roreri*) and Witches' broom (*Moniliophthora perniciosa*). Cocoa Swollen Shoot virus is the only known viral disease of cacao. The most damaging pests are mirids (several species) and cocoa pod borer (*Conopomorpha cramerella*) (Lass, 1987;

Flood *et al.*, 2004). List of major pest, diseases and their global crop loss is mentioned as follows in Table 2.1.

Table 2.1 Global loss caused by main cocoa pests and diseases

Disease	Pathogen/Pest	Region	Potential crop loss (tones)
Phytophthora pod rot	*Phytophthora spp.*	Ubiquitous	450,000
Witche's broom	*Crinipellis perniciosa*	South America and Caribbean	250,000
Frosty pod rot	*Moniliophthora roreri*	Central and South America	30,000
Capsids (Miridae)	Several species.	West Africa, Asia, Latin America	200,000
Swollen shoot virus	Virus	West Africa	50,000
Cocoa pod borer	*Conopomorpha crammerella*	Asia /Pacific	40,000
Vascular streak dieback	*Oncobasidium theobromae*	Asia and Pacific	30,000

(http://www.dropdata.org/cocoa/cocoa_prob.htm#severity, 5/07/2012)

2.3.1 Vascular streak dieback disease

In a detail of cocoa dieback, Turner (1967) recorded the occurrence to be widespread in 37 cocoa growing countries in Asia, Africa, the Caribbean, Central America and South America. Investigators in these countries attributed the dieback to

one or a combination of factors which included environmental, nutritional, tree age and vigor, varietal response, pests and diseases.

A dangerous dieback disease of cocoa differentiated from other types of dieback induced by environmental factors and insect attack was recognized in Papua New Guinea in the early 1960 by Shaw (1963) and Bridgland *et al.* (1966a, 1966b, 1967). This disease was referred to as "vascular streak dieback" (VSD) to differentiate it from other types of dieback by Keane (1972). The causal organism of the disease was established as a new species of fungus *Oncobasidium theobromae* (Talbot and Keane, 1971). Simultaneously this disease was reported from west Malaysia (Keane and Turner, 1971), Indonesia (Turner and Shepherd, 1978) and later from Burma (Lai, 1985) and southern Thailand (Keane and Prior, 1992).

In India Abraham (1981) and subsequently Chandramohan and Kaveriappa (1982) reported the presence of VSD of cocoa from Kottayam district of Kerala. The maximum occurrence of the disease was in Kottayam district followed by Ernakulam, Thiruvananthapuram, Kozhikkode, Idukki, and Pathanamthitta as reported by Abraham and Ravi (1991). This disease was not noticed in Thrissur and Palakkad districts. However, further disease survey conducted during has 1993 revealed the incidence of the disease in Thrissur district of Kerala (K. A. U., 1993, 1995).

2.3.2 Causal organism

Talbot and Keane (1971) were the first to describe the pathogen causing VSD of cocoa as a new genus and species of *Basidomycotina* (*Thulasnellales ceratobasidiaceae*) and named the fungus as *Oncobasidium theobromae*. They studied the fungus collected from Papua New Guinea: Fruit bodies white, membranous to subhypochnoid, occurring as small effused adherent patches on leaf scars and adjoining stem of cocoa with mycelium emerging from xylem vessels. Basal hyphae thin walled but firm, hyaline to yellowish , smooth not encrushed,

without clamps connections, with prominent dolipores, more or less horizontal and upto 10 µm wide , long celled (up to 200 µm), Branching at a wide angle; ascending hyphae narrower, up to 5-6 µm diameter, shorter celled hyline, binucleate. Cystidiate, at first often broad obovate, later elongating and becoming capitate-clavate (rarely sub cylindrical) with a subcylindrical base 6-8 µm wide and a more or less abruptly inflated apex 12-16 µm wide, the whole metabasidium (18-23) 26-36 µm long. Sterigmata constantly 4, stout, conical, straight or curved, 6-12 µm long, up to 4 µm wide at the base. Basidospores smooth, hyaline, thin walled, not amyloid, often multiguttulate, broad ellipsoid with one side flattened, 15-25 X 6.5-8.5 µm, conidial and sclerotial states not known to occur.

The morphological characters of VSD pathogen occurring in Malaysia and Indonesia were studied by many workers (Ahmad, 1982; Lam *et al.*, 1988 and Pawirosoemardjo *et al.* 1990). They reported similar morphological characters as described Talbot and Keane (1971) from Papua New Guinea. Keane and Prior (1992) reported that basidia collapsed immediately after the shedding of spores because of which only very few basidia were visible. Studies on VSD in Papua New Guinea and inoculation tests on seedlings with spores of the fungus confirmed *O. theobromae* as the causal pathogen of VSD (Prior, 1978).

2.3.3 Disease symptom

Symptomatology of VSD was first explained by Keane (1972) from Papua New Guinea. VSD has very characteristic symptoms which will be similar whether the disease occurred on the main stem of a seedling or on a branch of an older tree. The first symptom will be chlorosis of one leaf usually second or third flush from the tip (Plate. 1a). The pattern of chlorosis was very distinctive with islets of tissue remaining green (Plate. 1b). After 2-3 days the chlorotic leaf may fall down and subsequently the leaves above and below it turns chlorotic in the same way. Leaves shed in a distinctive pattern where the youngest and oldest leaves are intact, while all

the middle leaves fall down. Enlarged lenticels will be seen on the stem immediately below the petiole of the infected leaf causing roughening of bark. Leaf scars resulting from the fall of diseased leaves shall show three blackened spots when the dry surface was scrapped off.

The disease will spread to the lateral branches particularly those formed by the growth of axillary buds on the diseased stem and on such branches, the leaves will turn chlorotic and drop off in succession from the base. Leaves in the latest flush of the diseased seedlings or branches shall often show interveinal necrosis (oak leaf pattern) characteristics of calcium deficiency. Eventually, leaf fall occur right to the growing tip, followed by the death of the branch. From this branch the diseases spread to the other branches or the trunk and finally kill the whole plant.

Leaf scars resulting from the fall of chlorotic leaves will be covered by white, effused, adherent fruiting bodies of the fungus. These fruiting bodies will be seen only on the leaf scar and adjacent bark. If the bark of the diseased stem is peeled, the browning of the cambium could be seen. The underlying xylem discoloration with brown streak could be seen. Similarly, if the infected stem is split in to two, brown streaks could also be easily seen (Plate. 1cd).

Sporulation of *O. theobromae* has not so far been observed in culture. Inoculation studies were undertaken with spores obtained from sporophores present on infected stems in the field (Keane, 1972; Prior, 1978). Attempts to establish the fungus with cocoa callus tissue to produce spores were not successful (Prior, 1978). The disease is most damaging in seedlings less than 10 months old, which are killed by infection of the single stem. Only the most susceptible genotypes are killed by infections beginning in outer branches on older trees (Keane, 1981).

a: Infection on 2nd or 3rd flush behind the shoot tip

b: Distinctive green spots on leaf

c: Vascular streaking in xylem tissue

d: Black vascular traces in the tissue

Plate 1: Symptoms of vascular streak dieback

Symptomatology of the disease from Papua New Guinea, India, Malaysia and Indonesia were described by many scientists (Prior, 1980; Abraham, 1981; Ahmad, 1982 and Abraham and Ravi, 1991; KAU, 1995).

Prior (1980) reported that maximum disease occurrence was seen 3-5 months after seasonal rainfall. Ahmad (1982) suggested that there were different isolates of fungus present in Papua New Guinea and Malaysia but similarity of morphology of Malaysia isolates to *Oncobasidium theobromae* showed that both fungi belongs to the same genus. Seedlings inoculated with spores of the fungus developed typical symptoms of the disease within three months. Subsequent to Papua New Guinea, VSD occurred on the mainland of New Guinea and Papua and the island of New Britain (Keane, 1981).

Varghese *et al.* (1981) reported same differences in the symptoms of VSD occurring in Malaysia. Wood and Lass (1985) noted that in Malaysia, occurrence of interveinal necrosis was more common compared to yellow leaves with green islets as that in Papua New Guinea. Studies at Cadbury-KAU Cocoa Research Project also reveled few variations in symptoms of VSD from those reported from Papua New Guinea (KAU, 1995).

2.3.4 Losses

In Malaysia, VSD is the main disease of cocoa, which cause significant crop losses (Efron *et al.*, 2002; 2005; Guest and Keane, 2007). The losses due to VSD, especially in mature plant parts have been difficult to assess. According to Byrne (1976) the overall loss due to VSD alone was estimated between 25 to 40 per cent of total production. In Malaysia, considerable reduction in yields in new plantings occurred in areas severely affected by VSD (Shepherd *et al.*, 1976). Deaths due to VSD in seedlings in the nursery and in immature field plantings have been frequently observed in Malaysia (Ahmad *et al.*, 1982). In Papua New Guinea while the disease

was damaging in seedlings up to about 18 months old, only the most susceptible mature trees were killed (Keane, 1981). There were reports of the decline in yield of cocoa after the twelfth year of planting due to VSD, in unpruned stands in Papua New Guinea (Keane, 1981).

2.3.5 Management of Vascular Streak Dieback disease

The VSD disease management has been categorized in to following ways

2.3.5.1 Cultural practices

Clean nursery stock raised well away from diseased cocoa ensures that stock transplanted into the field is disease-free. Nursery stock can be further protected by growing it in a shade house or under a shelter that keeps leaves dry for all but a few hours following watering.

Soekirman and Agus (1992) reported that very high levels of infection and subsequent death have been observed in unprotected plants raised near or under older, diseased cocoa. Pruning diseased material about 30-cm below discolored xylem prevents further extension of infections and reduces inoculum levels by removing potential sites of sporulation. In a mature plantation in Java, where highly trained teams detected and pruned there infected branches every 2 weeks for nearly 2 years, the incidence of infected trees was kept below 1 per cent. In contrast, in an unpruned planting, disease incidence increased from about 30 to 90 per cent in 10 months. Dennis and Keane (1992) suggested that shade and canopy management to increase aeration and sun light on the foliage is of critical importance as sporulation and infection require moist conditions. These practices are best combined with integrated management practices designed to control *Phytophthora palmivora* and other pests and diseases.

2.3.5.2 Biological control

Oncobasidium theobromae is a vascular pathogen that infects immature plant leaves. Potential exists to investigate the use of epiphytic microbes to reduce leaf infection, and endophytic fungi and bacteria to protect against vascular colonization. Biological control agents could also be targeted at the basidiocarp stage.

2.3.5.3 Chemical control

According to Prior (1980) protective fungicides were unlikely to be effective in controlling VSD of cocoa in Papua New Guinea. However, Keane and Prior (1992) suggested that systemic fungicides should have particular value for the control of the disease. In spite of the obvious ineffectiveness of both protectant and systemic fungicides in the control of VSD, many studies were carried out to control the disease with fungicides. Ahmad (1982) observed that bitertanol at 1500 ppm gave significant reduction of VSD. Chung (1982) also reported the effectiveness of bitertanol in controlling the diseases.

In vitro screening studies with bitertanil, triadimefon, triadimenol, propiconazole, pp969 (all belonging to triazole group) and benomyl showed toxicity against *Oncobasidium* (Donough, 1984; Varghese *et al.*, 1985). Musa and Tey (1986) reported total inhibition of mycelial growth of *Oncobasidium* by benomyl @ 5 ppm.

Gurmit (1986) observed more than 90 per cent control for VSD in seedling with soil drenching of triadimefon at monthly intervals. From Papua New Guinea, Prior (1986) reported the effectiveness of propiconazole as spray and stem painting in reducing VSD incidence in cocoa seedlings. Sidhu (1987) reported that foliar spraying of triadimenol and pp969 at weekly and fortnightly intervals provided seedling protection against VSD in nursery. Holderness (1990) noticed that systemic fungicides *viz.* tebuconazole, hexaconazole and triademenol, when given as monthly

foliar spray, gave good protection against VSD of cocoa but they resulted in stunting of seedlings.

Abraham and Ravi (1991) reported that Bordeaux mixture and Kitazin as foliar spray were effective in checking the severity of VSD to a certain extent in older plants. Hee *et al.* (1992) reported that monthly soil drenching of flutriafol at 25-200 mg ai per plant controlled VSD and seedlings were found vegetatively more vigorous compared to untreated plants. Foliar spray of flutriafol at 200 ppm and triadimefon at 500 ppm also gave good disease control. Chemical control of VSD has been difficult mainly due to the fungus responsible being active within the vascular tissue of the host.

2.3.5.4 Quarantine

Prior (1980) suggested that the occurrence and distribution of VSD in Papua New Guinea showed, although it is common in some regions of the country and islands, it is not recorded in some of the other islands. Thus quarantine measures had been recommended as a method of control by enforcing a total ban in the movement of materials from disease infected areas to disease free areas. Byrne (1976) reported that in Malaysia, movement of cocoa vegetative planting material from VSD areas to VSD free areas is banned. Keane and Prior (1992) shows that extensive tests of seed collected from pods on infected branches have not been able to demonstrate transmission of the disease through seeds, suggesting that seed from the VSD infected areas could be moved to VSD free areas as planting material.

2.3.5.5 Disease resistance

The past studies largely concentrated on the management strategies that involved sanitation, appropriate farm management practices, chemical control and genetic resistance in the host plant. Prior (1978 and 1979) suggested that selection of resistant planting materials is perhaps the most practical approach to long term

control of VSD. The decline in the severity of VSD which occurred in epidemic proportions in the early 1960's in Papua New Guinea had been attributed to be partly due to the distribution of resistant clonal materials.

Screening for resistance in cocoa seedlings and clonal cuttings had been attempted by inoculating spore suspensions of *Oncobasidium theobromae* in the upper surface of a young unexpanded leaf and onto the stipules of the apical bud. Although considerable variation in resistance and susceptibility had been observed by this method, no cultivars had been observed to be completely resistant to VSD (Prior, 1978).

The recommendations that have been delivered are not widely adopted by the farmers and cocoa production losses to the two diseases are still high. Planting of cacao varieties resistant to VSD disease has been seen as the most economical approach to sustain production (Efron *et al.*, 2005). As such, development of cacao varieties resistant to VSD and combined with other desirable traits constitutes one of the main objectives of cacao breeding.

2.4 Molecular markers

Molecular markers are fragments of nuclear, mitochondrial or chloroplast DNA, which are linked with the gene of interest and hence acting as representative of the gene. Molecular marker analysis based on polymorphism in DNA, can be considered as objective measures of genetic variations and have catalyzed research in a variety of disciplines such as phylogeny, taxonomy, ecology, genetics and plant breeding. Markers are informative only if, they are polymorphic in populations. Level of polymorphism is an important determinant of what a marker is useful for. Different types of molecular markers with different properties exist, each with its own advantages and disadvantages (Karp *et al.*, 1997; Weising *et al.*, 2005).

However, it is extremely difficult to find molecular markers which could adequately meet all the ideal properties (Lowe *et al.*, 2004). Depending upon the type of the study to be undertaken, one can identify between varieties of marker systems that could fulfil the objective of the study (Weising *et al.*, 2005). Many authors also suggest the use of more than one type of molecular marker in a single experiment (Karp *et al.*, 1997). The DNA based marker systems are generally classified as hybridization-based (non-PCR) markers and PCR based markers (Joshi *et al.*, 1999). The value of PCR based molecular markers is influenced by several consideration such as the speed, cost and technical simplicity, but must be sufficiently informative to distinguish between the most individuals (Charters and Wilkinsons, 2000).

2.4.1 PCR- based molecular marker techniques

Applications of PCR based molecular markers include assessment of genetic variability and characterization of germplasm, identification and fingerprinting of genotypes, estimation of genetic distance, detection of monogenic and quantitative trait loci (QTL), marker assisted selection and in identification of resistant gene. The techniques used for cultivar identification are designed to detect the presence of specific DNA sequences or combination of sequences that uniquely identify the plant. In almost every major crop, molecular markers associated with every character and interests have been identified (Gurta *et al.*, 1999).

2.4.2 Polymerase Chain Reaction (PCR)

The Polymerase Chain Reaction (PCR) technique has been the basis of a growing range of new techniques for genome analysis, based on the selective amplification of genomic DNA fragments (Saiki *et al.*, 1988). Williams *et al.* (1990) reported the use of PCR with short oligonucleotide primers of arbitrary (random) sequence to generate markers, the basis of the Random Amplified Polymorphic DNA (RAPD). Welsh and McClelland (1990) also reported on Arbitrarily Primed

Polymerase Chain Reaction (AP-PCR). DNA Amplification Fingerprinting (DAF) was also reported as another technique of PCR used in various experiments (Caetano-Anolles *et al.*, 1991). The PCR reaction requires deoxynucleotides, DNA polymerase, primer, template and buffer containing magnesium (Taylor, 1991). Typical PCR amplification utilises oligonucleotide primers which hybridise to complementary strands. The product of DNA synthesis of one primer serves as template for another primer. The PCR process requires repeated cycles of DNA denaturation, annealing and extension with DNA polymerase enzyme, leading to amplification of the target sequence. This results in an exponential increase in the number of copies of the region amplified by the primer (Saiki *et al.*, 1988). The technique can be applied to detect polymorphism in various plants, animals, bacterial species and fungi.

The introduction of the PCR technique has revolutionized standard molecular techniques and was allowed for the proliferation of new tools for detecting DNA polymorphism (Hu and Quiros, 1991). The electrophoresis pattern of fragments generated by each primer for one isolate can be used as DNA fingerprints for assaying diversity (Tommerup *et al.*, 1998). Polymorphism between two individuals is generally scored as presence or absence (non-amplification) of a particular DNA fragment. The absence may result from deletion of a priming site or insertion rendering site too distant for successful amplification. Insertion can change the size of a DNA fragment without preventing its amplification (Williams *et al.*, 1990). PCR is simple, fast, specific, sensitive and the main advantage of this technique over others is its inherent simplistic analysis and the ability to amplify extremely, small quantities of DNA (Welsch *et al.*, 1991).

2.4.3 Molecular markers for identification of resistance genes

Identification of molecular markers linked to disease resistance genes facilitates marker-assisted selection (MAS) for achieving gene combinations in breeding programs (Sharp *et al.* 2001). Various molecular marker systems such as

RFLP, RAPD, ISSR, AFLP and microsatellites (SSRs) have been widely used to tag resistance genes in all the crops.

2.4.3.1 Random Amplified Polymorphic DNAs (RAPD)

Martin *et al.* (1991) used RAPD marker system to identify the markers linked to the *pto* locus giving resistance to the bacteria *Pseudomonas syringae* using the near isogenic lines in tomato. Similar marker system was used by Chalmers *et al.* (1992) to isolate markers linked to genetic factors contributing to the milling energy (ME) of the barley grain. They applied RAPD marker system for bulked segregant analysis (BSA), on doubled haploids. Again Barua *et al.* (1993) found that RAPD markers are the most efficient and cost effective means of isolating molecular markers linked to genes located on introgressed DNA segments. They identified RAPD markers linked to a *Rhynchosporium secalis* resistance locus in barley through BSA on near-isogenic lines.

Penner *et al.* (1993) observed ten polymorphic fragments between the bulks constructed for crown rust resistance but only one of the polymorphic DNA fragment was found to be tightly linked to the crown rust resistance gene. Similarly, Miklas *et al.* (1993) identified one RAPD marker named OPA-141100 linked to rust resistance in common bean. Poulsen *et al.* (1995) identified a 2.7 kb RAPD marker, linked to leaf rust resistance gene in barley generated by the primer OPU-02. Results of earlier studies indicate that, the RAPD markers linked to trait of interest can easily be identified using bulked segregant analysis and may be used effectively in breeding programmes.

Naqvi *et al.* (1995) used the bulked segregant RAPD analysis for rapid identification of DNA markers linked to*Pi-10t* blast resistance gene in rice. They used Pooled DNA extracts from five homozygous blast-resistant (RR) and five susceptible (rr) BC_3F_2 plants, derived from a CO39 × Tongil cross. Agwanda *et al.*

(1997) identified the RAPD markers for resistance to coffee berry disease, *Colletotrìchuin kahawae*, in arabica coffee. Random amplified polymorphic DNA (RAPD) has proved useful in tagging resistance genes in a number of crops, including apples (Gianfranceschi *et al.*, 1994; Yang & Krüger, 1994), barley (Poulsen *et al.*, 1995), Brassica napus (Foisset *et al.*, 1995), rice (Nair *et al.*, 1995), sunflower (Mouzeyar *et al.*, 1995) and wheat (Talbert *et al.*, 1996).

Sun *et al.* (1997) have used 340 RAPD primers to identify the molecular markers linked to the *Yr15* stripe rust resistance gene of wheat. Six polymorphic bands among the susceptible and the resistant lines were identified. The genetic linkage of the polymorphic markers was tested on segregating F2 population. Robert *et al.* (1999) identified molecular markers for the detection of the yellow rust resistance gene *Yr17* in wheat. They have used RAPD primers, for polymorphism, using a F2 progeny of the cross between VPM1 (resistant) and these (susceptible), they found RAPD marker OP-Y15580 was closely linked to the *Yr17* gene.

Sartorato *et al.* (2000) reported the RAPD and SCAR markers linked to resistance gene to angular leaf spot in common bean. They identified three RAPD markers in the F2 population, $OPN02_{890}$, $OPAC14_{2400}$ and $OPE04_{650}$. These markers were mapped in coupling phase at 5.9, 6.6 and 11.8 cM from the resistant gene respectively. The $OPN02_{890}$ fragment was transformed in to a SCAR marker. The polymorphism observed after amplification was identical to the one revealed with corresponding RAPD marker. Tullu *et al.* (2003) identify RAPD molecular markers linked to the anthracnose resistance gene in a recombinant inbred line (RIL) population developed from lentils, by a cross of eston lentil, the susceptible parent, and PI 320937 as resistant parent.

Chen *et al.* (2004) did RAPD analysis between a near-isogenic line (NIL) Yr5/6 x Avocet S carrying the resistance gene *Yr5* against wheat stripe rust and its susceptible parent Avocet S, using the *Yr5* gene donor parent *Triticum spelta* album

as control. Amplified product is separated on 4 per cent polyacrylamide gel electrophoresis, they suggested that using denaturing PAGE-silver staining could increase the level of DNA polymorphisms detected in wheat and also improve the repeatability of RAPD analysis. Kumar *et al.* (2006) screened 104 wheat recombinant inbred lines (RILs) from a cross between resistant parents (HD 29) and susceptible (WH 542) to karnal bunt (KB) disease using random amplified polymorphic DNA (RAPD) markers. Marker OPM-20 showed apparent association with resistance to KB.

Malik *et al.* (2007) reported the DNA marker for leaf rust disease resistance in wheat, PCR technique and bulked segregant analysis was used to identify DNA marker linked to leaf rust resistant gene in F2 segregating population. The F2 population derived from the cross involving leaf rust susceptible, SK-7 and resistant parent, PBG- 8881 with *Lr29* resistant gene was scored for disease resistance. The F2 population segregated into resistant and susceptible plants in a ratio of 3:1 showing monogenic inheritance. The primer 60-5 amplified a polymorphic molecule of 1100 base pairs from the genomic DNA of resistant plant. This DNA molecule can be used as DNA marker to identify leaf rust resistant plants in a breeding programme for developing rust resistant wheat cultivars.

Araújo *et al.* (2002) RAPD technique was used to identify molecular markers linked to blast resistance gene *Pi-ar* utilizing bulked segregant analysis in somaclone of rice cultivar araguaia, by using random primers for two parental DNAs from the resistant donor SC09 and 'Araguaia'. Random amplified polymorphic DNA (RAPD) markers have been widely used as effective tools for the indirect selection of disease resistance genes once the linkages between markers and the resistance genes have been established (Haley *et al.*, 1993; Miklas *et al.*, 1993; Carvalho *et al.*, 1998; Castanheira *et al.*, 1999).

2.4.3.2 Restriction Fragment Length Polymorphism (RFLPs)

Pineda *et al.* (1993) used a total 60 RFLP markers for identification of *H1* gene giving resistance to the potato cyst nematode *Globodera rostochiensis*. A molecular marker for the *H1* is useful for correlative screening tool for incorporation of resistance into new cultivars and for map-based cloning. By using RFLP marker Williams *et al.* (1990) did the same work for the cereal cyst nematode resistance gene (*Cre*) in wheat.

Wang *et al.* (1994) used RFLP marker for mapping of genes conferring complete and partial resistance to blast in a durably resistant rice cultivar. By using 127 RFLP markers they analyze the recombinant inbred (RI) lines produced from single seed descent for blast resistance in the greenhouse and the field plants.

Huang *et al.* (1997) had shown that pyramiding of bacterial blight resistance genes in rice with marker-assisted selection using RFLP and PCR. They developed PCR markers for the two recessive genes, *xa-5* and *xa-13*, and used these to survey a range of rice germplasm and the selection of parents in breeding programs aimed at transferring these bacterial blight resistance genes from one varietal background to another. Hittalmani *et al.* (2000) reported the fine mapping and DNA marker-assisted pyramiding of the three major genes for blast resistance in rice. They have found three major genes (*Pi*1, *Piz*-5 and *Pita*) for blast resistance on chromosomes 11, 6 and 12, respectively and were fine-mapped. The closely linked RFLP markers have been identified. Buna *et al.* (2001) use RFLP markers for the mapping of two new brown plant hopper resistance genes from wild rice. They use bulked segregant analysis to identify RFLP makers linked to the brown plant hopper resistance genes in B5 rice variety. RFLP method was used to rapidly identify the markers linked to the BPH resistance in B5 rice variety.

By using RFLP markers Fujji *et al.* (2000) precisely mapped the *Pb1* locus for the panicle blast resistance gene of a quantitative nature on rice chromosome 11. They used 21 RFLP markers for screening the resistant and susceptible parents for panicle blast resistance. Gillen and Bliss, (2005) use RFLP markers for the identification and mapping of markers linked to the *Mi* gene for root knot nematode resistance in peach.

2.4.3.3 Inter Simple Sequence Repeats (ISSR)

Ratnaparkhe *et al.* (1998, 1998a) reported that inter-simple-sequence-repeat (ISSR) polymorphisms are useful for finding markers associated with disease resistance gene clusters. The repeat $(AC)_8T$ amplified a marker, UBC- 825_{1200}, which was located 5.0 cM from the gene for resistance to fusarium wilt race 4 and was closer than other markers. These results shows that ISSR markers can provide important information for the design of other primers and that by making changes at the 3' and 5' anchors, close linkage to the desired gene can be found. They also demonstrated that ISSR markers are useful in gene tagging and identified a marker, UBC-855_{500}, linked to the gene for resistance to fusarium wilt race 4 in chickpea.

Santra *et al.* (2000) have reported identification and mapping of QTLs conferring resistance to ascochyta blight in chickpea. They found two quantitative trait loci (QTLs), QTL-1 and QTL-2, conferring resistance to ascochyta blight, were identified which accounted for 50.3 and 45.0 per cent of the estimated phenotypic variation in 1997 and 1998, respectively. Also they identified two RAPD markers flanked QTL-1 and were 10.9 cM apart while one ISSR marker and an isozyme marker flanked QTL-2 and were 5.9 cM apart. These markers can be used for marker-assisted selection for ascochyta blight resistance in chickpea breeding programs, and to develop durable resistant cultivars through gene pyramiding.

Raina *et al.* (2001) use ISSR and RAPD fingerprints as genetic markers for analysis of genetic diversity, varietal identification, and phylogenetic relationships in peanut (*Arachis hypogaea*) cultivars and wild species. Khan *et al.* (2005) reported that molecular mapping of stem and leaf rust resistance in wheat. A total of 1,330 RAPD and 100 ISSR primers and 33 SSR primer pairs selected on the basis of chromosomal locations of these genes were used. The ISSR marker UBC 840_{540} was found to be linked with *Lr3a* in repulsion at a distance of 6.0 cM. The advantages of ISSR markers are well documented in agronomic species (Godwin *et al.*, 1997). Genome analysis in woody perennials using ISSR has been shown to be possible in Douglas fir and sugi (Tsumura *et al.*, 1996), citrus (Fang *et al.*, 1998), and *Morus* (Awasthi *et al.* 2000).

Milad *et al.* (2011) reported that twenty-five ISSR and thirty eight RAPD primers were tested for polymorphism in parental genotypes and F2 population. Study indicates that four RAPD and two ISSR markers linked to the flag leaf senescence gene in wheat. They got two ISSR markers were linked to the QTL for the flag leaf senescence gene as indicator of drought tolerance. These markers can be used in wheat breeding programs, as a selection tool in early generations. ISSRs have been used successfully in genome mapping for a variety of crop species including maize, rice, barley and wheat (Ben El Maati *et al.*, 2004; Barakat *et al.*, 2010).

Deshmukh *et al.* (2011) reported ISSR and RAPD markers for identification of drought of tolerance in wheat (*Triticum aestivum* L.). They used 90 ISSR primers, out of these only 3 ISSR primers gave polymorphism in relation to the drought tolerance trait exhibiting 21.38 per cent polymorphism. Tahery, (2012) reported ISSR markers are linked to the root knot nematode resistance of *Hibiscus cannabinus*. They analyze sixty ISSR primers to genotype parental and F2 population DNAs and found thirteen polymorphic ISSR markers between the resistant and susceptible parents. Five ISSR markers appeared to be linked with RKN resistance.

2.4.3.4 Amplified Fragment Length Polymorphism (AFLP)

AFLP analysis is a genetic mapping technique that uses selective amplification of a subset of restriction enzyme-digested DNA fragments to generate a unique fingerprint for a particular genome. By using fluorescently based AFLP analysis Hartl *et al.*, (1999) did the screening of a total 7654 DNA fragments for linkage to wheat powdery mildew resistance gene *Pm1c* in common wheat.

In wheat, markers for rust resistance *Sr2* (Hayden *et al.*, 2004), *Sr39* (Gold *et al.*, 1999), *Lr19* (Prins *et al.*, 2001) and *Lr39* (Raupp *et al.*, 2001). Furthermore, there have been efforts to isolate and characterize leaf rust resistance genes by high resolution mapping for *Lr1* (Ling *et al.*, 2003) and map-based cloning for *Lr10* (Feuillet *et al.*, 2003), and *Lr21* (Huang *et al.*, 2003).

Mago *et al.* (2002) show that the identification and mapping of molecular markers linked to rust resistance genes located on chromosome 1RS of rye using wheat-rye translocation lines with AFLP, RFLP and RGA markers located on the short arm of chromosome 1 of rye which carries leaf (*Lr26*), stem (*Sr31/SrR*) and stripe (*Yr9*) rust resistance genes. Cai *et al.* (2003) used a combination of the amplified fragment length polymorphism (AFLP) technique and bulked segregant analysis (BSA) to a large F2 population in order to identify molecular markers linked to the *rhm* gene for resistance to Southern Corn Leaf Blight (SCLB). Ashutosh *et al.* (2007) identified the AFLP markers linked to the male fertility restorer gene of CMS *Brassica juncea* by using 64 primer combinations and converted it in to SCAR marker. They use the bulk segregant analysis method to segregate the sterile and fertile lines.

Amplified fragment length polymorphism in conjunction with BSA has been successfully used to identify markers linked to fertility restorer gene in petunia (Bentolila *et al.* 1998; Bentolila and Hanson, 2001), sorghum (Klein *et al.* 2001),

radish (Imai *et al.* 2003; Koizuka *et al.* 2003; Murayama *et al.* 2003), rapeseed (Janeja *et al.* 2003), sunflower (Horn *et al.* 2003) and sugar beet (Touzet *et al.* 2004; Hagihara *et al.* 2005). Shou *et al.* (2006) used AFLP analysis on two parents and their F2 resistant and susceptible bulks for identification of markers linked to bacterial wilt resistance gene in tomato. They got a total of 4200 distinguishable amplified bands. The DNA fragment AAG/CAT was found closely linked to one of the bacterial wilt resistant genes, with a genetic distance of 6.7 cM.

Brito *et al.* (2010) did the study to characterize the inheritance of coffee resistance gene(s) to race II of coffee leaf rust pathogen and to identify and map AFLP molecular markers linked to the coffee leaf rust resistance trait. Using AFLP markers, Thakur *et al.* (2014) were successful to identify the markers linked with the different alleles of the gene offering resistance to bacterial wilt disease in chili. They used the BSA on F2 population derived from a cross of NILs, susceptible Pusa Jwala and resistant Anugraha.

2.4.3.5 Simple Sequence Repeats (SSRs)

Microsatellites, or simple sequence repeats (SSRs), are stretches of DNA consisting of tandemly repeated short units of 1–6 base pairs in length. The uniqueness and the value of microsatellites arise from their multiallelic nature, codominant inheritance, relative abundance, extensive genome coverage and simple detection by PCR using unique primers, that flanks the microsatellite and hence the microsatellite locus (Powell *et al.* 1996). Xu *et al.* (1999) studied the RFLP, SSR and AFLP markers in maize for high resolution mapping of loci conferring resistance to sugarcane mosaic virus.

Yu *et al.* (1993) used simple sequence repeats as genetic marker for the identification of the chromosomal location of *Rsv* gene which confer resistance to soybean mosaic virus (SVM). They found SSR marker SM176 which is a soybean

heat shock protein gene is closely linked to the *Rsv* gene with 0.5 cM distance. Using 50 SSR primers Mittal and Boora, (2005) tagged the gene for leaf blight resistance in sorghum; they used the resistant and susceptible bulked DNA samples for screening, found 38 primes giving polymorphism for leaf blight disease.

Lillemo *et al.* (2008) used SSR markers to identify molecular markers associated with the resistance to powdery mildew in wheat. Two major QTLs were identified, one on chromosome 7D and the other on chromosome 1B, corresponding to the adult plant rust resistance loci *Lr34/Yr18* and *Lr46/Yr29,* respectively. Wu *et al.* (2014) identified and mapped powdery mildew resistance gene *VU-PM1* in the Chinese asparagus bean landrace ZN016 using SSR and SNP markers. The powdery mildew resistance gene was mapped to a locus on *LG9* flanked by SSR markers *CLM0305* and *CLM0260*, with a genetic distance of 2.0 cM and 5.0 cM.

2.4.4 Molecular markers in cocoa

The first type of molecular markers applied to cacao was isozymes, which have been used in genetic diversity and population structure studies (Lanaud, 1986). However, with the limited number of polymorphic enzymatic systems in cacao, their use in other genetic studies is restricted, apart from their potential use for germplasm characterization, population genetic studies and cultivar identification (Figueira, 2004).

Restriction fragment length polymorphisms (RFLPs) was the first DNA based marker that was applied to cacao in genetic diversity studies and construction of cacao genetic maps (Lanaud *et al.*, 1995; Crouzillat *et al.*, 1996). RFLPs are highly demanding technically, time-consuming, labour intensive and not amendable to automation. As a result, their use has been restricted to advanced laboratories. The next groups of DNA markers in cacao were developed based on polymerase chain reaction (PCR). These include random amplified polymorphic DNA (RAPD),

amplified fragment length polymorphism (AFLP), microsatellites or single sequence repeats (SSRs) and single nucleotide polymorphisms (SNPs). RAPD has been used in cacao genetic diversity studies (N'goran *et al.*, 1994) and AFLP, mostly used for genetic mapping (Clement *et al.*, 2003a; Queiroz *et al.*, 2003; Risterucci *et al.*, 2003).

In cocoa SSRs were first described by Lanaud *et al.* (1999). Since then, over 300 SSRs covering the 10 cacao linkage groups have been developed from various genomic DNA and expressed sequence tag libraries (Kuhn *et al.*, 2003; Pugh *et al.*, 2004; Lanaud *et al.*, 2004; Borrone *et al.*, 2007; Schnell *et al.*, 2007a). SSRs proved to be best markers because they are highly polymorphic, uniformly distributed throughout the genome, co-dominant, reproducible and easy to interpret. As such, SSR markers have been used widely in various genetic studies of cacao, including genetic mapping and identification of quantitative trait loci (QTLs) (Crouzillat *et al.*, 2000a, 2000b; Flament *et al.*, 2001; Queiroz *et al.*, 2003; Clement *et al.*, 2003a, 2003b; Pugh *et al.*, 2004; Brown *et al.*, 2005, 2007; Faleiro *et al.*, 2006), genetic diversity and population structure (Marita *et al.*, 2001; Sereno *et al.*, 2006; Motamayor *et al.*, 2008; Zhang *et al.*, 2008), parental analysis and identification of off-types (Motamayor and Lanaud, 2002; Motamayor *et al.*, 2002, 2003; Motilal and Butler, 2003; Turnbull *et al.*, 2004; Schnell *et al.*, 2004; 2005; Takrama *et al.*, 2005; Efombagn *et al.*, 2006) and MAS (Schnell *et al.*, 2007a, 2007b).

SNPs are the most common polymorphism detectable markers which occur in the coding and non-coding regions of DNA. SNP markers are increasingly being used in many crop species in preference to SSRs, particularly in crop breeding programs. SNPs provide many benefits over SSRs, including ease of analysis and unambiguous results across various platforms. In addition, as the most frequent DNA polymorphism, SNP markers allow for more detailed genetic maps (Segmagn *et al.*, 2006; Livingstone *et al.*, 2011). In cacao, a number of SNPs (mainly from candidate

genes), related to disease resistance and biotic and abiotic stress, have been identified (Borrone *et al.*, 2004; Kuhn *et al.*, 2005; Argout *et al.*, 2008; Lima *et al.*, 2009).

Several of these SNPs have been developed into single-strand conformational polymorphism (SSCP) markers that have been shown to successfully genotype cocoa plants (Borrone *et al.*, 2004; Kuhn *et al.*, 2005, 2008). These SSCP markers have been added to genetic linkage maps (Livingstone *et al.*, 2011). More SNP markers for cacao have been developed, allowing substantially more detailed genetic maps to be constructed from various cacao populations. In addition, SNPs have shown to perform nearly as well as SSRs in their ability to differentiate off-types among a cacao clonal population (Livingstone *et al.*, 2011). Thus, SNP markers hold a great potential for enhancing cacao breeding programs.

2.4.5 Inter simple sequence repeats (ISSR) markers in cocoa

ISSRs are DNA fragments of about 100-3000 base pairs located between adjacent, oppositely oriented microsatellite regions. ISSRs are amplified by PCR using microsatellite core sequences as primers with a few selective nucleotides as anchors into the non-repeat adjacent regions (16-18 bp). About 10 fragments from multiple loci are generated simultaneously, separated by gel electrophoresis and scored as the presence or absence of fragments of particular size. The ISSR primer is composed of microsatellite sequences either unanchored (Gupta *et al.*, 1994; Wu *et al.*, 1994) or anchored at the 5` or 3` end by two or four arbitrary nucleotides (Zietkiewicz *et al.*, 1994; Fang and Roose, 1997; Fang *et al.*, 1998). The sequence between the two binding sites in opposite orientation within suitable distance is amplified and loss or gains of binding sites are detected as band polymorphism (Yang *et al.*, 1996). The addition of a different base at the 5` or 3` end renders their binding sites more specific and reproducible (Barth *et al.*, 2002).

ISSR method has been used extensively to identify and determine relationships at the species and cultivar levels (Martins *et al.*, 2003). This method is widely applicable because it does not need sequence data for primer construction and is rapid, inexpensive and randomly distributed throughout the genome. The ISSR method has been reported to produce more complex marker patterns than the RAPD approach, which is advantageous when differentiating closely related cultivars (Parsons *et al.*, 1997; Chowdhury *et al.*, 2002). In addition, ISSR markers are more reproducible than RAPD markers (Goulão and. Oliveira, 2001), because ISSR primers, designed to anneal to a microsatellite sequence, are longer than RAPD primers, allowing higher annealing temperatures to be used. Also because of the multi locus fingerprinting profiles obtained, ISSR analysis can be applied in studies involving genetic identity, parentage, clone and strain identification, and taxonomic studies of closely related species. In addition, ISSRs are considered useful in gene mapping studies (Godwin *et al.*, 1997). Genome analysis in woody perennials using ISSR has been shown to be possible in cocoa (Charters and Wilkinson, 2000).

Julio *et al.* (2011) did the characterization of a *Theobroma cacao* L. collection at Tingo Maria using ISSR molecular markers. They testeed the potential of ISSR (Inter Simple Sequence Repeats) markers to differentiate 46 cacao accessions maintained in Tingo Maria - Perú, and the similarity relationships between the accessions. In this study, the results showed that ISSR, despite its dominance nature, could establish eye-catching associations, such as, the grouping of Trinitario accessions into a common cluster.

2.4.6 Simple sequence repeats (SSR) markers in cocoa

Microsatellites (SSRs) are present in the majority of eukaryotic genomes. Simple sequence repeat or microsatellite is the term used to refer tandemly repeated short nucleotide units between 1-5 bp in the genome (Staub *et al.*, 1996; Powell *et al.*, 1996). These repeats show a genome-wide distribution and can be placed in either

genes or non-coding regions of the nuclear genome or else in extra-nuclear genomes (Nunome *et al.*, 2003a, 2003b; Varshney *et al.*, 2005). In the genome, this distribution was reported to be collected around particular regions of the chromosomes such as centromeric areas. For the generation of SSR markers, sequence data would be required. Polymerase slippage during DNA replication, or slipped strand mispairing, is considered to be the main cause of variation in the number of repeat units of a microsatellite, resulting in polymorphism that can be detected by gel electrophoresis (Lanteri and Barcaccia, 2005).

SSR is a PCR based molecular method (Staub *et al.*, 1996) and the principle is the detection of polymorphism resulting from different numbers of repeat units in different individuals and is observed co-dominantly (Powell *et al.*, 1996; Jones *et al.*, 1997). The level of polymorphism is very high which makes SSR an ideal marker for mapping, diversity studies, fingerprinting and population genetics (Jones *et al.*, 1997; Mohan *et al.*, 1997). However, the major disadvantage is related with the development of SSR primers, that requires sequence information.

Brown *et al.* (2005) constructed the resistant gene mapping for witches broom disease in cocoa using the 170 SSR markers and they found two quantitative trait loci for resistance to witches broom disease, both showing important dominance effect. By using 13 SSR primers Lima *et al.* (2013) check the genetic molecular diversity to the resistance to witches broom disease in cocoa clones.

2.4.6.1 Development of SSR markers in cocoa

Microsatellites are powerful genetic markers due to several characteristics, including their abundance in eukaryotic genomes, high levels of polymorphism, Mendelian inheritance, co-dominance and locus-specificity (Pugh *et al.*, 2004; Merdinoglu *et al.*, 2005). In cocoa, microsatellites have been applied in studies of DNA fingerprint, genetic diversity, variety characterization and genetic mapping

(Charters and Wilkinson 2000; Faleiro *et al.*, 2004; Pugh *et al.*, 2004; Saunders *et al.*, 2001, 2004). Lanaud *et al.* (1999) developed the first group of simple sequence repeat (SSR) markers for T. cacao. More recently, Pugh *et al.* (2004) developed 387 new SSR markers for this species. However, all of these SSR loci were isolated using di nucleotide probes during the screening of the genomic library consequently; SSR loci consisting of repeats of tri- and tetra-nucleotides remain to be searched in the *T. cacao* genome. Araujo *et al.* (2007) develop a new group of SSR markers, including tri and tetra-nucleotide repeats. They had attempted to construct 13 genomic libraries, enriched for different SSR sequences, and subsequently to use these libraries to identify and characterize the new microsatellites.

Different methods have been used for the characterization of SSR loci. Initially, microsatellites were isolated from partial genomic libraries with small insert size. Large number of clones was screened by colony hybridization with repeat containing probes. Later, in order to reduce the time invested in microsatellite isolation and to significantly increase yield, library enrichment techniques were developed (Holton, 2001; Zane *et al.*, 2002). Microsatellite loci have been developed using RAPD-PCR of genomic DNA (Ender *et al.*, 1996; Lunt *et al.*, 1999; Liu *et al.*, 2008) and an AFLP-PCR approach called FIASCO (Fast Isolation by AFLP of Sequences Containing repeats) (Zane *et al.*, 2002; Sun *et al.*, 2008; Zang *et al.*, 2008).

2.4.6.2 Applications of SSR markers in cocoa breeding

In cocoa microsatellites have been widely used in many crop species due to their abundance, high degree of polymorphism, locus specificity, reproducibility, low amount of DNA required, suitability for multiplexing on automated systems and above all, their co-dominant mode of inheritance (Pugh *et al.*, 2004; Merdinoglu *et al.*, 2005). These characteristics make SSRs an attractive option for studying the disease resistance in the cacao. The availability of microsatellites open new avenues for tagging genes of economic importance, not only for marker-assisted selection, but

also for cloning genes leading to the development of transgenic plants for crop improvement (Pugh *et al.*, 2004).

Efombagn *et al.* (2006) reported that genetic diversity in cocoa germplasm of Southern Cameroon revealed by simple sequences repeat (SSRs) markers. They use 13 SSR markers. The gene diversity, genetic differentiation and genetic similarities were analyzed for the different populations. In total, 282 alleles were detected within all the populations studied. They found that the progenies of these parents have so far been poorly used in the cocoa farms surveyed.

Lima *et al.* (2010) studied the detection and analysis of SSR markers derived of cacao–*Moniliophthora perniciosa* expressed sequence tags (ESTs) in relation to cacao resistance to witches' broom disease (WBD). Forty-nine EST SSR primers were designed and evaluated in 21 cacao accessions, 12 revealed polymorphism, having 47 alleles in total, with an average of 3.92 alleles per locus. On the other hand, the 11 genomic SSR markers revealed a total of 47 alleles, with an average of 5.22 alleles per locus. Among the 12 polymorphic EST-SSR markers, two were mapped on the F_2 Sca 6 × ICS 1 population with reference for WBD resistance. Santos *et al.* (2012) develop the new polymorphic microsatellite loci for *Theobroma cacao* by isolation and characterization of microsatellites from enriched genomic libraries. They identified seventeen polymorphic microsatellite markers from enriched genomic libraries for cocoa.

2.4.6.3 Identification and nomenclature of microsatellite markers and designing of primer

Billote *et al.* (1999) described an easy method for developing microsatellite markers in tropical crops that has subsequently been used with success (Billote *et al.*, 2001; Aranzana *et al.*, 2002; Dirlewanger *et al.*, 2002). The main criteria for primer design were to produce well-matched primers that were 16-24 nucleotides long, had

an average GC content ranging between 40 and 50 per cent with an annealing temperature between 45°C and 55°C and were preferably G- or C- rich at the 3' end.

All loci are designated according to the nomenclature guidelines presented by Risterucci *et al.* (2000) and Lanaud *et al.* (2004). SSRs were denoted as mTcCIRX where 'm' corresponds to microsatellite, 'Tc' to *Theobroma cacao*, 'CIR' to CIRAD (Centre de Cooperation Internationale en Recherches Agronomique pour le Developpement) and 'X' to the microsatellite number, like that in SHRSTcX, 'SHRS' denotes the Subtropical Horticultural Research Station, 'Tc' to *Theobroma cacao* and 'X' is microsatellite number (Kuhn *et al.*, 2003).

2.5 Gene action

Genetic variation in disease resistance to vascular-streak dieback (VSD) was very important in the plant breeding programme. Since the early 1960's the heterogeneous resistance lowers the proportion of newly infected branches and also reduces the rate of invasion and subsequent sporulation of the fungus within infected branches (Keane 1981). According to Tan and Tan (1988) Gene effects were predominantly additive for most characters of the cocoa that indicates selection for progenies resistant to VSD is effective in the breeding population. There is strong evidence that resistance to VSD is in the form of horizontal resistance. It is polygenic and largely inherited as additive genes. The effect of gene is said to be additive when each additional gene enhances the expression of the trait by equal increments. Therefore, breeding for resistance is an effective means of controlling VSD.

To conclude, VSD is a very serious disease of cocoa, worldwide. Since the resistance is governed by polygenes with additive action, breeding is a promising strategy to combat the disease. Attempts to identify the molecular markers linked with resistance to this disease are scanty. Hence it is mandatory to take immediate action to validate the markers and to find the maximum number of markers for each

gene. ISSR and SSR are proven as the most suitable and repeatable system to tag the genes and the identified markers could be highly useful in marker assisted selection for resistance to vascular streak dieback in cocoa.

Material and Methods

3. MATERIALS AND METHODS

The study on "Identification of molecular marker linked to the resistance for vascular streak dieback disease in cocoa (*Theobroma cacao* L.)" was conducted at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Kerala Agricultural University during the period of 2012-2014. The materials used and methodologies adapted in the study are described in this chapter.

3.1. MATERIALS

3.1.1. Plant Materials

Plants of twelve cocoa genotypes *viz.*, VSD I- 4.6, VSD I- 4.11, VSD I- 5.8, VSD I- 6.9, G VI- 50, G VI- 52, G VI- 82, G VI- 100, G VI- 25, G VI- 53, G VI- 67, G VI- 144, having different levels of responses to vascular streak dieback disease (caused by *Oncobasidium theobromae*) maintained at Cocoa Research Centre, College of Horticulture, Vellanikkara, were used in this study (Table 3.1). The genotypes were selected on the basis of ten years of field observations for the incidence of VSD disease. Genotypes constitute the mapping population required for identification of molecular markers linked to the resistance for vascular streak dieback disease (VSD) by using SSR and ISSR markers.

3.1.2. Laboratory chemicals and glassware's

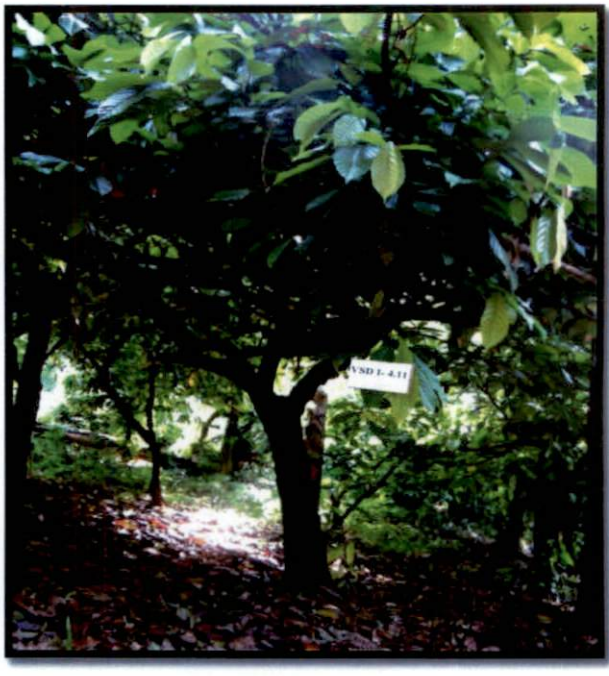
The chemicals used in this study were of good quality (AR grade) procured from Merck India Ltd., SRL, HIMEDIA and SISCO research Laboratories. The Taq DNA polymerase, Taq buffer and molecular weight marker (λ DNA */Hind III* + *Eco RI* double digest) were supplied by Banglore Genei. RNase A from Sigma, USA was used. The plastic wares used for the study were purchased from Tarsons India Ltd. and Axygen, USA.

Table 3.1 Details of the cocoa hybrids and clones used in the study

Sl. No.	Genotypes	Response to vascular streak dieback disease	Sources
1	VSD I- 4.6	Resistant	G II-19.5 x G VI-55
2	VSD I- 4.11	Resistant	G II-19.5 x G VI-55
3	VSD I- 5.8	Resistant	G VI-4 x G VI-55
4	VSD I- 6.9	Resistant	G VI-125 x G IV-18.5
5	G VI- 50	Susceptible	Clone ICS 6 Cocoa Germplasm, Kerala Agricultural University
6	G VI- 52	Susceptible	Clone Na 31 Cocoa Germplasm, Kerala Agricultural University
7	G VI- 82	Susceptible	Clone Landas 14 Cocoa Germplasm, Kerala Agricultural University
8	G VI- 100	Susceptible	Clone Landas 50 Cocoa Germplasm, Kerala Agricultural University
9	G VI- 25	Partially resistant	Clone T7/12 Cocoa Germplasm, Kerala Agricultural University
10	G VI- 53	Partially resistant	Clone MOQ 413 Cocoa Germplasm, Kerala Agricultural University
11	G VI- 67	Partially resistant	Introduction from Peru, Kerala Agricultural University
12	G VI- 144	Partially resistant	Local collection from Kottayam (Kerala State, India), Kerala Agricultural University

Further details on the clones available at ICGD webpage (International Cocoa Germplasm Database, http://www.icgd.rdg.ac.uk/)

VSD I- 4.6



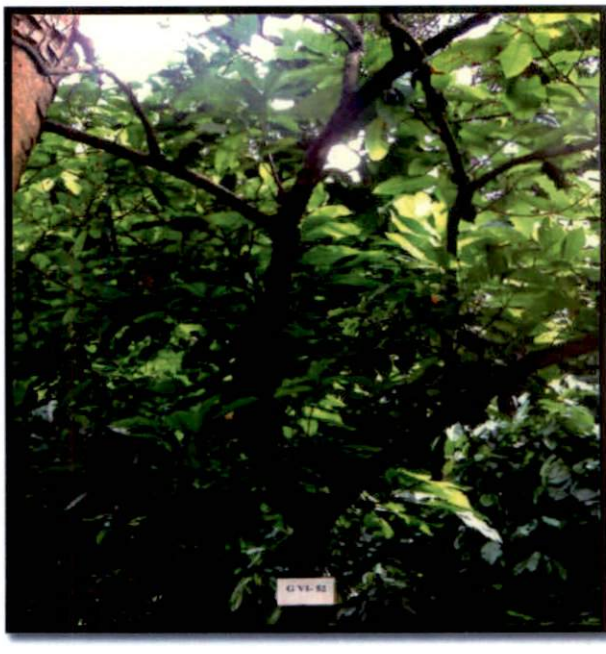
VSD I- 4.11

VSD I- 5.8

VSD I- 6.9

Plate 2: Resistant Plants

G VI- 50



G VI-52

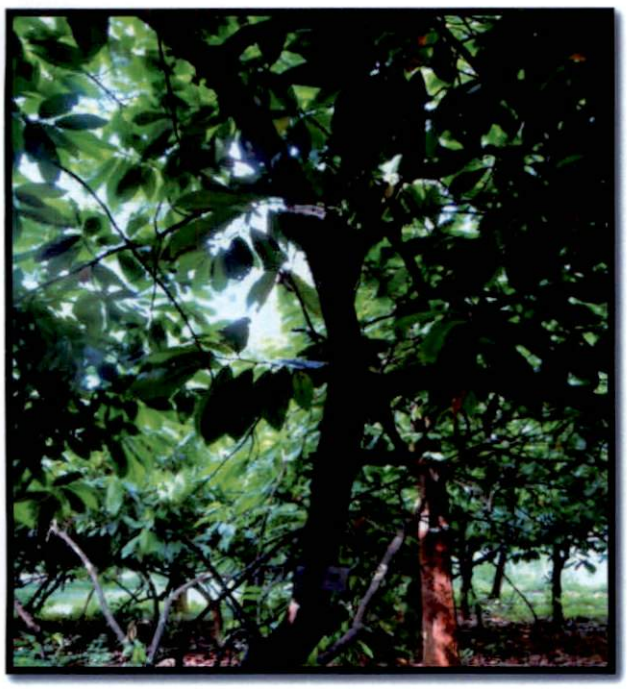
G VI-82

G VI- 100

Plate 3: Susceptible Plants

G VI- 25

G VI- 53



G VI- 67

G VI- 144

Plate 4: Partially resistant plants

3.1.3. Equipment and machinery

The equipments available at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture were used for the study. Centrifugation was done in KUBOTA 6500 high speed cooling floor model centrifuge. Dai Ki-S1010 (Dai Ki Scientific Co.) was used for the incubation of the DNA at 4° C . The PCR was done in Eppendorf Master Cycler (Eppendorf, USA) and agarose gel electrophoresis done in horizontal gel electrophoresis system (Bio-Rad). Gel DOC-It ™ Imaging system UVP (USA) was used for imaging the gel and gel picture were analysed using Quantity one software (Bio-Rad). The list of laboratory equipments used for the study is provided in Annexure I.

3.2. METHODS

3.2.1. Genomic DNA extraction

As reported in many other crops young needle shaped leaves were selected as the ideal part for extraction of the genomic DNA. Young tender, pale green leaves yielded good quality DNA in sufficient quantity.

Tender emerging leaves (first to third from the tip) were collected on ice early in the morning, from individual plants. The collected leaves were quickly covered in aluminum foils and transported to the laboratory in ice flask. The surface was cleaned by washing with sterile water and wiping with 70 per cent ethanol and stored at -80°C till being used. CTAB method developed by Doyle and Doyle (1987) was used for the extraction of genomic DNA.

Reagents

a. CTAB buffer (2X):
   - 2 per cent CTAB (W/V)
   - 100 mM Tris base (pH 8)

- 20 mM EDTA (pH 8)
- 1.4 M NaCl
- 1 per cent polyvinyl pyrrolidin (PVP)
- 0.2 per cent 2-β mercaptoethanol

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

c. Chilled isopropanol

d. Wash buffer
   - 76 per cent Ethyl alcohol
   - 10 mM ammonium acetate

e. Ethanol 70 and 100 per cent

f. TE buffer:
   - 10 mM Tris (pH 8)
   - 1 mM EDTA (pH 8)

g. Sterile distilled water

Reagent a. and f. were autoclaved and stored at room temperature.

List of chemicals given in annexure II.

Procedure

- Preheated 5-7.5 ml of CTAB isolation buffer (2X) in 50 ml Oakridge centrifuge tube to 60 °C in a water bath.

- Fresh leaf tissue (0.5-1.0 gm) was ground with a pinch of polyvinyl pyrrolidin (soluble) and 50µl of 2 per cent 2-β-mercaptoethanol in 60 °C CTAB isolation buffer in a preheated mortar and pestle.

- The samples were incubated at 60 °C for 30 (15-60) minutes with occasional gentle swirling.

- Equal volume of chloroform-isoamyl alcohol (24:1) mixture was added to the tube, mixed gently by inversion and centrifuged (Kubota 6500) at 12000 rpm for 20 minutes at room temperature.

- The content got separated in to three distinct phases.

Aqueous top layer	-	DNA with small quantity of RNA
Middle layer	-	Protein and fine particles
Lower layer	-	Chloroform, pigments and cell debris

- The top aqueous layer was transferred to a sterile centrifuge tube, equal volume of chloroform: isoamyl alcohol (24:1) was added and mixed by gentle inversions and subsequently centrifuged at 12,000 rpm for 20 minutes at room temperature.

- The aqueous phase has been transferred into a clean centrifuge tube and 0.6 volume (3 ml) of chilled isopropanol was added and mixed by gentle inversions till the DNA got precipitated. These tubes were kept at -20°C for half an hour for complete precipitation.

- After the expiry of time, tubes were centrifuged at 12,000 rpm for 15 minutes at 4°C and the supernatant was gently poured off.

- The DNA pellet was washed with 10-20 ml of wash buffer with centrifugation at 1000 rpm for 5 minutes.

- Then the supernatant carefully removed and again washed with 70 per cent ethanol and spun the tubes for 5 min at 10,000 rpm and ethanol was decanted.

- The pellet was air dried, dissolved in 50µl sterile distilled water and stored at -20°C

3.2.2. Assessing the quality of DNA by electrophoresis

The quality assessment of isolated DNA was done by Agarose Gel Electrophoresis (Sambrook *et al.*, 1989).

Reagents and Equipments

a. Agarose (Promega) 0.8 per cent (w/v)
b. 50X TAE buffer (pH 8.0)
   - Tris base -242 gm.
   - Glacial acetic acid -57.1 ml.
   - 05 mM EDTA -100 ml.
c. Tracking/loading dye (6X) – Banglore Genei
   - Bromophenol blue
   - Glycerol
d. Ethidium bromide (SRL) (stock 10 mg/ml; working concentration 0.5 µg/ml)
e. Electrophoresis unit- Bio-Rad power PAC 1000, gel casting tray, comb
f. UV transllluminator- (Herolab ^{R})
g. Gel documentation and analysis system- BioRad Gel DOC-It^{TM} imaging system.

Composition of reagents is provided in Annexure III.

Procedure for 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 in a glass beaker or conical flask by dissolving 0.8 g agarose 100 ml 1X TAE buffer (2 ml from 50X TAE buffer stock was make up to 100 ml with distilled water). Micro waved for 45 to 60 seconds until agarose was dissolved and solution was clear.

- Solution was allowed to cool to 42 to 45 ^{0}C under room condition and at this point 5µl ethidium bromide from 10 mg/ml stock solution was added and mixed well.

- This warm gel solution was poured into the casting tray to a depth of 5 mm. and the gel was allowed to solidify for about 30-45 minutes at room temperature.

- The comb and the tape used for sealing the gel tray were gently removed and the tray was placed in electrophoresis chamber. The gel was covered with electrophoresis 1X TAE buffer till the wells are submerged.

- Samples for electrophoresis were prepared by adding 1 µl of 6X gel loading dye for every 5µl of DNA sample and by mixing them well. Loaded 6µl DNA and dye mixture per well. A suitable molecular weight marker (λ DNA *Eco*RI / *Hind* III double digest- Banglore Genei) was also loaded in one lane.

- Electrophoresis was carried out at 70 V until dye has migrated two-third the length of the gel.

- Intact DNA appeared as orange fluorescent bands. The degraded ones appeared as a smear because of the presence of a large number of DNA fragments which differed only in few bases. The presence of protein was observed as a thick white patch which got restricted in the wells itself. The RNA contamination was observed as thick band with size less than 100 bp.

3.2.3 Gel documentation

The gel containing electrophoresed DNA was viewed under UV transilluminator for presence of DNA. The DNA fluoresces under UV light due to ethidium bromide dye. The image was documented on gel documentation system (BioRad Gel DOC-It^{TM} imaging system). The gel profile was examined for intactness, clarity of DNA and contamination with RNA and protein.

3.2.4 Purification of DNA

The DNA which had RNA as contaminant (as observed from the electrophoresis) was purified by RNase treatment and subsequent precipitation (Sambrook *et al.*, 1989).

Reagents

I. Chloroform: Isoamyl alcohol (24:1 v/v)

II. Chilled isopropanol (100 %)

III. 70 per cent ethanol

IV. 1 per cent RNase

One per cent solution was prepared by dissolving RNase (Sigma, USA) in TE buffer at 100^{0}C for 15 minutes to inactivate residual DNase. The solution was cooled to room temperature, dispensed into aliquots and stored at -20^{0}C.

Procedure

- For 50 µl DNA sample, 1 µl of 1 per cent RNase solution was added and incubated at 37° C in dry bath for 40 minutes
- The total volume was made up to 250 µl with distilled water.

- Equal volume of chloroform: isoamyl alcohol (24: 1) mixture was added and mixed gently.
- Centrifuged at 12000 rpm for 15 minutes at 4°C.
- The aqueous phase was transferred into a fresh micro centrifuge tube and equal volume of chloroform: isoamyl alcohol (24: 1) was added.
- Centrifuged at 12,000 rpm for 15 minutes 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 2-3 gentle inversion till the DNA precipitated. For complete precipitation kept it at -20°C for half an hour.
- Centrifuged at 10,000 rpm for 15 minutes at 4° C.
- DNA pellet was washed with 70 per cent ethanol and centrifuged at 10,000 rpm for 10 minutes at 4° C.
- The pellet was air dried and dissolved in 50 µl sterile distilled water and stored at -20° C.
- The samples were loaded on 0.8 per cent agarose gel at constant voltage of 70V to test the quality and to find whether there is any shearing during RNAse treatment.

3.2.5 Assessing the quality and quantity of DNA using spectrophotometer (NanoDrop ND-1000)

The purity of DNA was further checked using Nano Drop spectrophometer model NanoDrop-1000. Nucleic acid shows absorption maxima at 260 nm whereas proteins show peak absorbance at 280 nm. Absorbance has been recorded at both wavelengths and the purity was indicated by the ratio OD_{260}/OD_{280}. A value between 1.8 and 2.0 indicated that the DNA is pure and free from proteins and RNA. When the ratio was <1.8 it means that the sample is RNA contaminated and the ratio >2.0 had shown that sample is protein contaminated.

The quantity of DNA in the pure sample was calculated using the relation.

1OD at 260 nm = 50 µg DNA/ml

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

Procedure followed for checking the quantity using NanoDrop spectrophotometer

- NanoDrop spectrophotometer was connected and complete installed with the operating software ND-1000.
- The option nucleic acid was selected in the software.
- With the sampling arm open, 1µl grade I water was pipetted onto the lower measurement pedestal.
- Sampling arm was closed and spectral measurement was initiated using the operating software. The sample column was automatically drawn between the upper and lower measurement pedestals and the spectral measurement was made.
- The reading was set to zero with sample blank
- Subsequently, 1µl of sample was pipetted out onto the measurement pedestal and 'measure' option was selected.
- When the measurement has been completed, the sampling arm was opened and the sample was wiped from both the upper and lower pedestals using a soft laboratory tissue paper.
- Simple wiping has prevented the sample carryover in successive measurements for the samples varying by more than 1000 fold in concentration.

3.3 Molecular markers used for the study

Two types of markers, ISSR (Inter Simple Sequences Repeats) and SSR (Simple Sequence Repeats) were used in this study. Under each marker analysis system, DNA from all of the twelve genotypes of cocoa were amplified simultaneously with the selected primer. This has helped to obtain the amplification pattern for all genotypes with a specific primer in a marker system, simultaneously.

3.3.1 DNA amplification conditions

The PCR conditions required for effective amplification in ISSR and SSR analyses included appropriate proportions of the components of the reaction mixture. The reaction mixture included template DNA, assay buffer A or B, $MgCl_2$, Taq DNA polymerase, dNTPs and primers. The aliquot of this master mix were dispensed into 0.2 ml PCR tubes. The PCR was carried out in Veriti Thermal Cycler (Applied Biosystems, USA).

Another important factor, which affected the amplification, was the temperature profile of thermal cycles. The thermocycler was programmed for desired number of cycles and temperatures for denaturation, annealing and polymerisation.

3.3.2 ISSR (Inter Simple Sequence Repeat) analysis

Good quality genomic DNA (25-30 ng/µl) isolated from cocoa leaf samples were used in the ISSR analysis. ISSR primers with good resolving power were used for amplification of DNA, after an initial screening.

PCR amplification was performed in a 20 µl reaction mixture and the composition of the reaction mixture consisted of,

a) Genomic DNA (30 ng)	- 2.0 µl
b) 10X Taq assay buffer B	- 2.0 µl
c) $MgCl_2$	- 2.0 µl
c) dNTPs mix (10 mM each)	- 1.5 µl
d) Taq DNA polymerase (3U)	- 0.4 µl
e) Primer (10 pM)	- 1.5 µl
f) Autoclaved distilled water	- 10.6 µl
	Total volume - 20.0 µl

The PCR amplification was carried out with the following thermal profile

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

3.3.2.1 Screening of ISSR primers and analysis

Thirty six primers (ISSR Technologies) were screened for ISSR analysis and are listed in Table 2. Primers were selected from literatures based on previous studies in ISSR analysis in cocoa (Julio *et al.*, 2011).

The amplified products were electrophoresed, along with 1000 bp DNA ladder (Sigma, USA) on 1.5 per cent agarose gel using 1X TAE buffer stained with ethidium bromide. The profile was visualized under UV (312 nm) transilluminator and documented for further analyses. The documented ISSR profiles were carefully examined for the polymorphism among amplicons.

Table 3.2 Details of ISSR primers used in this study

Sl. No.	Primer	Nucleotide Sequence
1	UBC 354	5'- AGAGAGAGAGAGAGAGY-3'
2	UBC 807	5'- AGAGAGAGAGAGAGAGT- 3'
3	UBC 811	5'- GAGAGAGAGAGAGAGAC- 3'
4	UBC 812	5'- GAGAGAGAGAGAGAGAA-3'
5	UBC 813	5'- CTCTCTCTCTCTCTCTT- 3'
6	UBC 814	5'- CTCTCTCTCTCTCTCTA- 3'
7	UBC 815	5'- CTCTCTCTCTCTCTCTG- 3'
8	UBC 817	5'- CACACACACACACACAA- 3'
9	UBC 818	5'- CACACACACACACACAG- 3'
10	UBC 820	5'- GTGTGTGTGTGTGTGTC- 3'
11	UBC 823	5'- TCTCTCTCTCTCTCTCC- 3'
12	UBC 825	5'- ACAGACACACACACACT- 3'
13	UBC 826	5'- ACACACACACACACACC- 3'
14	UBC 827	5' - ACACACACACACACACG- 3'
15	UBC 835	5'- AGAGAGAGAGAGAGAGYC- 3'
16	UBC 836	5'- AGAGAGAGAGAGAGAGYA- 3'
17	UBC 840	5'- GAGAGAGAGAGAGAGAYT- 3'
18	UBC 841	5'- GAGAGAGAGAGAGAGAY-3'
19	UBC 843	5'- CTCTCTCTCTCTCTCTRA- 3'
20	UBC 844	5'- CTCTCTCTCTCTCTCTRC- 3'
21	UBC 845	5'- CTCTCTCTCTCTCTCTRG- 3'
22	UBC 846	5'- CACACACACACACACART-3'
23	UBC 848	5'- CACACACACACACACARG-3'
24	UBC 854	5'- TCTCTCTCTCTCTCTCRG- 3'

25	UBC 855	5'- ACACACACACACACACYT-3'
26	UBC 856	5'- ACACACACACACACACYA- 3'
27	UBC 857	5' - ACACACACACACACACYG- 3'
28	UBC 865	5' - CCGCCGCCGCCGCCGCCG-3'
29	UBC 866	5'- CTCCTCCTCCTCCTCCTC- 3'
30	UBC 873	5'- GACAGACAGACAGACA-3'
31	UBC 880	5'- GGAGAGGAGAGGAGA-3'
32	UBC 890	5'- VHVGTGTGTGTGTGTGT- 3'
33	UBC 892	5'-TAGATCTGATATCTGAATTCCC-3'
34	UBC 895	5'- AGAGTTGGTAGCTCTTGATC-3'
35	UBC 899	5'-CATGGTGTTGGTCATTGTTCCA -3'
36	UBC 900	5'-ACTTCCCCACAGGTTAACACA-3'
37	UBC S2	5'- CTCTCTCTCGTGTGTGTG- 3'
38	ISSR 2	5'- ATTATTATTATTATTCAT-3'
39	ISSR 3	5'- TTATTATTATTATTACTT-3'
40	ISSR 4	5'- ATTATTATTATTATTGTT-3'
41	ISSR 5	5'- ATTATTGTTGTTGTTTTC-3'
42	ISSR 6	5'- TTATTATTATTATTATAA-3'
43	ISSR 7	5'- ATTATTGTTGTTGTTGTA-3'
44	ISSR 8	5'- ATTATTATTATTATTGTA-3'
45	ISSR 9	5'- TTATTATTATTATTATTACT-3'
46	ISSR 03	5'- CTCTCTCTCTCTCTCTTG-3'
47	ISSR 04	5'- GAGAGAGAGAGAGG-3'
48	ISSR 05	5'- CACACACACACACAC-3'
49	ISSR 06	5'- GTGTGTGTGTGTCC-3'
50	ISSR 08	5'- TGTGTGTGTGTGTGYCATGCACATTGTGT-3'
51	ISSR 09	5'- TGTGTGTGTGTGTGGCACATGCARTGTGT-3'

52	ISSR 010	5'- GTGTGTGTGTGTGTGTGTGTGTGTG-3'
53	Oligo ISSR 04	5'- ACACACACACACACC- 3'
54	Oligo ISSR 05	5'- CTCTCTCTCTCTCTTG- 3'
55	Oligo ISSR 06	5'- GAGAGAGAGAGAGAC- 3'
56	Oligo ISSR 07	5'- CTCTCTCTCTCTCTTG- 3'
57	Oligo ISSR 08	5'- GAGAGAGAGAGAGAT- 3'
58	Oligo ISSR 15	5'- TCCTCCTCCTCCTCC- 3'
59	Oligo SPS 03	5'- GACAGACAGACAGACA- 3'
60	Oligo SPS 08	5'- GGAGGAGGAGGA- 3'
61	R 11	5'- GATCATCATCATCATCATCATCATCATC-3'
62	P1	5'- GACACGACACGACACGACAC-3'
63	P2	5'- GAAGTGGGGAAGTGGG-3'
64	S1	5'- GTTGTTGTTGTTGTT-3'
65	S2	5'- CTGACTGACTGACTGA-3'
66	S3	5'- CCACCACCACCACCA-3'
67	S4	5'- GTCGTCGTCGTCGTC-3'
68	HB 10	5'-GAGAGAGAGAGACC-3'
69	HB 12	5'- CACCACCACGC-3'
70	S07	5'- TCCGGATGCTG-
71	17899A	5'- CACCACCACGC-3'

3.3.3 SSR (Simple Sequence Repeat) analysis

Good quality genomic DNA (20 to 25ng/µl) isolated from cocoa leaf samples was used in the SSR analysis. SSR primers supplied by Sigma, USA were used for amplification of DNA. These SSR primers were selected as per the high PIC values reported in the previous studies (Obando, 2009; Epaina, 2012; Thondaiman *et al.*, 2013). The amplification was carried out in Veriti Thermal Cycler (Applied

Biosystems, USA). PCR amplification was performed in a 20 µl reaction mixture which consisted of,

a) Genomic DNA (30ng)	-	2.0µl
b) 10X Taq assay buffer A	-	2.0µl
c) dNTPs mix (10mm each)	-	1.5µl
d) Taq DNA Polymerase (1U)	-	0.3µl
e) Forward Primer (10pM)	-	0.75µl
f) Reverse Primer (10pM)	-	0.75µl
g) Autoclaved Distilled Water	-	12.7 µl
Total volume	-	20.0µl

The thermal cycling was carried out with the following programme

Initial denaturation	-	94^0C for 3 minute	
Denaturation	-	94^0C for 1 minute	35 cycles
Primer annealing	-	53^0C to 55^0C for 1 minute	
Primer extension	-	72^0C for 1 minute	
Final extension	-	72^0C for 5 minutes	
Incubation	-	4^0C for infinity to hold the sample	

3.3.3.1 Screening of SSR Primers and analysis

Seventeen SSR primer combinations were screened by PCR and their sequence are listed in Table 3. The amplified products were run along with marker (1000 bp ladder) on two per cent agarose gel using 1X TAE buffer and stained with ethidium bromide. The profile was visualized under UV (312 nm) transilluminator and documented. The documented SSR profiles were carefully examined for the polymorphism in banding pattern among the accessions with respect to the disease response.

Table 3.3 Details of SSR primer combinations used in this study

Sr. No.	Primer	Nucleotide Sequence
1	mTcCIR6	**F** 5'- TTCCCTCTAAACTACCCTAAAT-3' **R** 5'-TAAAGCAAAGCAATCTAACATA-3'
2	mTcCIR10	**F** 5'-CCGAATTGACAGATGGCCTA -3' **R** 5'-CCCAAGCAAGCCTCATACTC -3'
3	mTcCIR11	**F** 5'- TTTGGTGATTATTAGCAG-3' **R** 5'-GATTCGATTTGATGTGAG-3'
4	mTcCIR12	**F** 5'- TCTGACCCCAAACCTGTA-3' **R** 5'-ATTCCAGTTAAAGCACAT-3'
5	mTcCIR15	**F** 5'- CAGCCGCCTCTTGTTAG-3' **R** 5'-TATTTGGGATTCTTGATG-3'
6	mTcCIR18	**F** 5'-GATAGCTAAGGGGATTGAGGA-3' **R** 5'-GGTAATTCAATCATTTGAGGATA-3'
7	mTcCIR33	**F** 5'-TGGGTTGAAGATTTGGT-3' **R** 5'-CAACAATGAAAATAGGCA-3'
8	mTcCIR25	**F** 5'- CTTCGTAGTGAATGTAGGAG- 3' **R** 5'-TTAGGTAGGTAGGGTTATCT- 3'
9	mTcCIR102	**F**5'-TTGTGAAAAGATTGCGA- 3' **R**5'- TTGCTTGTTATTGCTACTAT- 3'
10	mTcCIR121	F5'- CATGTGCATTTAGGTGTC- 3' R5'- TCTGGCTTCTTAGTGATAC- 3'
11	mTcCIR146	F5'- GCAAGGTCTTTTTACGAT- 3' R5'- ATGGACACGTCTAAGTTG- 3'
12	mTcCIR115	**F** 5'-GTGATTCAAATTCAAATATG-3' **R** 5'-AATAGCAAGAGAGTGATGAG-3'
13	mTcCIR158	**F** 5'-TGTAGGTTATGCAGCGTGTTC-3' **R** 5'-GATGAGGGGTGTAGCTGTTG-3'
14	mTcCIR168	**F** 5'-GGTAGTATTGAGGTGCGTAT-3' **R** 5'-GTGAATGAATGGATGTGAAA-3'
15	mTcCIR222	**F** 5'-CTACAGAAAATAGGCAATA-3' **R** 5'-TCATTGTATTATCAGGTAGA-3'
16	SHRSTc 49	**F**5'- ATCGCAGCAAACTCCCTCTC- 3' **R**5'- TTCTCTTCCCACCAAGTCCC- 3'
17	SHRSTc 51	**F**5'- CTGTTTTTGCCTCCCTTGTTCT- 3'

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		R5'- ATTGCTGGTTGTTCTCCATCCT- 3'
18	SHRSTc 52	**F5**'-TTTTAGAGCATCCACTTCCCT- 3' **R5**'-CCATTCTTTCCACACTGAGAG- 3'
19	SHRSTc 53	**F5**'- TTCCCTTTCTTTCTCTCTCTC- 3' **R5**'- AGTCGTTGCTACTGCTGG- 3'
20	SHRSTc 64	**F5**'- TCCTACATTCCTGCACCC- 3' **R5**'- TCGAGAGAAAAGCTCTTACACT- 3'
21	SHRSTc 66	**F5**'- ACAGGAATCCCCATCAGCGA- 3' **R5**'- GCAATCGACAGGCATGAGAGAG- 3'
22	mAoR 03	**F5**'- CAGAACCGTCACTCCACTCC- 3' **R5'**- ATCCAGACGAAGAAGCGATG- 3'
23	mAoR 06	**F5**'- CAAAACTAGCCGGAATCTAGC- 3' **R5**'- CCCCATCAAACCCTTATGAC- 3'
24	mAoR 07	**F5'**- AACCTTCACTCCTCTGAAGC - 3' **R5**'- GTGAATCCAAAGCGTGTG- 3'
25	mAoR 11	F5' ATCCAACAGCCACAATCCTC - 3' **R5**'- CTTACAGCCCCAAACTCTCG- 3'
26	mAoR 17	**F5'**- GCAATGTGCAGACATGGTTC - 3' **R5**'- GGTTTCGCATGGAAGAAGAG- 3'
27	mAoR 29	F5'-GGAGAAGAAAAGTTAGGTTTGAC - 3' **R5**'-: CGTCTTCTTCCACATGCTTC- 3'
28	mAoR 42	**F5**'- ACTGTCACGTCAATGGCATC - 3' **R5**'- GCGAAGGTCAAAGAGCAGTC- 3'
29	mAoR 46	**F5**'- CGGCGTCGTTAAAGCAGT - 3' **R5**'-TCCTCCTCCGTCTCACTTTC- 3'
30	mAoR 48	**F5**'- CAGCGAGTGGCTTACGAAAT - 3' **R5**'- GACCATGGGCTTGATACGTC- 3'
31	mAoR 52	**F5**'- GCTATGACCCTTGGGAACTC - 3' **R5**'- GTGACACAACCAAAACCACA- 3'
32	mAoR 55	**F5**'- TGACTTTCAAATGCCACAAC - 3' **R5**'-CTCAAGCTTTCATGGGGATT- 3'
33	SSRKAU2	**F** 5'-TTCAGGTATGTCTCACACCA-3' **R** 5'-TTGCAAGAACACCTCCCTTT-3'
34	SSRKAU3	**F** 5'-GGGTTATCAATGATGCAATGG-3' **R** 5'-CCTTTATGTCAGCCGGTGTT-3'
35	SSRKAU4	**F** 5'-TGCCAATCCACTCAGACAAA-3'

		R 5'-TGGATTCACCAAGGCTTCTT-3'
36	SSRKAU10	**F** 5'-GCGATGAGGATGACATTGAG-3' **R** 5'-TTTACAGGCTGTCGCTTCCT-3'
37	SSRKAU11	**F** 5'-TGTTGGTTGGAGAAACTCCC-3' **R** 5'-AGGCATTTAAACCAATAGGTAGC-3'
38	SSRKAU12	**F** 5'-TCCTCAAGAAATGAAGCTCTGA-3' **R** 5'-CCTTGGAGATAACAACCACAA-3'
39	SSRKAU13	**F** 5'-GGAATAACCTCTAACTGCGGG-3' **R** 5'-CGATGCCTTCATTTGGACTT-3'
40	SSRKAU15	**F** 5'-TGTTGCTCGAACTCTCCAAA-3' **R** 5'-CATAGGAGAGGTAACCCGCA-3'
41	SSRKAU19	**F** 5'-ATTGTACAAAGACCCGTGGC-3' **R** 5'-GTTGCACACTGGATCAATGC-3'
42	SSRKAU20	**F** 5'-AGGGTCCTTCGTTTGGAACT-3' **R** 5'-GCATTCCACTTGTGAAGCAT-3'
43	SSRKAU21	**F** 5'-GGTCCAGTTCAATCAACCGA-3' **R** 5'-TGAAGTCGTCTCATGGTTCG-3'
44	SSRKAU22	**F** 5'-GCAGAGGATATTGCATTCGC-3' **R** 5'-CAAACCGAACTCATCAAGGG-3'
45	SSRKAU24	**F** 5'-CCGAGGCGAATCTTGAATAC-3' **R** 5'-GCACCATCTCTTGTGCCTCT-3'
46	SSRKAU25	**F** 5'-CTCGTCTTTAGGTATCAATGGAGAT-3' **R** 5'-TCAATGCTACTCAATGGCTCA-3'

3.3.4 Native polyacrylamide gel electrophoresis (PAGE)

Reagents

1. **30% Acrylamide** (100ml)
   - a) Acrylamide 29g
   - b) Bis-acrylamide 1g
   - c) Distilled water 100ml

Filtered through 0.45µm filter and stored in brown bottle at 4°C.

2. **10% Ammonium acetate**
   - a) APS 100mg
   - b) Double distilled water 1 ml (Stored at –20°C)

3. **10X TBE**
   - a) Tris Base 108g
   - b) Boric Acid 55g
   - c) EDTA 7.45g

Made up the volume to 1 liter with double distilled water

Filtered through 0.45 µm and stored at room temperature

4. **Gel loading buffer dye for 50 ml**
   - a) 80% Glycerol 47ml
   - b) Bromophenol Blue 125mg
   - c) Xylene Cyanol 125mg
   - d) 1M Tris (pH.7.4) 0.5ml
   - e) 5M NaCl 0.1ml
   - f) 0.5M EDTA 1.0ml
   - g) 10% SDS 0.5ml

Procedure

- The gel apparatus was prepared by cleaning from any debris
  The polyacrylamide gel solution was mixed in a beaker. The composition is given below

Reagents	8%	10%	12%
30% Acrylamide: Bis (29:1) (ml)	3.2	4.0	4.8
5 X TBE (ml)	2.4	2.4	2.4
Distilled H_2O (ml)	6.4	5.6	4.8
Ammonium per sulphate (µl)	200	200	200
TEMED (µl)	10	10	10

- Immediately, poured the gel mixture slowly into the previously prepared gel form, until the liquid level reaches the top of the upper glass plate.
- Comb was inserted between the two glass plates and clamped in place with a small spring clip.
- Little extra gel mixture over the junction between the comb was dribbled in the glass plates, using a pipette, to compensate for volume shrinking upon gelation and small leaks. Gel was kept on stand for 1-2 hours.
- Comb was removed by gently wriggling it and lifting out.
- Unpolymerized acrylamide out of the wells washed by squirting with distilled water.
- Lower and upper tank was filled with 1 X TBE.
- A bent syringe needle was used to perforate and removed air bubbles trapped in the space where the bottom spacer was originally located.
- DNA samples were loaded with Glycerol Dye Mix in the wells and started the electrophoresis run.
- After the electrophoresis was finished, turn off the power supply.
- Gel was stained in silver stain and photograph was taken.

3.3.5 Silver staining protocol

Reagents

10 per cent Acetic Acid (Fixer solution)

a) Acetic acid - 10ml

b) Distilled water - 90 ml

Mixed well and stored at room temperature

0.1 per cent Silver nitrate (Stainer)

0.1 gm silver nitrate dissolved in 100 ml of water and stored in brown colored bottle at room temperature.

Developer Solution

a) Sodium carbonate - 4.5 gm

b) Formaldehyde - 200 µl

c) Sodium thiosulphate - 30 µl

Dissolved the chemicals in 100 ml of chilled distilled water

Procedure

- After the run gel was removed from glass plate and transferred in 10 per cent acetic acid solution for 20 minutes.
- After 20 minutes gel was washed for three times with distilled water.
- Immediately transferred the gel in 0.1 per cent silver nitrate solution for 20 minutes.
- After 20 minutes gel was quickly washed with distilled water three times.
- Then gel was shifted in to developer solution till the band appear
- With satisfactory band development staining procedure stopped by transferring the gel in 10 per cent acetic acid solution for 10 minutes.
- Then the gel was transferred in to distilled water for 5 min.
- Photograph was taken.

3.3.6 Identification of polymorphism

For every ISSR and SSR primer, the marker profiles were generated through electrophoresis of the PCR products on 1.5 and 2.0 per cent agarose gel respectively. The profiles were examined in relation to the reported disease response and any band which is present in all the resistant clones and missing in the susceptible clones and vice versa was considered as polymorphic.

3.3.7 Elution of polymorphic band and cleaning (NucleoSpin® Gel and PCR Clean-up kit protocol)

a) Excision of DNA fragment / solubilise gel slice

Using a clean scalpel, the DNA fragment was excised from the agarose gel. All the excess agarose was removed. Weight of the gel slice was taken and transfered it to a clean tube. For each 100 mg of 2 per cent agarose gel 200 µl Buffer NTI was added. For gels containing > 2 per cent agarose, the volume of Buffer NTI was doubled. Sample was incubated for 5–10 min at 50 °C. The sample was briefly vertexed every 2–3 min until the gel slice is completely dissolved.

b) Binding of DNA

NucleoSpin® Gel and PCR Clean-up Column was placed into a collection tube (2 mL) and 700 µl sample was loaded. Centrifuged the sample for 30 s at 11,000 x g. Bottom liquid was discarded and placed the column back into the collection tube. Loaded remaining sample and centrifugation step was repeated.

c) Washing of silica membrane

700 µl of Buffer NT3 was added to the NucleoSpin® Gel and PCR Clean-up Column. Centrifuged for 30 s at 11,000 x g. Bottom flow-through was discarded and placed the column back into the collection tube.

d) Drying of silica membrane

Column was centrifuged for 1 min at 11,000 x g to remove Buffer NT3 completely. Spin column was avoided to contact with the flow-through while removing it from the centrifuge and the collection tube. Residual ethanol from Buffer NT3 is reported to inhibit enzymatic reactions. So, care was taken to obtain the total removal of ethanol by incubating the columns for 2–5 min at 70 °C prior to elution.

e) Elution of DNA

NucleoSpin® Gel and PCR Clean-up Column was placed into a new 1.5 mL micro centrifuge tube. 15–30 µl Buffer NE was added and incubated at room temperature (18–25 °C) for 1 min. Centrifuged for 1 min at 11,000 x g.

3.4 Sequencing the eluted product from the polymorphic bands

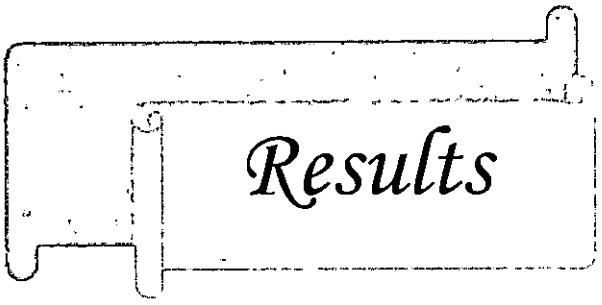
The eluted DNA product was subjected to PCR reamplification. Nested PCR has been done like ISSR protocol (3.3.2). After amplification PCR product was run on 2 per cent agarose gel electrophoresis. In the agarose gel the product showing single band was sent for sequencing at SciGenom lab. Cochin.

3.5 Sequence analysis and submission to GenBank

The sequence generated from this study was submitted to GenBank at http://www.ncbi.nlm.nih.gov/guide/howto/submit-sequence-data/ website, using the software BankIt.

3.5.1 BLASTn

The sequence generated from this study was analyzed using the nucleotide BLAST at NCBI.


Results

4. RESULTS

The results of the experiments carried out in the study entitled "Identification of molecular markers linked to the resistance for vascular streak dieback disease in cocoa (*Theobroma cacao* L.)" during 2012-2014 at Center for Plant Biotechnology and Molecular Biology, College of Horticulture, Kerala Agricultural University are presented in this chapter.

4.1 Plant material

Twelve cocoa clones having various responses to vascular streak dieback disease and maintained at Cocoa Research Centre, College of Horticulture, Kerala Agricultural University were used in the study. Detail of genotypes given in chapter 3.1.1. The genotypes were selected on the basis of field screening experiments started in 1998-1999. Details on the clones and their disease response are furnished in Table 3.1.

4.2 Molecular Analyses

4.2.1 Isolation, purification and quantification of DNA

Initially the CTAB method of (Roger and Bendich, 1994) was used for the isolation of genomic DNA. The quality of DNA was not satisfactory with presence of high amount of polyphenolic compounds and polysaccharides. Subsequently an improved semi automated rapid method in cocoa, as reported by Bhattacharjee *et al.* (2004) was also tried but the results were not better than the CTAB method.

Subsequently, the genomic DNA isolation through the modified CTAB method (Doyle and Doyle, 1987) attempted. The isolated DNA showed high levels of RNA contamination (Fig. 4.1). Treatment with β-mercaptoethanol was given for removing the color of DNA due to polyphenols. The chloroform:isoamyl alcohol

(24:1) treatment was given three times and the DNA was washed with ammonium acetate. RNase treatment and further precipitation gave sufficient quantity of good quality DNA from leaf sample. The agarose gel electrophoresis had shown clear and discrete band with no RNA contamination (Fig. 4.2) and spectrophotometric analysis gave the acceptable ratio of UV absorbance ($A_{260/280}$) between 1.8 and 2.0 (Table 4.1).

4.3 Molecular marker analysis

The protocol for different marker assays, ISSR and SSR were validated with bulked DNA of cocoa accessions. Different primers were screened with the bulked genomic DNA, using the validated protocols.

4.3.1 Inter Simple Sequence Repeat (ISSR) analysis

Seventy one ISSR primers were used in the initial screening based on their ability to amplify the bulked genomic DNA, with the thermal settings mentioned earlier under the material and methods section (3.3.2) gave varying amplification pattern. Based on the amplification pattern, 13 primers were selected for ISSR analysis (Table 4.2). Details on the selected primers are provided in Table 4.3.

The analysis of result on individual primers is detailed here under.

4.3.1.1 UBC 811

Averages of eight amplicons were obtained in each accession, on DNA amplification with the primer UBC 811. The pattern of amplification is shown in Fig. 4.3. The molecular weight of the bands varied from 450 bp to 2.5 kb. Amplicons obtained with this primer were distinctly polymorphic for the resistance to VSD. The polymorphic bands of 0.950 kb was present in resistant and in partially resistant lines but absent in susceptible lines. This primer was able to differentiate resistant and susceptible lines.

Table 4.1 Quality and quantity of DNA isolated from the cocoa genotypes, assessed by Nano Drop spectrophotometer

Genotype	UV absorbance at 260 nm (A_{260})	UV absorbance at 280 nm (A_{280})	$A_{260}/_{280}$	Quantity (ng/µl)
VSD I- 4.6	4.303	2.254	1.90	215.15
VSD I- 4.11	10.872	5.760	1.88	543.58
VSD I- 5.8	10.422	5.569	1.87	521.10
VSD I- 6.9	3.783	1.926	1.96	189.15
G VI-50	3.001	1.606	1.87	150.06
G VI- 52	3.978	2.127	1.87	199.35
G VI- 82	5.727	3.200	1.79	286.35
G VI- 100	5.459	3.016	1.81	272.95
G VI- 25	2.237	1.230	1.82	111.85
G VI- 53	2.236	1.219	1.83	111.80
G VI- 67	5.422	3.037	1.79	271.10
G VI- 144	10.708	5.677	1.88	535.40

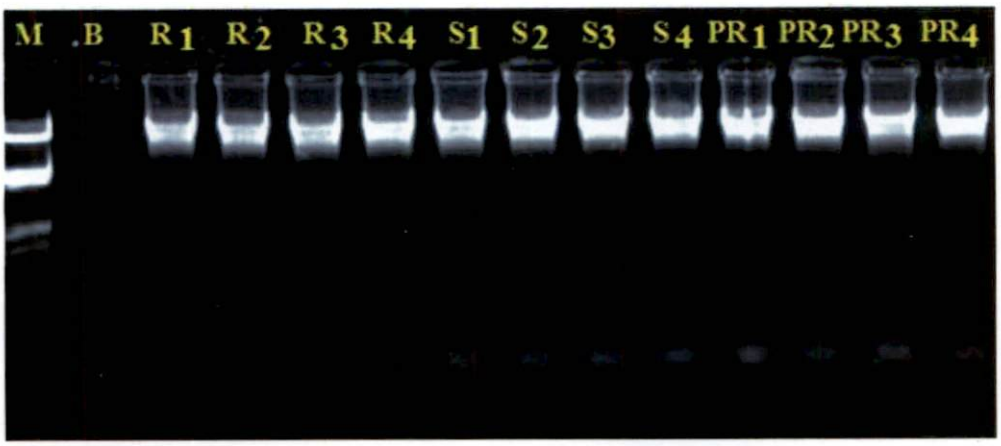


Fig 4.1 The gel profile obtained by electophoresis of DNA samples isolated using the Doyle and Doyle method, before RNase treatment

M: Molecular weight marker λ DNA (Eco RI/ *Hind* III digest), Lane B: Blank, **R1**: VSD I- 4.6, **R2**: VSD I- 4.11, **R3**: VSD I- 5.8, **R4**: VSD I- 6.9, **S1**: G VI- 50, **S2**: G VI- 52, **S3**: G VI- 82, **S4**: G VI- 100, **PR1**: G VI- 25, **PR2**: G VI- 53, **PR3**: G VI- 67, **PR4**: G VI- 144.

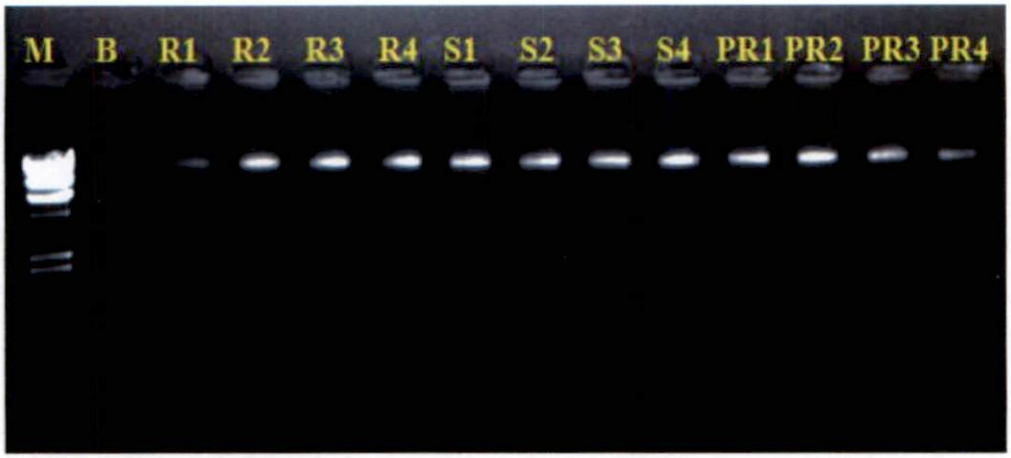


Fig 4.2 The gel profile obtained by electophoresis of DNA samples isolated using the Doyle and Doyle method, after RNase treatment

M: Molecular weight marker λ DNA (Eco RI/ *Hind* III digest), Lane B: Blank, **R1**: VSD I- 4.6, **R2**: VSD I- 4.11, **R3**: VSD I- 5.8, **R4**: VSD I- 6.9, **S1**: G VI- 50, **S2**: G VI- 52, **S3**: G VI- 82, **S4**: G VI- 100, **PR1**: G VI- 25, **PR2**: G VI- 53, **PR3**: G VI- 67, **PR4**: G VI- 144.

4.3.1.2 UBC 815

Using the ISSR primer UBC 815, on an average twelve amplicons were obtained in each accession. The pattern of amplification is shown in Fig. 4.4. The molecular weight of the amplicons varied from 0.250 to 2.0 kb. On careful analysis, amplicons obtained with this primer were found polymorphic for the VSD resistance. The polymorphic bands were observed at 750 bp in resistant lines but were absent in susceptible and in partially resistant lines. This primer was found to be a good candidate with potential to differentiate between resistant and susceptible lines.

4.3.1.3 UBC 826

ISSR analysis of the DNA samples using the primer UBC 826 generated at an average of nine amplicons per accession on agarose gel. The molecular weight of the amplicons varied from 400 bp to 1.50 kb. The pattern of amplification is shown in Fig. 4.5. Amplicons obtained with this primer were polymorphic and the amplicon is also linked with the VSD resistance. The polymorphic bands were present at 650 bp in resistant lines. But that polymorphic band was present in one susceptible line (S2) and in one partially resistant line also. Since this marker is present in all the resistant lines and are absent in all the susceptible and partially resistant lines, with exception in two lines, it may be associated with a gene with minor contribution to resistance. The polygenic nature of the resistance is pointed in this marker.

4.3.1.4 UBC 827

On an average sixteen amplicons were obtained after DNA amplification with the primer UBC 827 (Fig. 4.6). The molecular weight of the bands ranged from 0.250 to 2.500 kb. Amplicons obtained with this primer were distinctly polymorphic. The polymorphic bands were present at 2.5 kb in all the susceptible lines and two partially resistant lines but also in one resistant line (R4). This primer may be associated with a gene that offers susceptibility to VSD to some extent.

4.3.1.5 UBC 835

On an average eighteen amplicons were obtained with the primer UBC 835. The pattern of amplification is shown in Fig. 4.7. The molecular weight of the amplicons ranged from 200 bp to 1.5 kb. Through this marker large no of amplicons were generated, they were monomorphic for the character VSD resistance.

4.3.1.6 UBC 841

Using the primer UBC 841, in every accession ten clear amplicons were observed after agarose gel electrophoresis. The pattern of amplification is shown in Fig. 4.8. The molecular weight of the bands varied from 0.250 to 1.5 kb. Only two polymorphic bands were observed in the susceptible lines but were not in VSD resistant lines.

4.3.1.7 UBC 844

On an average twelve clear amplicons were developed in every accession using the primer UBC 844 (Fig. 4.9). The molecular weight of the amplicon varied from 600 bp to 2.00 kb. All amplicons generated from this primer were monomorphic.

4.3.1.8 UBC 846

Amplification with this primer has generated on an average fourteen amplicons of which three were polymorphic (Fig. 4.10). The molecular weight of the amplicon varied from 0.300 to 2.5 kb. However none of these polymorphic amplicons were associated with the gene containing resistance to VSD.

4.3.1.9 UBC 855

Amplification with this primer has generated on an average ten amplicons in all the accession. The molecular weight of the band varied from 0.400 to 1.5 kb (Fig. 4.11). All amplicons generated from this primer was monomorphic.

4.3.1.10 UBC 857

The ISSR primer UBC857 has generated an average of eleven clear amplicons on 1.5 per cent agarose gel electrophoresis (Fig. 4.12). The molecular weight of the band varied from 200 bp to 1.6 kb. Amplicons generated from this primer were polymorphic with a band of 450 bp in all the resistant lines but was absent in all the susceptible lines. This clear polymorphism between the resistant and susceptible lines was highly repeatable and was useful for identification of a marker associated with resistant trait for VSD in cocoa.

4.3.1.11 UBC 866

When the genotypes were screened with the ISSR primer UBC866, an average ten amplicons were obtained in each accession. The pattern of amplification is shown in Fig. 4.13. Amplicons obtained with this primer were polymorphic. The molecular weight of the bands varied from 0.400 to 1.500 kb. Two polymorphic bands present at 1.300 kb (marked 'a') and 1.5 kb (marked 'b') in three susceptible lines and three partially resistant line but absent in all the resistant lines were found to be repeatable and linked with the gene offering resistance to VSD.

4.3.1.12 ISSR 7

When the genotypes have been screened with the ISSR primer ISSR7 an average of eight clear amplicons were observed on the 1.5 per cent agarose gel. The molecular weight of the band varied from 350 bp to 2.800 kb (Fig. 4.14). All amplicons generated with this primer were monomorphic; with no association with VSD response.

Table 4.2 Details of amplification with the 71 primers for ISSR assay in cocoa genotypes

Sr. No	Primers	Amplification pattern			
		No. of Bands	type of bands		Remarks
			Distinct	Faint	
1	UBC 354	0	0	0	Not selected
2	UBC 807	6	2	4	Not selected
3	UBC 811	8	4	4	Selected
4	UBC 812	0	0	0	Not selected
5	UBC 813	5	2	3	Not selected
6	UBC 814	0	0	0	Not Selected
7	UBC 815	12	5	7	Selected
8	UBC 817	0	0	0	Not Selected
9	UBC 818	6	0	6	Not Selected
10	UBC 820	4	1	3	Not Selected
11	UBC 823	0	0	0	Not Selected
12	UBC 825	6	1	5	Not Selected
13	UBC 826	9	5	4	Selected
14	UBC 827	16	7	9	Selected
15	UBC 835	18	3	15	Selected
16	UBC 836	5	2	3	Not Selected
17	UBC 840	6	2	4	Not Selected
18	UBC 841	10	3	7	Selected
19	UBC 843	0	0	0	Not Selected
20	UBC 844	12	8	4	Selected
21	UBC 845	5	1	4	Not Selected
22	UBC 846	14	5	9	Selected

23	UBC 848	0	0	0	Not Selected
24	UBC 854	5	3	2	Not Selected
25	UBC 855	10	8	2	Selected
26	UBC 856	2	1	1	Not Selected
27	UBC 857	11	6	5	Selected
28	UBC 865	0	0	0	Not Selected
29	UBC 866	10	8	2	Selected
30	UBC 873	1	1	0	Not Selected
31	UBC 880	7	2	5	Not Selected
32	UBC 890	1	1	0	Not Selected
33	UBC 892	0	0	0	Not Selected
34	UBC 895	6	5	1	Not Selected
35	UBC 899	0	0	0	Not Selected
36	UBC 900	0	0	0	Not Selected
37	UBC S2	6	4	2	Not Selected
38	ISSR 2	0	0	0	Not Selected
39	ISSR 3	0	0	0	Not Selected
40	ISSR 4	0	0	0	Not Selected
41	ISSR 5	4	2	2	Not Selected
42	ISSR 6	0	0	0	Not Selected
43	ISSR 7	11	7	4	Selected
44	ISSR 8	0	0	0	Not Selected
45	ISSR 9	0	0	0	Not Selected
46	ISSR 03	0	0	0	Not Selected
47	ISSR 04	0	0	0	Not Selected
48	ISSR 05	0	0	0	Not Selected
49	ISSR 06	0	0	0	Not Selected

50	ISSR 08	0	0	0	Not Selected
51	ISSR 09	0	0	0	Not Selected
52	ISSR 010	0	0	0	Not Selected
53	Oligo ISSR 04	4	0	4	Not Selected
54	Oligo ISSR 05	0	0	0	Not Selected
55	Oligo ISSR 06	5	4	1	Not Selected
56	Oligo ISSR 07	3	0	3	Not Selected
57	Oligo ISSR 08	0	0	0	Not Selected
58	Oligo ISSR 15	0	0	0	Not Selected
59	Oligo SPS 03	0	0	0	Not Selected
60	Oligo SPS 08	0	0	0	Not Selected
61	R 11	3	2	1	Not Selected
62	P1	4	3	1	Not Selected
63	P2	5	2	3	Not Selected
64	S1	0	0	0	Not Selected
65	S2	0	0	0	Not Selected
66	S3	0	0	0	Not Selected
67	S4	0	0	0	Not Selected
68	HB 10	5	3	2	Not Selected
69	HB 12	14	8	6	Selected
70	S07	5	4	1	Not Selected
71	17899A	0	0	0	Not Selected

Table 4.3 Details of ISSR primers selected for identification of resistant gene

Sl. No.	Primer	Annealing temperature (°C)	Nucleotide Sequence
1	UBC 811	43.3	5'- GAGAGAGAGAGAGAGAC-3'
2	UBC 815	44.9	5'- CTCTCTCTCTCTCTCTG-3'
3	UBC 826	53.3	5'- ACACACACACACACACC-3'
4	UBC 827	54.9	5' -ACACACACACACACACG-3'
5	UBC 835	45.6	5'- AGAGAGAGAGAGAGAGYC-3'
6	UBC 841	46.0	5'- GAGAGAGAGAGAGAGAY-3'
7	UBC 844	46.5	5'CTCTCTCTCTCTCTCTRC-3'
8	UBC 846	53.7	5'- CACACACACACACACART-3'
9	UBC 855	51.9	5'- ACACACACACACACACYT-3'
10	UBC 857	52.1	5'- ACACACACACACACACYG-3'
11	UBC 866	55.0	5'- CTCCTCCTCCTCCTCCTC-3'
12	ISSR 7	44.9	5'- ATTATTGTTGTTGTTGTA-3'
13	HB 12	31.0	5'-CACCACCACGC-3'

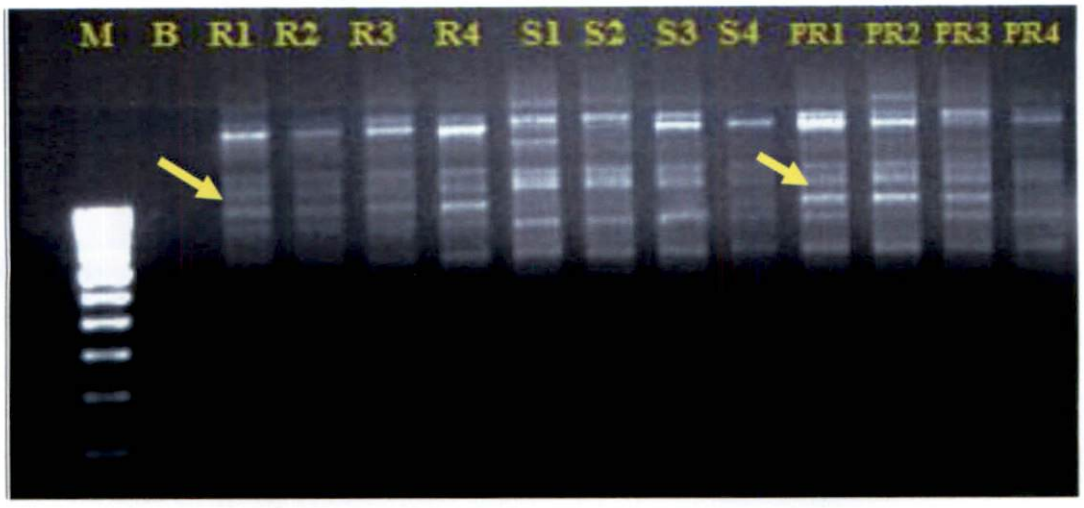


Fig. 4.3 Amplification pattern of twelve cocoa accessions generated with ISSR primer UBC 811

M: 1000 bp Molecular marker, Lane B: Blank, **R1**: VSD I- 4.6, **R2**: VSD I- 4.11, **R3**: VSD I- 5.8, **R4**: VSD I- 6.9, **S1**: G VI- 50, **S2**: G VI- 52, **S3**: G VI- 82, **S4**: G VI- 100, **PR1**: G VI- 25, **PR2**: G VI- 53, **PR3**: G VI- 67, **PR4**: G VI- 144.

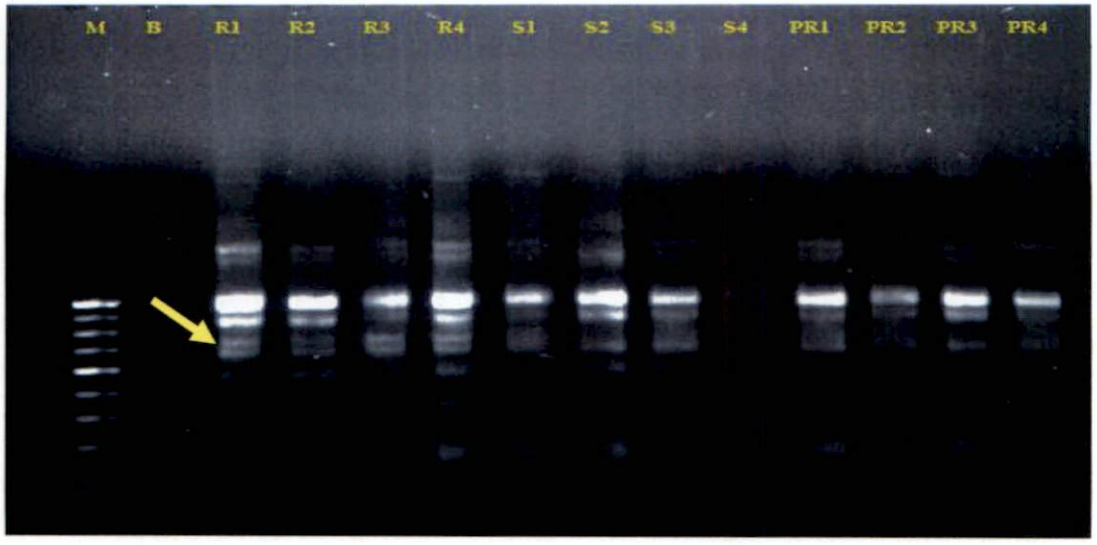


Fig. 4.4 Amplification pattern of twelve cocoa accessions generated with ISSR primer UBC 815

M: 1000 bp Molecular marker, Lane B: Blank, **R1**: VSD I- 4.6, **R2**: VSD I- 4.11, **R3**: VSD I- 5.8, **R4**: VSD I- 6.9, **S1**: G VI- 50, **S2**: G VI- 52, **S3**: G VI- 82, **S4**: G VI- 100, **PR1**: G VI- 25, **PR2**: G VI- 53, **PR3**: G VI- 67, **PR4**: G VI- 144.

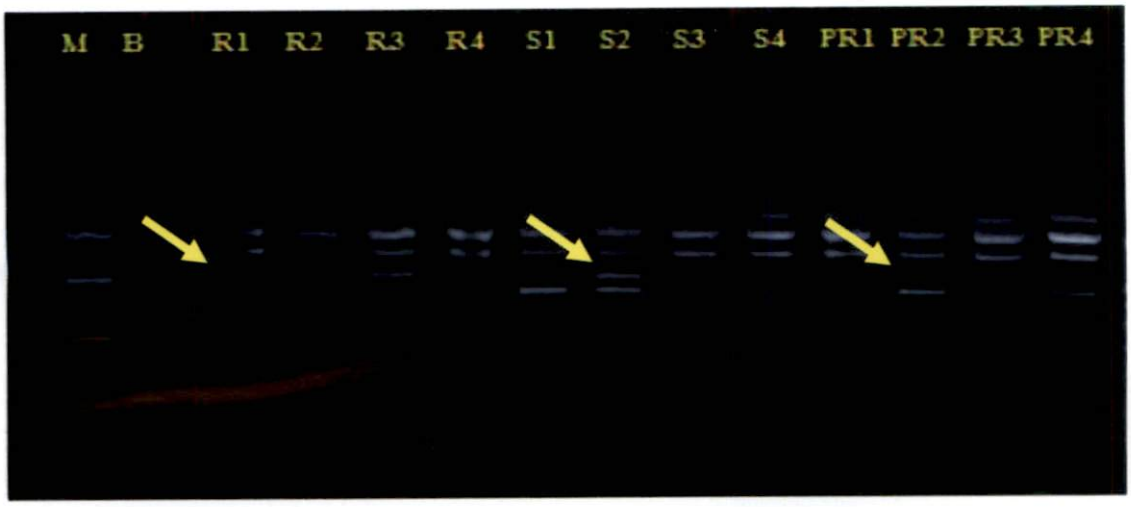


Fig. 4.5 Amplification pattern of twelve cocoa accessions generated with ISSR primer UBC 826

M: 1000 bp Molecular marker, Lane B: Blank, **R1**: VSD I- 4.6, **R2**: VSD I- 4.11, **R3**: VSD I- 5.8, **R4**: VSD I- 6.9, **S1**: G VI- 50, **S2**: G VI- 52, **S3**: G VI- 82, **S4**: G VI- 100, **PR1**: G VI- 25, **PR2**: G VI- 53, **PR3**: G VI- 67, **PR4**: G VI- 144.

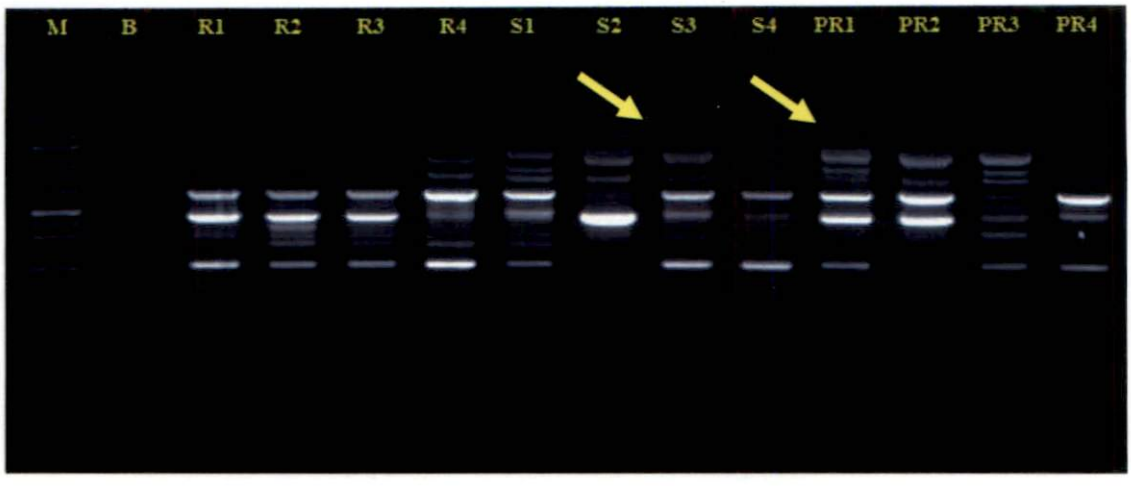


Fig. 4.6 Amplification pattern of twelve cocoa accessions generated with ISSR primer UBC 827

M: 3 kb Molecular marker, Lane B: Blank, **R1**: VSD I- 4.6, **R2**: VSD I- 4.11, **R3**: VSD I- 5.8, **R4**: VSD I- 6.9, **S1**: G VI- 50, **S2**: G VI- 52, **S3**: G VI- 82, **S4**: G VI- 100, **PR1**: G VI- 25, **PR2**: G VI- 53, **PR3**: G VI- 67, **PR4**: G VI- 144.

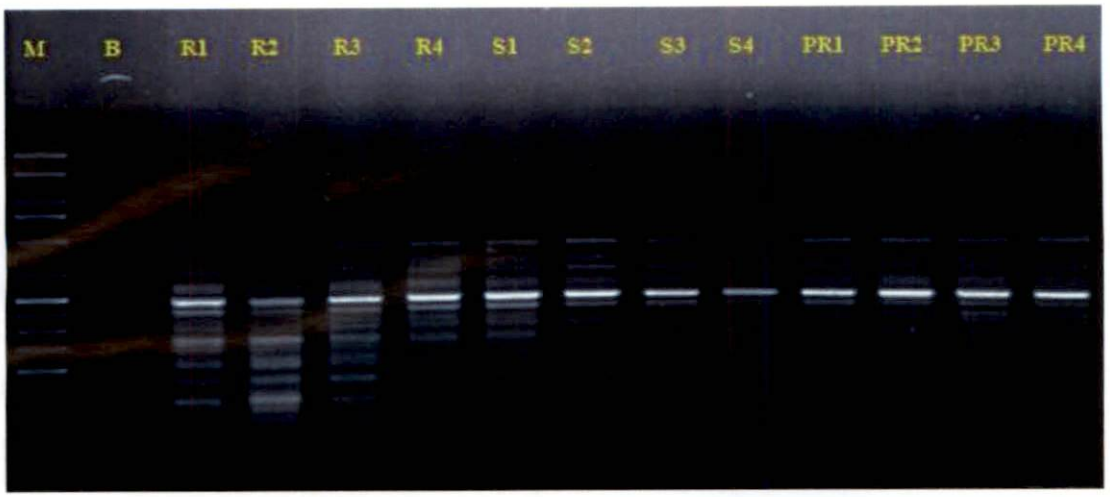


Fig. 4.7 Amplification pattern of twelve cocoa accessions generated with ISSR primer UBC 835

M: 3 kb Molecular marker, Lane B: Blank, **R1**: VSD I- 4.6, **R2**: VSD I- 4.11, **R3**: VSD I- 5.8, **R4**: VSD I- 6.9, **S1**: G VI- 50, **S2**: G VI- 52, **S3**: G VI- 82, **S4**: G VI- 100, **PR1**: G VI- 25, **PR2**: G VI- 53, **PR3**: G VI- 67, **PR4**: G VI- 144.

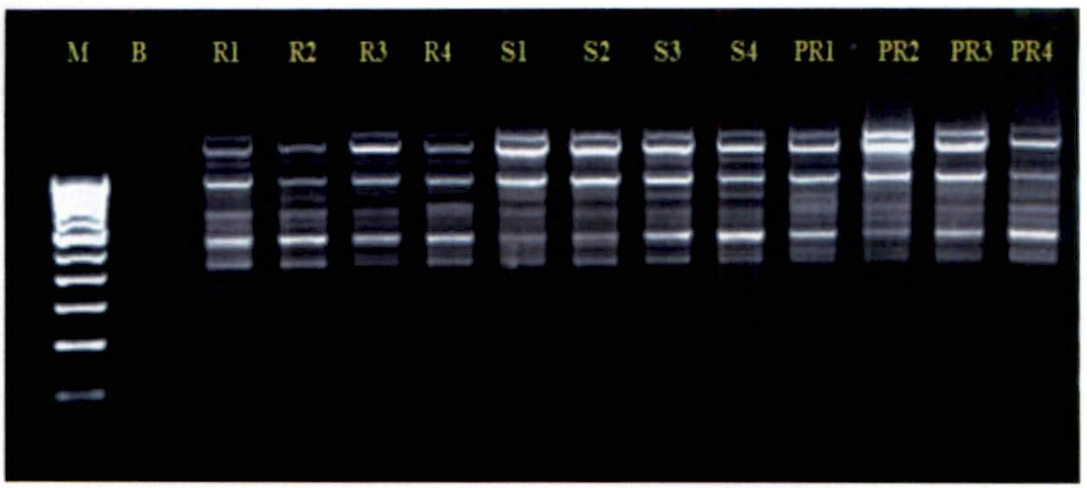


Fig. 4.8 Amplification pattern of twelve cocoa accessions generated with ISSR primer UBC 841

M: 1000 bp Molecular marker, Lane B: Blank, **R1**: VSD I- 4.6, **R2**: VSD I- 4.11, **R3**: VSD I- 5.8, **R4**: VSD I- 6.9, **S1**: G VI- 50, **S2**: G VI- 52, **S3**: G VI- 82, **S4**: G VI- 100, **PR1**: G VI- 25, **PR2**: G VI- 53, **PR3**: G VI- 67, **PR4**: G VI- 144.

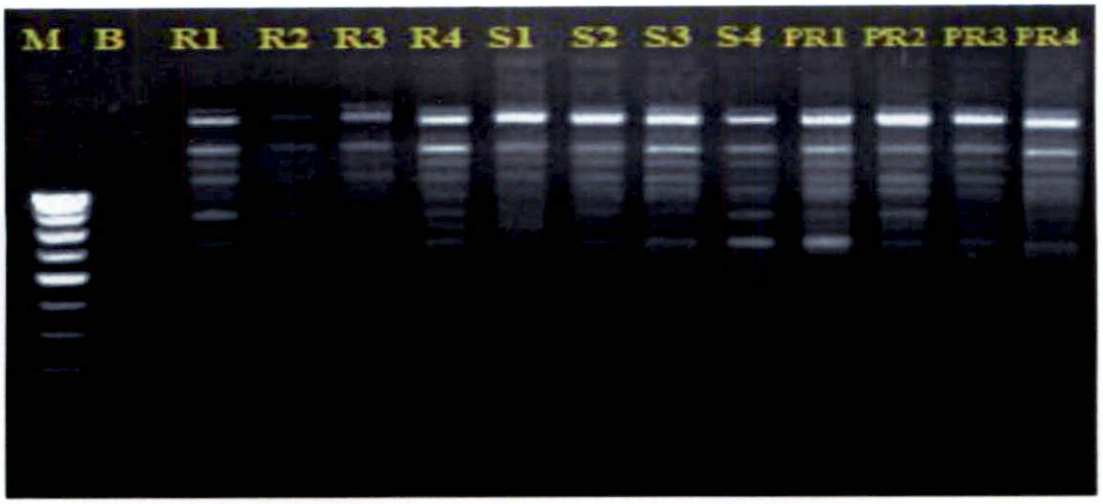


Fig. 4.9 Amplification pattern of twelve cocoa accessions generated with ISSR primer UBC 844

M: 1000 bp Molecular marker, Lane B: Blank, **R1**: VSD I- 4.6, **R2**: VSD I- 4.11, **R3**: VSD I- 5.8, **R4**: VSD I- 6.9, **S1**: G VI- 50, **S2**: G VI- 52, **S3**: G VI- 82, **S4**: G VI- 100, **PR1**: G VI- 25, **PR2**: G VI- 53, **PR3**: G VI- 67, **PR4**: G VI- 144.

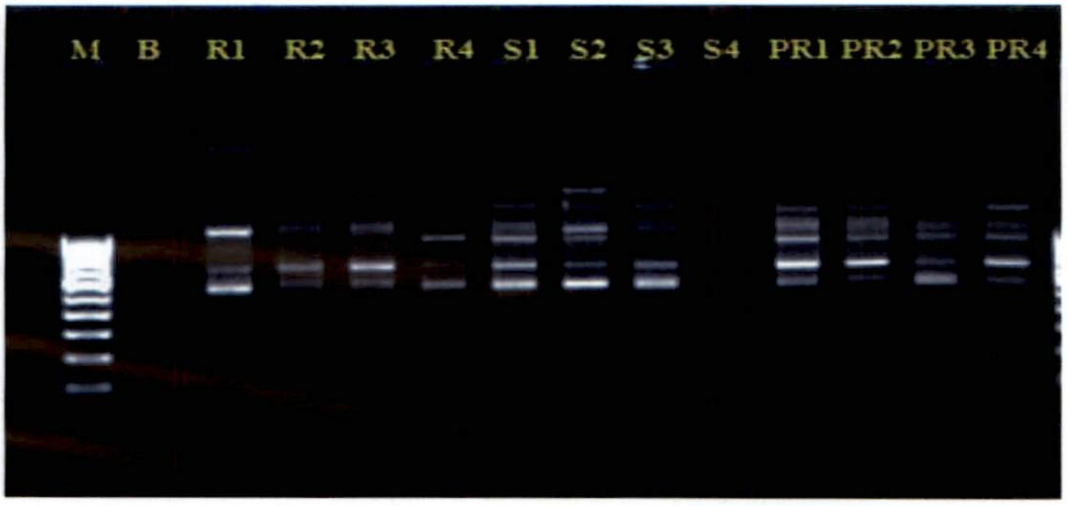


Fig. 4.10 Amplification pattern of twelve cocoa accessions generated with ISSR primer UBC 846

M: 1000 bp Molecular marker, Lane B: Blank, **R1**: VSD I- 4.6, **R2**: VSD I- 4.11, **R3**: VSD I- 5.8, **R4**: VSD I- 6.9, **S1**: G VI- 50, **S2**: G VI- 52, **S3**: G VI- 82, **S4**: G VI- 100, **PR1**: G VI- 25, **PR2**: G VI- 53, **PR3**: G VI- 67, **PR4**: G VI- 144.

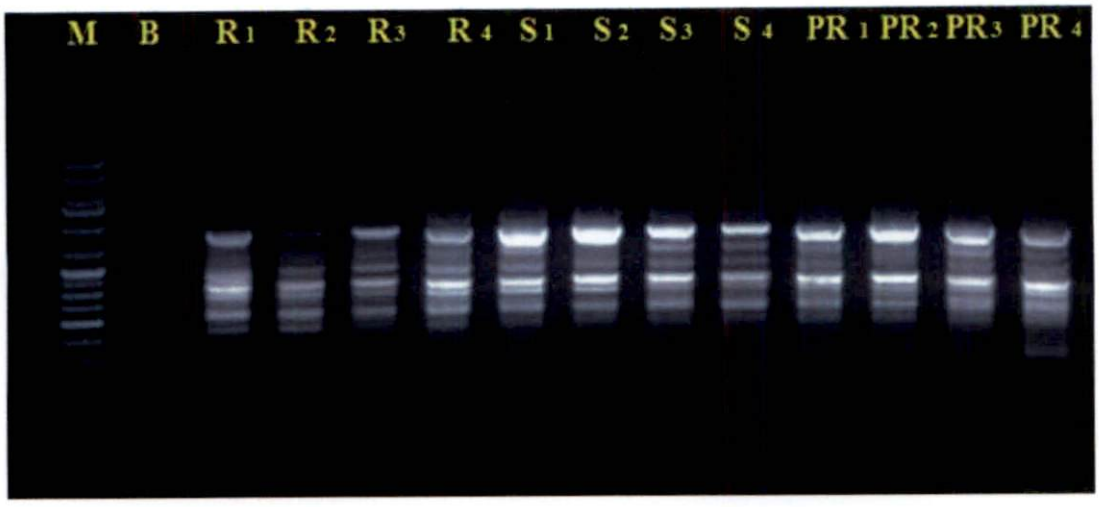


Fig. 4.11 Amplification pattern of twelve cocoa accessions generated with ISSR primer UBC 855

M: 3 kb Molecular marker, Lane B: Blank, **R1**: VSD I- 4.6, **R2**: VSD I- 4.11, **R3**: VSD I- 5.8, **R4**: VSD I- 6.9, **S1**: G VI- 50, **S2**: G VI- 52, **S3**: G VI- 82, **S4**: G VI- 100, **PR1**: G VI- 25, **PR2**: G VI- 53, **PR3**: G VI- 67, **PR4**: G VI- 144.

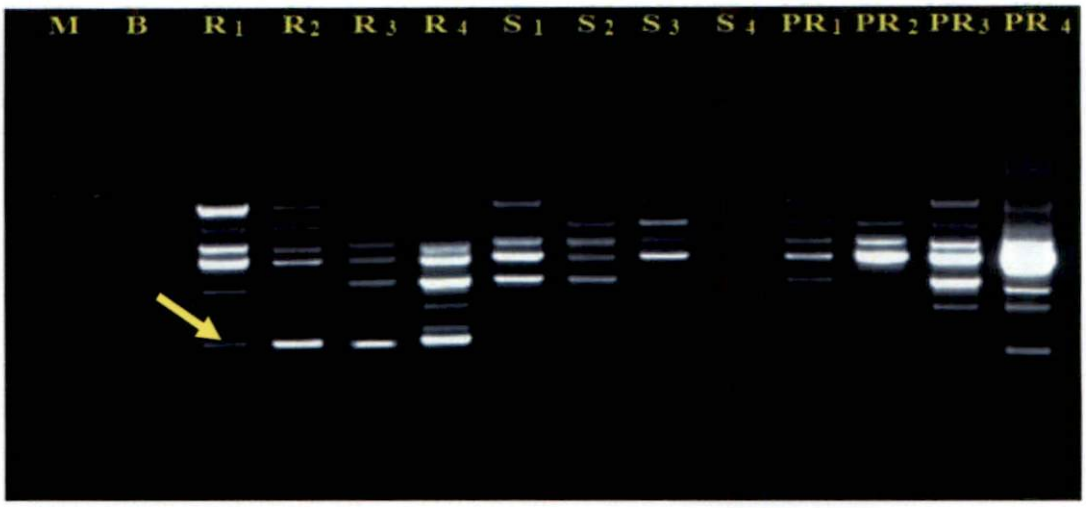


Fig. 4.12 Amplification pattern of twelve cocoa accessions generated with ISSR primer UBC 857

M: 3 kb Molecular marker, Lane B: Blank, **R1**: VSD I- 4.6, **R2**: VSD I- 4.11, **R3**: VSD I- 5.8, **R4**: VSD I- 6.9, **S1**: G VI- 50, **S2**: G VI- 52, **S3**: G VI- 82, **S4**: G VI- 100, **PR1**: G VI- 25, **PR2**: G VI- 53, **PR3**: G VI- 67, **PR4**: G VI- 144.

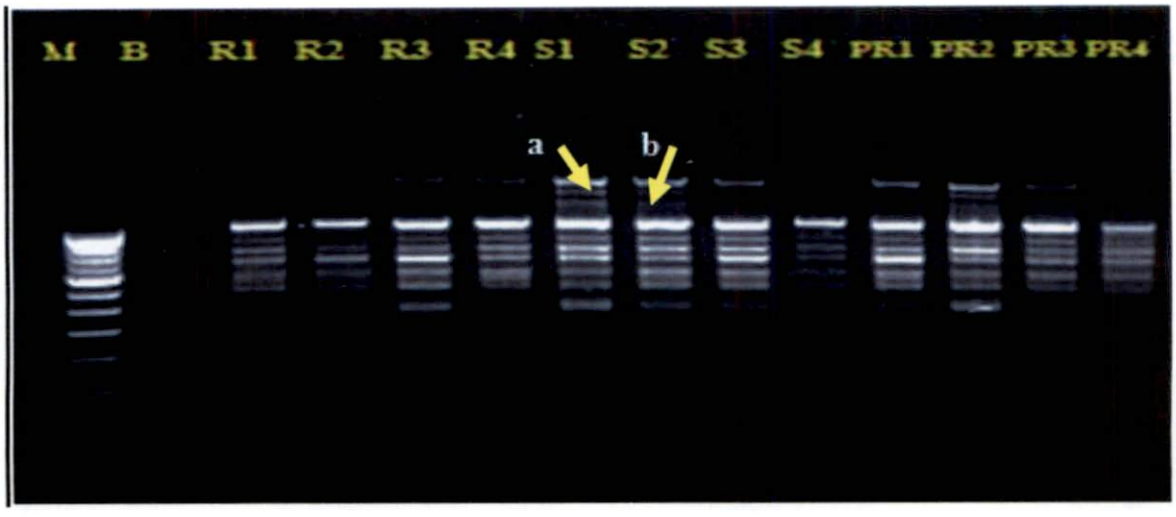


Fig. 4.13 Amplification pattern of twelve cocoa accessions generated with ISSR primer UBC 866

M: 1000 bp Molecular marker, Lane B: Blank, **R1**: VSD I- 4.6, **R2**: VSD I- 4.11, **R3**: VSD I- 5.8, **R4**: VSD I- 6.9, **S1**: G VI- 50, **S2**: G VI- 52, **S3**: G VI- 82, **S4**: G VI- 100, **PR1**: G VI- 25, **PR2**: G VI- 53, **PR3**: G VI- 67, **PR4**: G VI- 144.

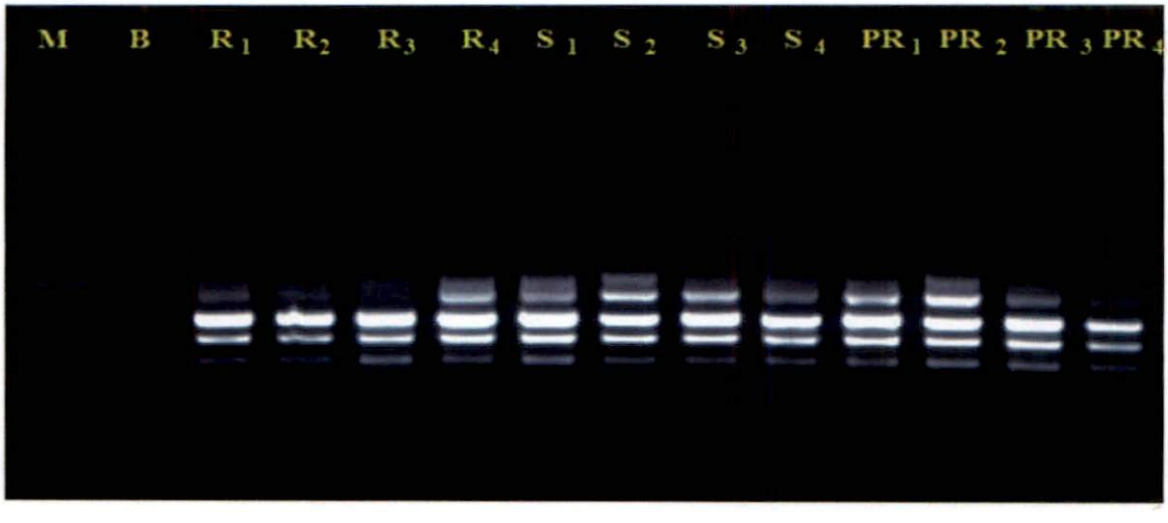


Fig. 4.14 Amplification pattern of twelve cocoa accessions generated with ISSR primer ISSR 7

M: 3 kb Molecular marker, Lane B: Blank, **R1**: VSD I- 4.6, **R2**: VSD I- 4.11, **R3**: VSD I- 5.8, **R4**: VSD I- 6.9, **S1**: G VI- 50, **S2**: G VI- 52, **S3**: G VI- 82, **S4**: G VI- 100, **PR1**: G VI- 25, **PR2**: G VI- 53, **PR3**: G VI- 67, **PR4**: G VI- 144.

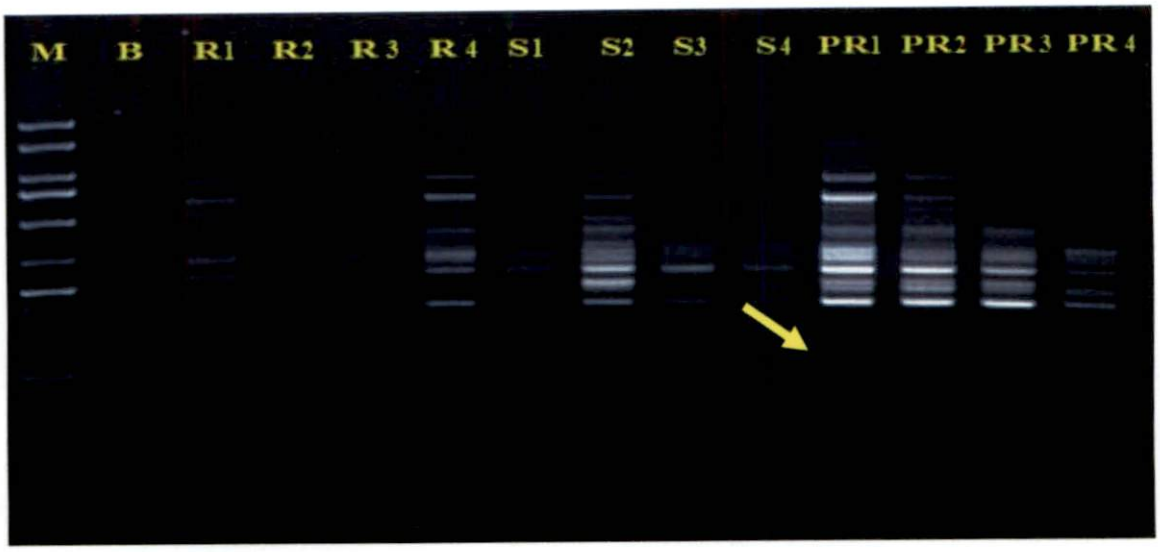


Fig. 4.15 Amplification pattern of twelve cocoa accessions generated with ISSR primer HB 12

M: 3 kb Molecular marker, Lane B: Blank, **R1**: VSD I- 4.6, **R2**: VSD I- 4.11, **R3**: VSD I- 5.8, **R4**: VSD I- 6.9, **S1**: G VI- 50, **S2**: G VI- 52, **S3**: G VI- 82, **S4**: G VI- 100, **PR1**: G VI- 25, **PR2**: G VI- 53, **PR3**: G VI- 67, **PR4**: G VI- 144.

4.3.1.13 HB 12

When the accession were screened with the ISSR primer HB 12, on an average fourteen clear amplicons were generated in all accession. Although this primer has generated polymorphic amplicons at 500bp and 550 bp. The molecular weight of the band varied from 500 bp to 2.800 kb (Fig. 4.15). There was no linkage for the amplicon with the VSD resistance gene. The marked amplicon in the figure was present in partially resistant lines but were absent in all resistant and susceptible lines.

4.3.2 Simple Sequence Repeat (SSR) analysis

Forty six primer sets were initially screened for their capability to amplify the SSR regions in the genomic DNA of cocoa, with the thermal settings mentioned earlier (3.3.3). The amplification observed is presented in Table 4.4. Based on the best amplification pattern, five primer sets (Table 4.5) were selected for further analysis.

4.3.2.1 mTcCIR 10

SSR assay using the primer set mTcCIR 10; followed by electrophoresis on two per cent agarose gel has generated a maximum of four amplicons in the accession under study. The molecular weight of the band varied from 250 bp to 1 kb (Fig. 4.16). The amplicons generated were monomorphic with no association with VSD resistance.

4.3.2.2 mTcCIR 12

When the accessions were analysed using the SSR primer mTcCIR 12, bands of three distinct sizes were observed across all the accessions (Fig. 4.17). The molecular weight of the bands varied from 200 bp to 300 bp. This primer is highly polymorphic but failed to establish any linkage with the VSD resistance. Since these amplicons had only slight variation in their sizes, it was difficult to establish their possible association with the VSD on a two per cent agarose gel. Hence PAGE analysis was carried out (Fig. 4.18). But fails to establish any positive linkage.

4.3.2.3 mTcCIR 33

When the accessions were screened with the SSR primer mTcCIR 33, amplification with four different sizes was generated across the accessions (Fig. 4.19) and the sizes of amplicon varied from 300 to 800 bp. There was strict similarity in the amplification pattern generated by mTcCIR12 and mTcCIR33. This primer is highly polymorphic but failed to establish any association with VSD. Since the analysis was difficult on two per cent agarose gel, PAGE analysis of the PCR product was also done (Fig. 4.20) which also failed to establish linkage.

4.3.2.4 SHRSTc 53

When the DNA from the cocoa accessions were PCR amplified using SSR primer SHRSTc 53 and electrophoresed on two per cent agarose gel, bands with three different sizes were generated across the accessions. The sizes of the amplicon varied from 200 to 400 bp (Fig. 4.21). Since the initial screening on four resistant and four susceptible lines failed to generate any polymorphism, Partially resistant lines were not analysed further.

4.3.2.5 SHRSTc 64

When the accessions were analysed using the SSR primer SHRSTc 64, two amplicons were generated across the accession. A faint band of size 170 bp and a distinct band of size 220 bp were generated (Fig. 4.22). Since the initial analysis using four resistant and four susceptible accessions failed to generate any kind of polymorphism, the partially resistant lines were not further analysed.

Table 4.4 Details of the amplification pattern generated with the 46 primers screened for SSR assay on cocoa genotypes using the bulked DNA.

Sl. No	Primer	Amplification pattern			
		No. of Bands	Type of band		Remarks
			Distinct	Faint	
1	mTcCIR 6	1	1	0	Not Selected
2	mTcCIR 10	4	4	0	Selected
3	mTcCIR 11	1	1	0	Not Selected
4	mTcCIR 12	3	2	1	Selected
5	mTcCIR 15	3	1	2	Selected
6	mTcCIR 18	1	1	0	Not Selected
7	mTcCIR 33	3	2	1	Selected
8	mTcCIR 25	1	1	0	Not Selected
9	mTcCIR 102	1	1	0	Not Selected
10	mTcCIR 115	1	1	0	Not Selected
11	mTcCIR 121	1	1	0	Not Selected
12	mTcCIR 146	1	1	0	Not Selected
13	mTcCIR 158	1	1	0	Not Selected
14	mTcCIR 168	1	1	0	Not Selected
15	mTcCIR 222	1	1	0	Not Selected
16	SHRSTc 49	1	1	0	Not Selected
17	SHRSTc 51	1	0	1	Not Selected
18	SHRSTc 52	1	1	0	Not Selected
19	SHRSTc 53	2	2	0	Selected
20	SHRSTc 64	2	2	0	Selected
21	SHRSTc 66	1	1	0	Not Selected

22	mAoR 03	1	1	0	Not Selected
23	mAoR 06	0	0	0	Not Selected
24	mAoR 07	0	0	0	Not Selected
25	mAoR 11	0	0	0	Not Selected
26	mAoR 17	0	0	0	Not Selected
27	mAoR 29	1	1	0	Not Selected
28	mAoR 42	0	0	0	Not Selected
29	mAoR 46	0	0	0	Not Selected
30	mAoR 48	0	0	0	Not Selected
31	mAoR 52	1	0	1	Not Selected
32	mAoR 55	1	0	1	Not Selected
33	SSRKAU 2	0	0	0	Not Selected
34	SSRKAU 3	0	0	0	Not Selected
35	SSRKAU 4	1	0	1	Not Selected
36	SSRKAU 10	0	0	0	Not Selected
37	SSRKAU 11	1	1	0	Not Selected
38	SSRKAU 12	0	0	0	Not Selected
39	SSRKAU 13	1	0	1	Not Selected
40	SSRKAU 15	0	0	0	Not Selected
41	SSRKAU 19	0	0	0	Not Selected
42	SSRKAU 20	0	0	0	Not Selected
43	SSRKAU 21	0	0	0	Not Selected
44	SSRKAU 22	1	0	1	Not Selected
45	SSRKAU 24	0	0	0	Not Selected
46	SSRKAU 25	0	0	0	Not Selected

Table 4.5 Details of SSR primers selected after screening

Sl. No.	Name of Primer	Annealing temperature (°C)	Sequence
1	mTcCIR 10	63.7	**F** 5'- CCGAATTGACAGATGGCCTA -3' **R** 5'- CCCAAGCAAGCCTCATACTC -3'
2	mTcCIR 12	51.7	**F** 5'- TCTGACCCCAAACCTGTA -3' **R** 5'- ATTCCAGTTAAAGCACAT -3'
3	mTcCIR 33	54.5	**F** 5'- TGGGTTGAAGATTTGGT -3' **R** 5'- CAACAATGAAAATAGGCA -3'
4	SHRSTc 53	53.3	**F** 5'- TTCCCTTTCTTTCTCTCTCTC -3' **R** 5'- AGTCGTTGCTACTGCTGG -3'
5	SHRSTc 64	54.0	**F** 5'- TCCTACATTCCTGCACCC -3' **R** 5'- TCGAGAGAAAAGCTCTTACACT -3'

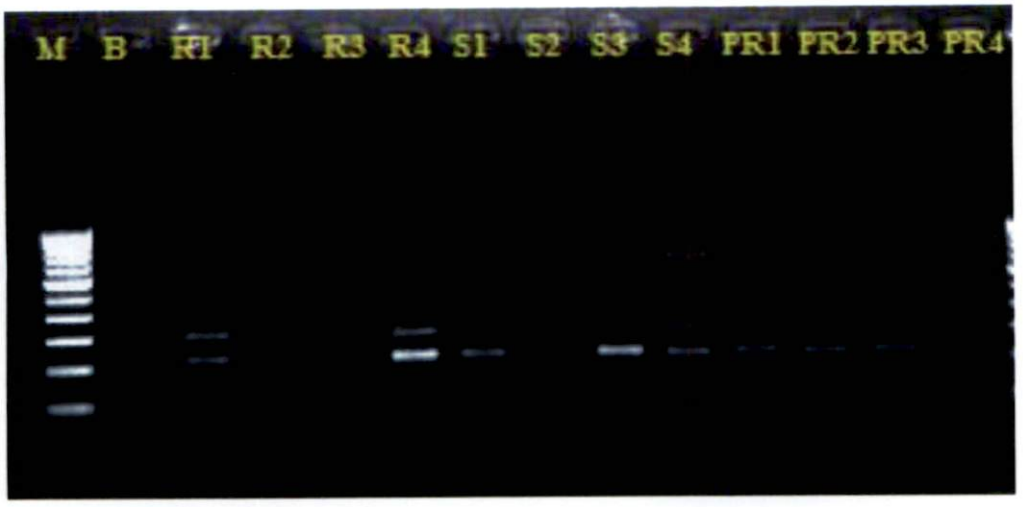


Fig. 4.16 Amplification pattern of twelve cocoa accessions generated with SSR primer mTcCIR 10

M: 1000 bp Molecular marker, Lane B: Blank, **R1**: VSD I- 4.6, **R2**: VSD I- 4.11, **R3**: VSD I- 5.8, **R4**: VSD I- 6.9, **S1**: G VI- 50, **S2**: G VI- 52, **S3**: G VI- 82, **S4**: G VI- 100, **PR1**: G VI- 25, **PR2**: G VI- 53, **PR3**: G VI- 67, **PR4**: G VI- 144.

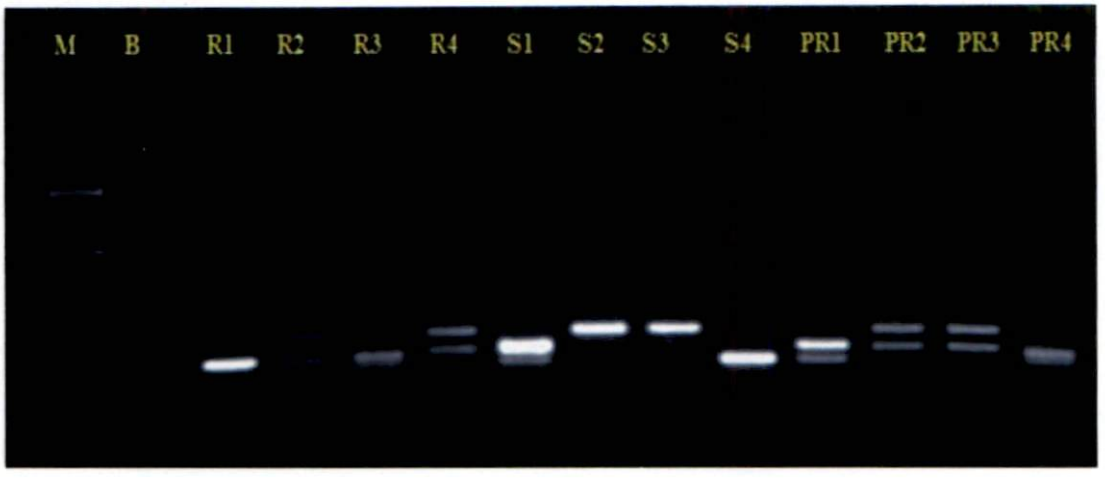


Fig. 4.17 Amplification pattern of twelve cocoa accessions generated with SSR primer mTcCIR 12

M: 1000 bp Molecular marker, Lane B: Blank, **R1**: VSD I- 4.6, **R2**: VSD I- 4.11, **R3**: VSD I- 5.8, **R4**: VSD I- 6.9, **S1**: G VI- 50, **S2**: G VI- 52, **S3**: G VI- 82, **S4**: G VI- 100, **PR1**: G VI- 25, **PR2**: G VI- 53, **PR3**: G VI- 67, **PR4**: G VI- 144.

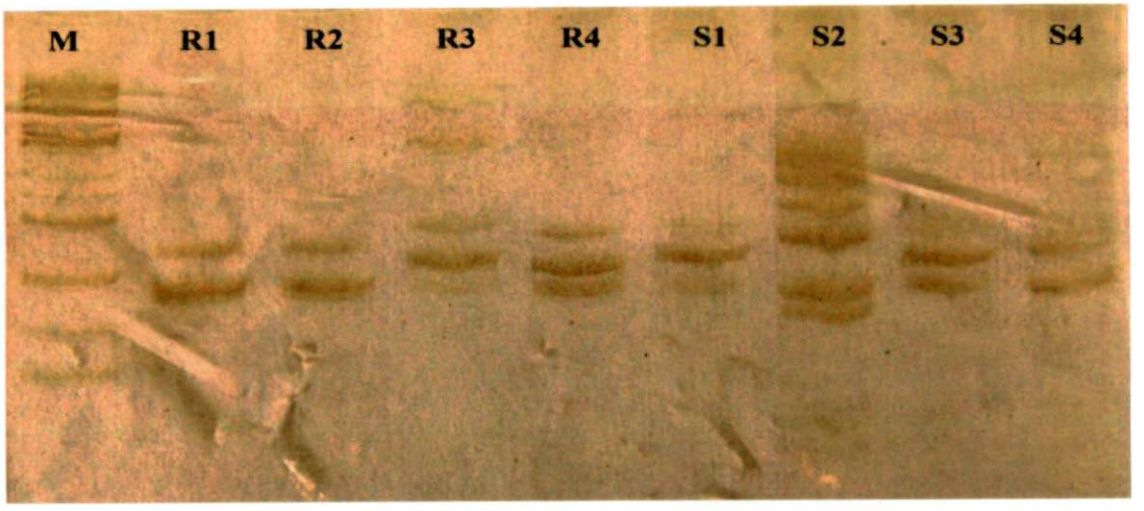


Fig. 4.18 Amplification pattern of eight cocoa accessions generated with SSR primer mTcCIR 12 on PAGE

M: 1000 bp Molecular marker, **R1**: VSD I- 4.6, **R2**: VSD I- 4.11, **R3**: VSD I- 5.8, **R4**: VSD I- 6.9, **S1**: G VI- 50, **S2**: G VI- 52, **S3**: G VI- 82, **S4**: G VI- 100.

Fig. 4.19 Amplification pattern of twelve cocoa accessions generated with SSR primer mTcCIR 33

M: 1000 bp Molecular marker, Lane B: Blank, **R1**: VSD I- 4.6, **R2**: VSD I- 4.11, **R3**: VSD I- 5.8, **R4**: VSD I- 6.9, **S1**: G VI- 50, **S2**: G VI- 52, **S3**: G VI- 82, **S4**: G VI- 100, **PR1**: G VI- 25, **PR2**: G VI- 53, **PR3**: G VI- 67, **PR4**: G VI- 144.

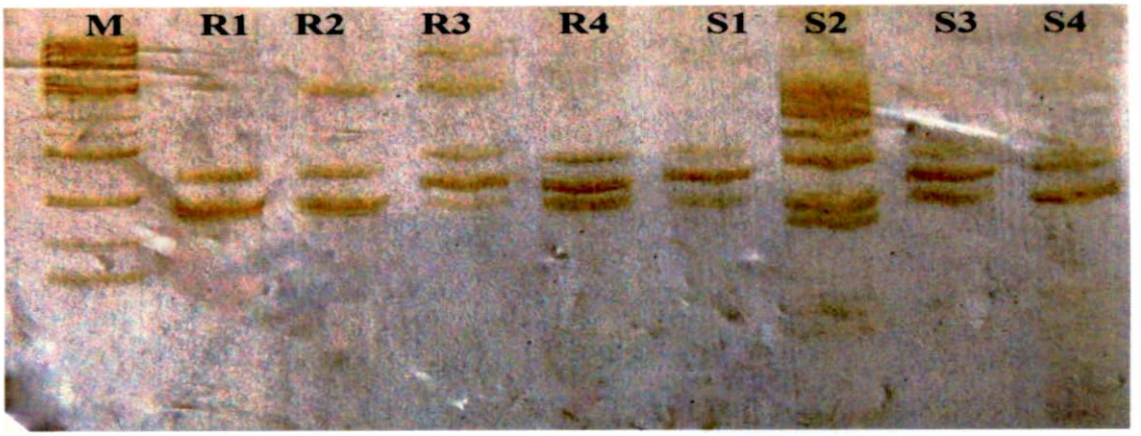


Fig. 4.20 Amplification pattern of eight cocoa accessions generated with SSR primer mTcCIR 33 on PAGE

M: 1000 bp Molecular marker, **R1**: VSD I- 4.6, **R2**: VSD I- 4.11, **R3**: VSD I- 5.8, **R4**: VSD I- 6.9, **S1**: G VI- 50, **S2**: G VI- 52, **S3**: G VI- 82, **S4**: G VI- 100.

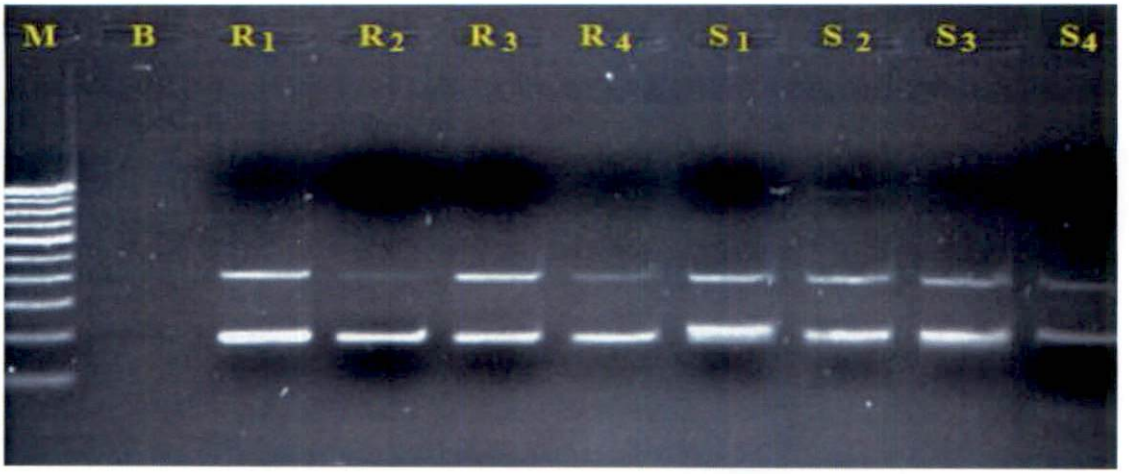


Fig. 4.21 Amplification pattern of eight cocoa accessions generated with SSR primer SHRSTc 53

M: 1000 bp Molecular marker, B: Blank, **R1**: VSD I- 4.6, **R2**: VSD I- 4.11, **R3**: VSD I- 5.8, **R4**: VSD I- 6.9, **S1**: G VI- 50, **S2**: G VI- 52, **S3**: G VI- 82, **S4**: G VI- 100.

Fig. 4.22 Amplification pattern of eight cocoa accessions generated with SSR primer SHRSTc 64

M: 1000 bp Molecular marker, B: Blank, **R1**: VSD I- 4.6, **R2**: VSD I- 4.11, **R3**: VSD I- 5.8, **R4**: VSD I- 6.9, **S1**: G VI- 50, **S2**: G VI- 52, **S3**: G VI- 82, **S4**: G VI- 100.

4.3.3 Sequencing and analysis of polymorphism exhibited in resistant genotypes

For every ISSR primer, the marker profiles generated through electrophoresis of the PCR products on 1.5 per cent agarose gel were photographed and carefully examined. The polymorphic bands were identified in relation to the disease response.

The 450 bp sized polymorphic band generated from UBC 857 was eluted using Nucleospin® Gel and PCR clean up kit (Macherey-Nagel, Germany). The quality and quantity of the eluted and purified DNA were confirmed using agarose gel electrophoresis and was further subjected to reamplification (Fig. 4.23) to confirm that the band contains the amplified products from a single region of the genome. If the DNA eluted from a single polymorphic band was found to yield more than one bands on nested PCR, it was clear that the band contains DNA fragments amplified from different regions of genome and hence cannot be used for direct sequencing on NGS platform.

Since the single band generated using the nested PCR had shown its suitability for direct sequencing, the product was sent for sequencing with the primer UBC 857. The sequencing has given 246 nucleotides. The sequence generated was 5'-**TAGTACATGATTAGAGCACTAGCAAAATAGCAAAGCTGATCAAAAACATTCA ACTATTTCCCTCCATATGTCGCAGAAAAAAAAAAAGAAAAGTTTGTGTTCAAG GAATCTGTTACCCCTTCAAGAAAGTTATTCCTATAGCGAAAAAAAAGATTAAC TTTTAGCTACTGTATTCTTTAAGCAGACCCCAAATGTGGGAACATGAGTGCAA GCATGCATGAGTGTGTTTGTGCTGTTCTTAGGAAA-3'**. Sequence was subjected to *in silco* analysis, using BLAST (Basic Local Alignment Search Tool). In BLASTn, analysis this sequence was found to have 94 per cent similarity with the clone of SSR primer mTcCIR 42 of *Theobroma cacao* with accession number AJ271944.1 (Fig. 4.25 a-c).

Using the mTcCIR 42 forward primer 5'- TTGCTGAAGTATCTTTTGAC-3' and reverse primer 5'- GCTCCACCCCTATTTG-3' twelve genotypes were further

screened at 55° annealing temperature. The analysis has given four kinds of distinct band across the accession (Fig. 4.24). In all the resistant clones and in the third partially resistant clone, the SSR had yielded a distinct band of size 650 bp (marker a). In all the susceptible and other partially resistant lines, except in the third susceptible clone, there were no bands of size above 500 bp. The 1.7 kb band generated in the first resistant and third susceptible plants had no association with resistance or any resistance related parameters (marker b). The partially resistant clones were characterized due to presence of a unique band of size 400 bp (marker c). In the susceptible accessions the marker was slightly heavier at 450 bp (marker d) and absent as in the third susceptible clone. With these clear amplification patterns, this SSR was found to be highly successful to differentiate the VSD resistant, susceptible and partially resistant cocoa clones.

The ISSR primers UBC 811, UBC 815, UBC 826, UBC 857 and UBC 866 gave repeatable and reliable polymorphic amplicons were able to differentiate resistant and susceptible cocoa accession against VSD. The sequence of polymorphic bands has shown that mTcCIR 42 is a SSR highly linked with disease and it was confirmed by screening. Hence mTcCIR 42 is recommended for MAS for cocoa breeding for resistance to VSD.

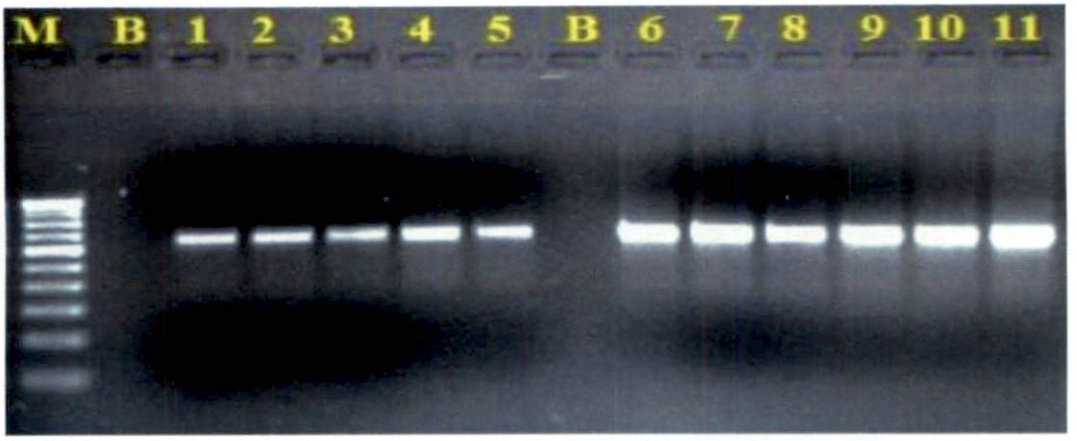


Fig. 4.23 The profile of reamplified PCR generated by the primer UBC 857

M: 1000 bp Molecular marker/ Ladder, B: Blank, 1-5 and 6-11 at 5 µl and 10 µl concentration of DNA eluted from UBC 857

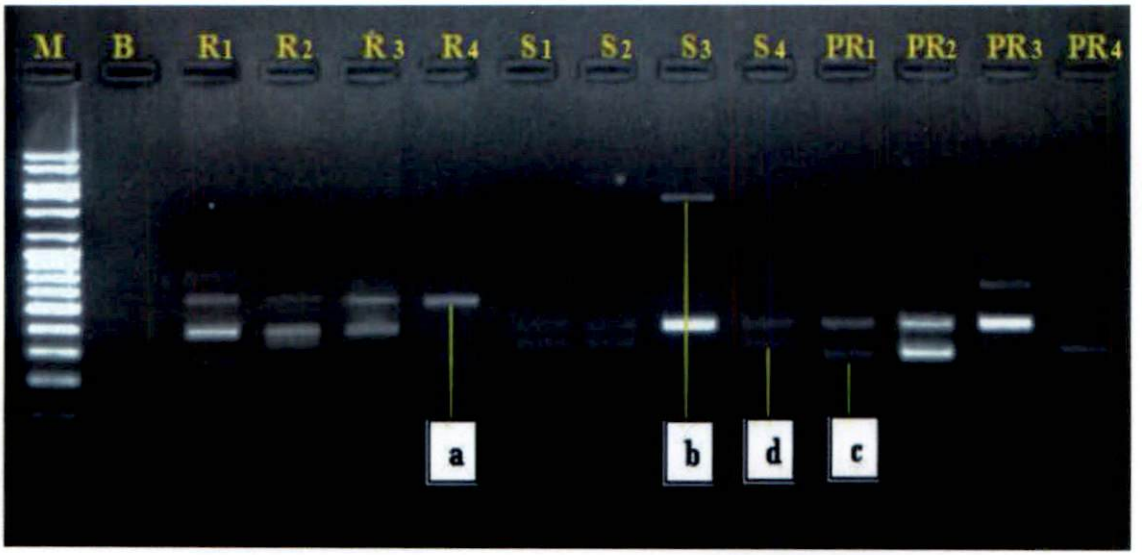
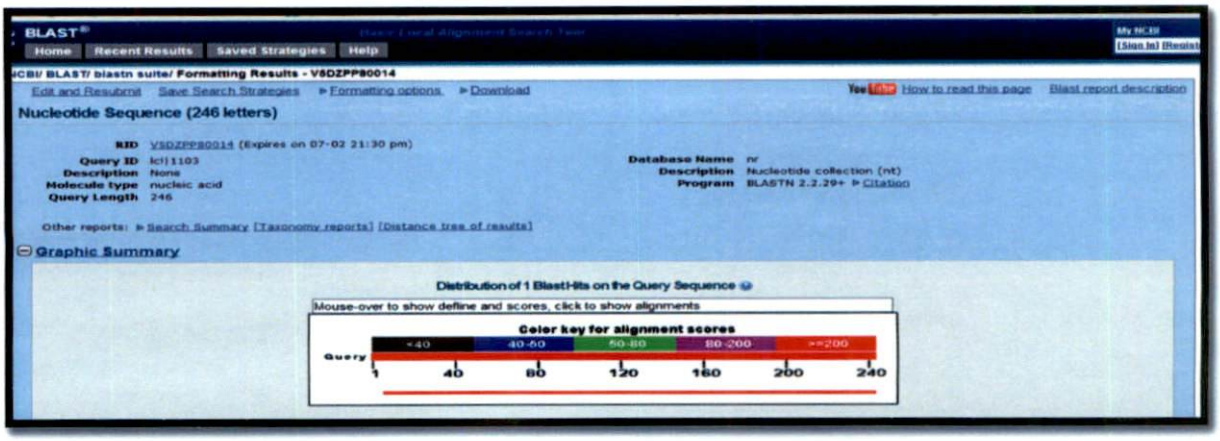
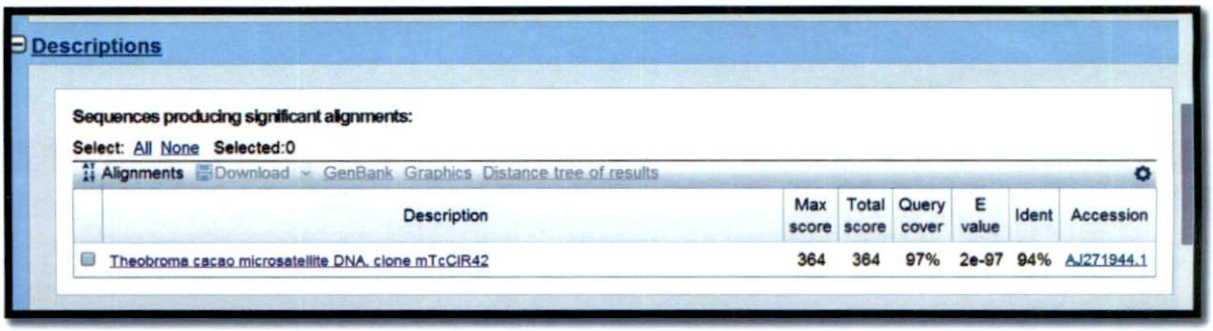


Fig. 4.24 Amplification pattern on twelve cocoa accessions using the SSR primer mTcCIR 42

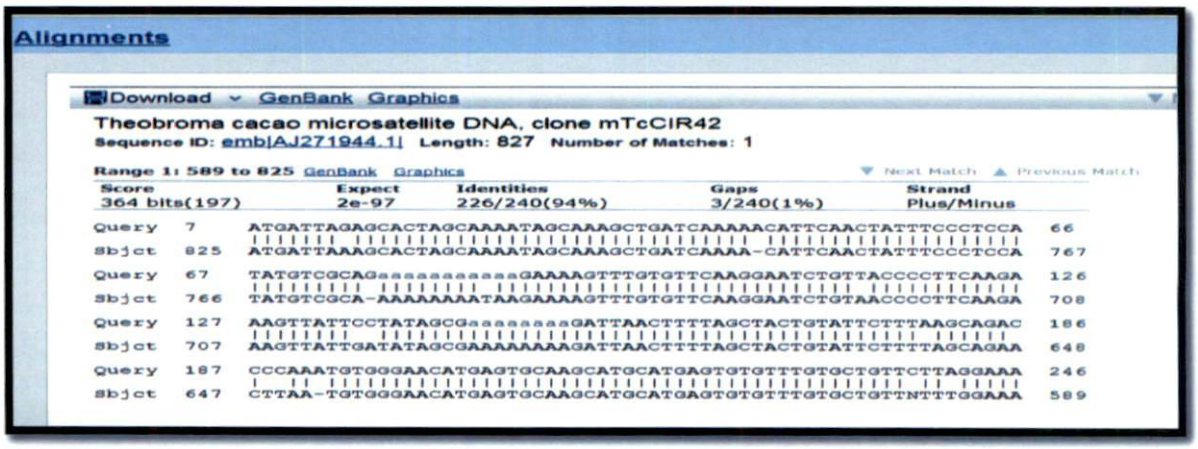
M: 3 kb Molecular marker, Lane B: Blank, **R1**: VSD I- 4.6, **R2**: VSD I- 4.11, **R3**: VSD I- 5.8, **R4**: VSD I- 6.9, **S1**: G VI- 50, **S2**: G VI- 52, **S3**: G VI- 82, **S4**: G VI- 100, **PR1**: G VI- 25, **PR2**: G VI- 53, **PR3**: G VI- 67, **PR4**: G VI- 144.



a: Graphical summary of nucleotide sequence



b: Sequence similarity



c: Alignment

Fig. 4.25 Result of nucleotide BLAST of the 246 bp sequence generated using the polymorphic band obtained with primer UBC 857.

Discussion

5. DISCUSSION

Theobroma cacao L. also called cacao tree, golden tree or cocoa tree is a small evergreen tree in the family *Malvaceae* native to the deep tropical regions of Central and South America with chromosome number 2n=20. Cacao is the only commonly cultivated species among the 22 species of *Theobroma.* The generic name is derived from the Greek word "Food of the God"; from *theos*, meaning "God," and *broma*, meaning "Food".

Cocoa is a beverage crop introduced in India in the early 1965. It is a native of South America and widely cultivated in Ghana, Nigeria, Sierraleon, Cameroon, Brazil, Equador, West Indies and Malaysia. In India cocoa is cultivated in about 22,000 hectares as a mixed crop in coconut and arecanut gardens. Kerala accounts for 79 per cent of the total area and 71 per cent of the total production followed by Karnataka (Indiastat, 2014). Cocoa is highly cross pollinated and growing of different varieties adjacent to each other is beneficial to achieve maximum fruit set and yield realization.

Vascular Streak Dieback (VSD) caused by the fungus *Oncobasidium theobromae*, is a serious disease of cocoa in all the cocoa growing countries all over the world. This disease is characterized by a progressive desiccation of branches beginning at the tip of the branches and gradually spreading downwards. In 1971, Keane and Turner reported the presence of a particular form of dieback of cocoa in West Malaysia which was similar to 'vascular streak dieback' (VSD) caused by *Oncobasidium theobromae* recorded from Papua New Guinea (Keane, 1972). Since reported from Papua New Guinea, the VSD has emerged as the most devastating disease of cocoa in the world. In India, Abraham (1981) followed by Chandramohan and Kaveriappa (1982) were first to report the presence of VSD from the Kottayam district of Kerala state and thereafter, the spread of the disease to the commercial cocoa tracts was very fast, affecting more than 50 per cent crop losses. Cocoa

Research Centre, Kerala Agricultural University, India has developed many high yielding and VSD resistant hybrids and the performance of these hybrids is being evaluated for the past one and half decade. Among the parents used in the extensive crossing programmes, clones GVI 55, GVI 18.5 and GVI 126 were the best source of resistance with maximum recovery of good hybrids having VSD resistance (Minimol and Amma, 2013). More recently, five best performing hybrids S IV1.26, S IV2.29, S IV4.29, S IV6.18 and VSD I31.8 are identified to be released for commercial cultivation (Amma, 2013).

Though the planting with high yielding and resistant cultivars is identified as the only sustainable alternative to counter this disease (Efron, 2000; Efron *et al.*, 2005), the conventional breeding is crippled with the absence of a reliable methodology to screen out for the presence of the resistance gene, without which the breeding in this perennial crop continues to be time demanding. The confirmation of transfer of gene is at most important in crop breeding and availability of a tool to quickly detect the gene will reduce the breeding cycle length. Molecular markers are already proven good to detect the genes of interest. The present study aims at developing a reliable molecular marker linked with the gene offering resistance to VSD in cocoa, using SSR and ISSR marker systems.

In the present study, twelve cocoa clones VSD I-4.6, VSD I-4.11, VSD I-5.8, VSD I-6.9, G VI-50, G VI-52, G VI-82, G VI-100, G VI-25, G VI-53, G VI-67 and G VI-144, with different responses to VSD and maintained at Cocoa Research Centre, College of Horticulture, Kerala Agricultural University, were used. The genotypes were selected on the basis of field screening experiments conducted from 1998-1999 (Minimol and Amma, 2013).

5.1 DNA isolation

Regardless of the population or the DNA marker system, the DNA isolated should be having very good quality. Several methods of DNA extraction suitable for

DNA marker analyses have been developed (Murray and Thompson, 1980; Dellaporta *et al.*, 1983; Couch and Fritz, 1990; Lanaud *et al.*, 1995; Rogers and Bendich, 1994; Perry *et al.*, 1998; Bhattacharjee *et al.*, 2004). The objective was to develop a simple, rapid procedure that yields DNA of good quality and quantity from small amounts of starting material.

Unlike in crops such as rubber (Thulaseedharan *et al.*, 2012) or arabidopsis (Kasajima *et al.*, 2004), where the DNA extraction is easy, cocoa tissues contain high levels of mucilage, tannins, and other polyphenolic compounds. When cells are disrupted, these cytoplasmic compounds come into contact with DNA, RNA and all other cellular components. In their oxidized forms, polyphenols covalently bind to DNA giving it a brown color and making it useless for most research applications (Loomis, 1974).

Several published extraction methods have been tried and found unsuitable for cocoa, due to the high levels of charged polysaccharides and polyphenolic compounds within the tissues. Other published protocols developed specifically for cocoa or plants with high amounts of polyphenolic compounds are complex, require extensive centrifugation steps, often with the use of expensive cesium chloride gradients and specialized equipments (Couch and Fritz, 1990; Figuiera *et al.*, 1992; Lanaud *et al.*, 1995). After trying several modified protocols, the present DNA extraction method was developed for cocoa for further marker analysis.

Modified Doyle and Doyle (1987) method was found to yield good quality DNA. In the standard protocol C:I treatment was given once but in modified method tried three times to remove the excess mucilage. The DNA pellet was progressively washed with the wash buffer (76 per cent ethanol and 10mM ammonium acetate) to remove the stickiness. Treatment with the wash buffer progressively removes the brown color and stickiness of the DNA. In the CTAB method DNA isolated was unable to dissolve in the water due to its stickiness but the DNA from modified Doyle

and Doyle method obtained was easily dissolved in water. The concentration obtained using the CTAB method was very low i.e. 10 ng/µl with RNA and protein contamination, where as in the modified Doyle and Doyle method gave 540 ng/µl DNA without protein and minute quantity of RNA. RNA was further removed by the RNase treatment.

The protocols tried mostly produced DNA with brown color. The brown coloration in the DNA pellets was mostly due to polyphenol and mucilage contamination (Couch and Fritz, 1990). Plants can vary considerably in the amount and number of secondary metabolites they produce, it is unlikely that any one technique for DNA extraction can be developed (Loomis, 1974). This developed DNA extraction protocol can be used to isolate nuclear DNA from a variety of other plant species especially high in polyphenols, tannins and mucilage.

Unsheared and uncontaminated DNA was essential for the subsequent genetic analysis of cocoa varieties. The preparation of a crude nuclear pellet eliminated polysaccharides (Figueira *et al.*, 1992) by preventing co-extraction of DNA and sugars, and also eliminated contamination by non-nuclear DNA. Using the technique described above, it was possible to prepare several DNA samples, rapidly and efficiently, from interflush leaves of mature plants, or from seedling leaves. The major problem encountered in the isolation and purification of high molecular weight DNA from plant species is the degradation of DNA due to endonuclease, polyphenols and other secondary metabolites that directly and indirectly interfere with subsequent enzymatic reactions. The addition of antioxidant, β-mercaptoethanol and PVP (poly vinyl pyrrolidone) during grinding was found effective in obtaining good quality DNA. Additionally the commercial available extraction kits are also recommended for the extraction of DNA from cocoa (Haymes *et al.*, 2004).

5.1.1 Purification and quantification of DNA

Zimmermann *et al.* (1998) reported the methods for purifying the nucleic acids from cell extracts through the techniques such as extraction/precipitation, chromatography, centrifugation and affinity separation.

In the present study, the quality of DNA was tested by subjecting it to agarose gel electrophoresis as well as spectophotometric method. DNA was visualized on 0.8 per cent agarose gel under UV light by ethidium bromide staining. A DNA sample is reported as of high quality if it had a band of high molecular weight with little smearing and a low amount of RNA (Wettasingf and Peffley, 1998). The DNA extracted showed high amount of RNA as a smear below it. To remove RNA, RNaseA was used. Use of RNase A has been reported by several workers (Gallego and Martinez, 1996; Raval *et al.*, 1998). In the present investigation, the RNase treated DNA sample on electrophoresis showed a high molecular weight DNA, which formed a single band just below the well. This indicted that the DNA under test was of good quality.

In the spectrophotometeric method, the ratio of optical density at 260 and 280 nm was worked out to test the quality. The absorbance ratio was calculated as OD at 260/280, for the various samples. Those samples with ratio between 1.8 and 2.0 were considered to be of high quality. All the samples under the study recorded a ratio between 1.8 and 2.0 (Thakur *et al.*, 2014).

5.2 Molecular marker analysis

5.2.1 ISSR analysis

In the present study 71 ISSR primers have been screened and thirteen ISSR primers selected on the basis of screening. From the thirteen primers UBC 811, UBC 815, UBC 826, UBC 857 and UBC 866 are capable to yield the polymorphic band which is linked with resistance to VSD. The ISSR analysis detected a more number

of bands per primer and a less number of monomorphic markers, indicating high intraspecific genetic variability of the accessions. For other crops, this analysis also showed high polymorphism levels, ranging from 70.5 to 98 per cent (Li and Ge, 2001, Wu *et al.*, 2009, Santos *et al.*, 2011). Although ISSR analysis is not recommended for taxonomic studies of phylogeny, the high number of polymorphic bands made grouping for geographical origin and parentage possible (Wu *et al.*, 2009).

The marker system called ISSR (Inter Simple Sequence Repeats) is a PCR based method that asses variation in the numerous microsatellite regions dispersed throughout the genome. In this technique reported by Zietkiewicz *et al.*, (1994), primers based on microsatellites are utilized to amplify inter simple sequence repeat sequences in the DNA. When the primer successfully locates two microsatellite regions within an amplifiable distance away on the two strands of the template DNA, the PCR reaction 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.

ISSR is highly sensitive, reproducible and dominant marker system and has been successfully applied in genetic and evolutionary studies of many species, including fingermillet (Salimath *et al.*, 1995), wheat (Nagaoka & Ogihara, 1997), rice (Joshi *et al.*, 2000), *Vigna* (Ajibade *et al.*, 2000). The ISSR marker requires small amounts of DNA and does not require information on DNA sequence. ISSR primers are designed from SSR motifs and can be undertaken for any plant species containing a sufficient number and distribution of SSR motifs in the genome (Gupta *et al.*, 1996; Buhulikar *et al.*, 2004). ISSR targets the highly variable microsatellite regions of the nuclear genome providing a large number of polymorphic fragments (Gupta *et al.*, 1994). Therefore this technique has been used extensively to evaluate genetic diversity both within and between plant populations in angiosperms and

gymnosperms (Osborn *et al.*, 2005). The high level reproducibility of ISSR markers is already well established in many crops and hence these markers offer the potential for direct usage in MAS (Bornet and Branchard, 2001; Reddy *et al.*, 2002).

The ISSR markers are found useful in the characterization of *Theobroma cacao* L. collection at Tingo Maria (Julio *et al.*, 2011). Costa *et al.* (2012) evaluated the genetic diversity in improved (IG) and unimproved germplasm (UIG) of yellow Passion fruit, based on ISSR (Inter Simple Sequence Repeat) markers in the study they demonstrated the potential of ISSR to determine molecular polymorphism in yellow passion fruit and that breeding has narrowed the genetic variability. ISSR markers can be valuable tools to study intraspecific genetic diversity in yellow passion fruit. Mora *et al.* (2007) reported AFLP and ISSR profiles for 94 isolates of *Moniliophthora roreri* from across its geographic range in Central/South America.

Rivas *et al.*, (2013) studied the ISSR markers for the population structure and genetic diversity in natural populations of *Theobroma subincanum* Mart. Giustina *et al.*, (2013) also used natural populations of *Theobroma speciosum.* ISSR molecular markers were able to detect polymorphism in the populations analyzed and proved to be reproducible for the species. This method is efficient to detect genetic variability within and between populations of *T. subincanum.* The usefulness of this type of marker has been previously described for both cultivated and wild species (Rossi *et al.*, 2009; Almeida *et al.*, 2009; Brandão *et al.*, 2011). This marker also presents high reproducibility and potential to be used in species without detailed DNA sequence information (Rossi *et al.*, 2009; Almeida *et al.*, 2009; Brandão *et al.*, 2011).

The inter-simple sequence repeat (ISSR) markers have been widely used in studies on genetic diversity and variability of wild populations. They are better than the others since they do not require prior information of the DNA sequence, have low development costs, and have high transferability to other plant species, and generate a greater number of polymorphic fragments (Barth *et.al.*, 2002; Brandão *et al.*, 2011)

UBC 811 has yielded distinct polymorphic band of size 950 bp. This band was present in all the resistant and three partially resistant clones, but were absent in the susceptible clones. This ISSR primer is tightly linked with the VSD disease resistant gene. Since it is missing in all the susceptible lines and present in few of the partially resistant lines, it is clear that many genes are contributing for resistance. Many diseases are reported to be polygenic in nature (Parlevliet *et al.*, 1985; Caranta *et al.*, 1997; Niks *et al.*, 2000; Lindhout, 2002). Identification of all the markers linked to all the genes will be a great challenge (Epaina, 2012). The gene for which the marker represented in this primer is having substantial contribution to resistance but contribution from other gene will also be mandatory to obtain the complete resistance. Another primer UBC 815 produces the distinct polymorphic amplicon of 750 bp size in resistant clones alone. Apart from the 750 bp all other amplicons are monomorphic. This marker appears to be a very important and since it is present in all the resistant accessions, it could be observed that this marker is associated with a resistance. More over it could be observed that this gene has some kind of segregating action. This kind of segregating gene action is previously reported by Tan and Tan (1988). This gene could be targeted for developing resistant line in marker assisted selection.

UBC 826 also given the polymorphism at 600 bp in all resistant line but in one susceptible accession and partially resistant line also had the specific marker. This banding pattern generated by the primer UBC 826 shows that this marker is associated with a gene which had low level of contribution to the resistance. This may be the reason why the marker has appeared in the susceptible line also. Obviously the presence of this marker in all the resistant line shows that this gene is an indispensible part of the gene groups which are contributing towards the resistance to VSD in cocoa. It should be concluded that mere presence of this gene alone will not offer any resistance or the level of resistance offered by the gene for which this

marker is linked will be minimal. The polygenic nature of gene action in cocoa is already reported by Niks *et al.* (2000), Lindhout, (2002).

VSD resistance is polygenic with additive gene action and high heritability (Tan and Tan, 1988; Van der Vossen, 1997). Since the heritability is high, hybrid progenies of resistant parents are most likely to be resistant (Keane and Prior, 1992) and we had the similar observations during the hybridization programme to develop VSD resistant cocoa hybrids (Minimol and Amma, 2013). The involvement of more than one gene for resistance may the reason for the failure of the markers suggested by Epaina (2012) in the population used in this study.

Another primer UBC 857 is showing the clear amplicon at 450 bp in all resistant lines which is absent in all susceptible and in one partially resistant line. UBC857 has produced the marker linked with the most important gene that contributes to the resistance to VSD. Since the marker is present in all the resistant line and absent in all the susceptible lines and in partially resistant lines, it is clear that this is the major contributing gene for the resistance. Its presence itself leads to high level of resistance and others will be contributing towards its expression. Through the statistical analysis it is well proven that in a polygenic gene action, the level of contribution given by each gene differs a lot (Tan and Tan, 1988). UBC 866 primer produces the two extra amplicons larger than one kb in two susceptible and in one partially resistant line.

5.2.2 SSR analysis

Microsatellites, or simple sequence repeats (SSRs), are becoming increasingly attractive ma rkers in molecular breeding, diversity assessment and fingerprinting (Morgante and Olivieri, 1993; Powell *et al.*, 1996). SSRs are short tandemly repeated sequence motifs of approximately 1-8 bp in length, which are scattered throughout the genome and can vary between individuals in repeat length. 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). A number of SSR markers have been developed for cacao (www.cacaogenomedb.org). SSRs show polymorphism higher than other markers and coupled with other desirable features, they have been used widely in various genetic studies of cacao, including construction of various genetic and QTL maps (Clement *et al.*, 2003a, 2003b; Brown *et al.*, 2005, 2007; Faleiro *et al.*, 2006).

In the present study SSR assay was done with 46 primer sets which are reported to yield good polymorphism in cocoa (Obando, 2009; Epaina, 2012; Thondaiman *et al.*, 2013). Though the various primer sets screened were found to yield polymorphism among the clones at various levels, none of them was successful to give a clear distinction among the resistant and susceptible. The failure may have been due to both experimental and genetic factors. Similar observations have been made in genetic studies using SSR markers in many crop species (Gupta and Varshney, 2000).

Even though the primers for SSR assay were selected based on their high PIC values as reported previously, none was successful to generate a marker linked with the resistance. A total of 46 SSRs were pre-screened using the bulked DNA of resistant and susceptible progenies. Three primers produced unambiguous amplification profiles and polymorphism was selected. Others produced ambiguous amplification profiles or failed to amplify the expected fragments. This demonstrated that not all SSRs work equally well for all cacao populations.

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

SSR markers for cacao were first described by Lanaud *et al.*, (1999). Since then, over 300 SSR markers covering the 10 linkage groups have been developed from various genomic DNA and expressed sequence tag (EST) libraries mainly by CIRAD, France, and USDA-ARS, SHRS, USA (Risterucci *et al.*, 2000; Kuhn *et al.*, 2003; Lanaud *et al.*, 2004; Pugh *et al.*, 2004; Borrone *et al.*, 2007). Details of the SSR markers can be viewed at the Cacao Genome Database at www.cacaogenomedb.org (Schnell *et al.*, 2007a). These SSR markers have been used in various genetic studies of cacao, including genetic mapping and identification of QTLs (Crouzillat *et al.*, 2000a, 2000b; Flament *et al.*, 2001; Clement *et al.*, 2003a, 2003b; Queiroz *et al.*, 2003; Pugh *et al.*, 2004; Brown *et al.*, 2005, 2007, 2008; Faleiro *et al.*, 2006;), genetic diversity and population structure (Marita *et al.* 2001; Sereno *et al.*, 2006; Motamayor *et al.*, 2008; Zhang *et al.*, 2008), parental analysis and identification of off-types (Motamayor *et al.* 2002, 2003; Motilal and Butler, 2003; Schnell *et al.*, 2004, 2005; Turnbull *et al.*, 2004; Takrama *et al.*, 2005; Efombagn *et al.*, 2006) and marker assisted selection (Schnell *et al.*, 2007a, 2007b).

5.3 Sequence analysis of polymorphic ISSR marker

The distinct marker generated using the ISSR primer UBC 857 of size 450 bp that was linked with the VSD resistance. Flis *et al.*, (2005) reported that the UBC 857 ISSR primer is linked to the *Ry-f*$_{sto}$ gene in *Solanum stoloniferum* for extreme resistant to *Potato virus Y.* The other ISSR markers were found to be linked with loci against Potato virus S (Marczewski, 2001; Marczewski *et al.*, 2002b) and Potato leaf roll virus (Marczewski *et al.*, 2002a), mapped to potato chromosome-VIII and XI, respectively. Paplauskienė and Dabkevičienė (2012) reported that clover seed yield is one of the most variable parameters, which were linked to two markers, generated with the AC motif primers UBC 827 (850 bp) and UBC 857 (500 bp). The highest number of fragments (10) generated with the UBC 857 primer was found for the individuals of var. 'Liepsna' (Paplauskienė and Dabkevičienė, 2008). The primers UBC 825, UBC 827 and UBC 857 were also successfully used in the genetic

diversity tests of *Arabidopsis thaliana* L. (Barth *et al.*, 2002). The UBC 857 produces the highest level of polymorphism (100 per cent) and the highest Rp (10.22) in the Genetic Characterization of Hazelnut (*Corylus avellana* L.) (Kafkas and Dogan, 2009). Choudhary *et al.*, (2014) checked the molecular variability of plantain ecotypes from the genus *Musa* (Musaceae) using the UBC 857 and other ISSR primers they got highest polymorphism using UBC 857.

The polymorphic amplicon from UBC 857 was eluted and subjected to reamplification. The product was electrophoresed to confirm that it is yielding a single band. The product from the nested PCR was directly used for sequencing. The sequence have yielded 246 nucleotides, which on standard nucleotide BLAST had shown 94 per cent identity with the *Theobroma cacao* microsatellite DNA clone of mTcCIR 42 SSR (NCBI accession number AJ271944).

Subsequently, the clones were subjected to SSR assay using the primer set for mTcCIR 42 at an optimized annealing temperature of 55°C. The assay has generated distinctly polymorphic banding pattern to differentiate the resistant cocoa lines from the susceptible and partially resistant lines. In all the resistant clones and in the third partially resistant clone, the SSR had yielded a distinct band at 650 bp. In all the susceptible and other partially resistant lines, except in the third susceptible clone, there were no bands above 500 bp sizes. The heavy band (1.7 kb) seen in the first resistant and third susceptible plants had no association with resistance or any resistance related parameters. The partially resistant clones were characterized with the presence of a unique marker at 400 bp. In the susceptible accessions the marker was slightly heavier at 450 bp or absent as in the third susceptible clone. With these clear marker patterns, this SSR was found to be highly successful to differentiate the VSD resistant, susceptible and partially resistant cocoa clones. The assay was repeated several times using the primer set mTcCIR 42 and the banding pattern was found to be highly reproducible.

Future line of work includes the identification of markers linked with the other genes contributing towards the VSD resistance, since the resistance is polygenic. The validated SSR and ISSR markers may be used in marker assisted breeding for VSD resistance. Since the gene action is additive, QTL mapping for VSD resistance has done. Further, these markers have to be used in the nursery screening for early detection of VSD resistant seedlings.

Summary

6. SUMMARY

The study entitled "Identification of molecular markers linked to the resistance for vascular streak dieback disease in cocoa (*Theobroma cacao*)" was carried out at the Center for Plant Biotechnology and Molecular Biology, College of Horticulture Vellanikkara during the period 2012-2014. The objective of the study was to identify SSR and ISSR markers linked to the gene offering resistance to the vascular streak dieback disease in cocoa. Genotypes of cocoa *viz.*, VSD I- 4.6, VSD I- 4.11, VSD I- 5.8, VSD I- 6.9, G VI- 50, G VI- 52, G VI- 82, G VI- 100, G VI- 25, G VI- 53, G VI- 67, G VI- 144 with varying response to the VSD disease and maintained at the Cocoa Research Centre, College of Horticulture, Kerala Agricultural University, were employed in this study. Morphological characters and response to disease as scored at Cocoa Research Centre from 1988-1999 onwards.

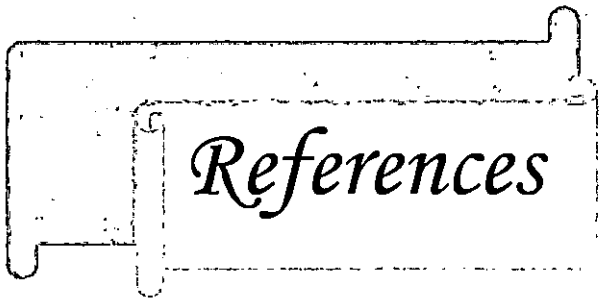
The salient findings of the study are as follows:

1. The protocol suggested by Doyle and Doyle (1987) with slight modification (Chloroform Isoamyl alcohol treatment was given three times to remove the protein contamination and washing with 76 per cent ethanol plus 10mM ammonium acetate) was found ideal for isolation of genomic DNA from the cocoa genotypes. The RNA contamination was completely removed through RNase treatment.

2. The quality and quantity of DNA was analysed using NanoDrop^{R} ND-1000 spectrophotometer. The absorbance ratio ranged from 1.80-1.89, which indicated good quality DNA and the recovery was high with 540 ng/µl of DNA.

3. Protocol for ISSR and SSR assay in coca were standardised with the various quantities of DNA, PCR mixtures and conditions for DNA amplification.

4. Two molecular marker systems namely, ISSR and SSR were employed for characterisation of the selected genotypes for VSD resistance. A total of 71 ISSR primers and 46 SSR primer pairs were screened for their ability to amplify the DNA fragments. Out of these, 11 ISSR primers and 6 SSR primer pairs were selected based on the number of bands and nature of amplicons.
5. Out of thirteen, five primers UBC 811, UBC 815, UBC 826, UBC 857 and UBC 866 have produced distinct polymorphism in relation to VSD resistance.
6. SSR assay was done with 6 primer sets which are reported to yield good polymorphism in cocoa. Though the primer sets screened were found to yield polymorphism among the clones at various levels, no polymorphism in relation to VSD disease response was obtained.
7. The distinct marker generated using the ISSR primer UBC 857 that was linked with the VSD resistance was eluted and reamplified. The 246 nucleotide sequences obtained after sequencing were analyzed using NCBI BLASTn.
8. Sequence had shown 94 per cent similarity with cocoa clone generated using the primer mTcCIR42 (GenBank accession number AJ271944.1) identified that this SSR is associated with the resistance gene for VSD disease.
9. Twelve genotypes were further screened with mTcCIR 42 and this screening has yielded distinctly polymorphic banding pattern to differentiate the resistant cocoa lines from the susceptible and partially resistant lines.
10. The SSR and ISSR markers identified through this study are linked with various genes involved in resistance to VSD in cocoa and they could be employed in breeding programmes aimed to develop VSD resistance and high yielding cocoa clones.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Annexure

ANNEXURE I

List of laboratory equipments used for the study

Refrigerated centrifuge	:	Kubota 6500, Japan
Horizontal electrophoresis system	:	BioRad, USA
Thermal cycler	:	Veriti Thermal Cycler (Applied Biosystem, USA)
Gel documentation system	:	BioRad, USA
Nanodrop® ND-1000 spectrophotometer	:	Nanodrop®Technologies Inc. USA

ANNEXURE II

Reagents required for DNA isolation

Reagents:

1. 2x CTAB extraction buffer (100 ml)

CTAB	:	2g
(Cetyl trimethyl ammonium bromide)		
Tris base	:	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. Chloroform- Isoamyl alcohol (24:1 v/v)

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

3. Chilled isopropanol

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

4. Wash buffer

Ethyl alcohol 76 ml and distilled water 24 ml.

Ammonium acetate 0.077 g

Mix the 0.077 gm ammonium acetate in 100 ml 76% ethyl alcohol and mix well.

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.

IDENTIFICATION OF MOLECULAR MARKER LINKED TO THE RESISTANCE FOR VASCULAR STREAK DIEBACK DISEASE IN COCOA (*Theobroma cacao* L.)

By

EKATPURE SACHIN CHANDRAKANT
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ABSTRACT OF THE THESIS

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Kerala Agricultural University, Thrissur

CENTRE FOR PLANT BIOTECHNOLOGY AND MOLECULAR BIOLOGY
COLLEGE OF HORTICULTURE
VELLANIKKARA, THRISSUR – 680 656
KERALA, INDIA

2014

ABSTRACT

Cocoa is the third important beverage crop next to coffee and tea and is the third highest traded commodity in the world after coffee and sugar. Current world production of cocoa is about 4 million tonnes but India contributes only 0.02 per cent. In Kerala, area under the cocoa is 12,480 ha. with the production of 6.14 metric tonnes and productivity 490 kg/ ha (Directorate of Cashewnut and Cocoa Development, 2013).

Vascular streak dieback (VSD), caused by the fungus *Oncobasidium theobromae* and first reported in India in 1981, is a very serious disease of cocoa that has already spread to all the cocoa growing tracts of India. The characteristic symptoms include a green-spotted chlorosis and fall of leaves beginning on the second or third flush behind the stem apex, raised lenticels, and darkening of vascular traces at the leaf scars and infected xylem. Eventually complete defoliation occurs and, if the fungus spreads to the trunk, the tree will die.

The confirmation of transfer of gene is at most important in crop breeding and availability of a tool to quickly detect the gene will reduce the breeding cycle length. Molecular markers are already proven good to detect the genes of interest. The present study aims at developing a reliable molecular marker linked with the gene offering resistance to VSD in cocoa, using SSR and ISSR marker systems.

Extensive germplasm collection and evaluation have been done at Kerala Agricultural University, India and tolerant cocoa clones were identified. Using these clones, 45000 hybrids have been bred and were screened for resistance using natural inoculum under high humidity and 1177 field established resistant hybrids are being evaluated for the past 15 years. Already, KAU has released three hybrids and 7 selections of cocoa with field tolerance to VSD.

In the present investigation, twelve genotypes of cocoa, having different responses to VSD namely, VSD I-4.6, VSD I-4.11, VSD I-5.8, VSD I-6.9 (resistant), G VI-50, G VI-52, G VI-82, G VI-100 (Susceptible), G VI-25, G VI-53, G VI-67 and G VI-144 (partially resistant) were employed. The genotypes were selected on the basis of field screening experiments started in 1998-1999 at Cocoa Research Station, Kerala Agricultural University.

Genomic DNA isolated using modified CTAB method suggested by Doyle and Doyle (1987). ISSR assay on the total genomic DNA of twelve cocoa clones using 71 primers had shown that the primers UBC811, UBC815, UBC826, UBC857 and UBC866 are capable to yield the polymorphic bands, in relation to VSD resistance, whereas, SSR assay with 46 primer sets has failed to generate any marker for the same.

The most distinct polymorphic marker generated in the resistant lines by the ISSR primer UBC857 was eluted. This DNA was further subjected to PCR analysis using the same primer, to verify its suitability for direct sequencing. The direct sequencing has yielded 246 nucleotides, which on BLASTn had shown 94 per cent identity with the *Theobroma cacao* microsatellite DNA clone of mTcCIR42 SSR (NCBI accession number AJ271944).

Subsequently, the clones were subjected to SSR assay using the primer mTcCIR42. The assay has generated distinctly polymorphic banding pattern that differentiated the resistant lines from the susceptible and partially resistant ones. Since highly successful in differentiating the resistant, susceptible and partially resistant cocoa clones, these markers are recommended for use in marker assisted breeding for VSD resistance.