DNA FINGERPRINTING OF SELECTED COCOA (*Theobroma cacao* L.) VARIETIES OF KERALA AGRICULTURAL UNIVERSITY

By

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(2017-11- 007)

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VELLANIKKARA, THRISSUR – 680656

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THESIS

Submitted in partial fulfilment of the requirement for the degree of

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CENTRE FOR PLANT BIOTECHNOLOGY AND MOLECULAR BIOLOGY

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VELLANIKKARA, THRISSUR – 680656

KERALA, INDIA

2020

DECLARATION

I, hereby declare that this thesis entitled "**DNA fingerprinting of selected cocoa (*Theobroma cacao* L.) varieties of Kerala Agricultural University"** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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CERTIFICATE

Certified that the thesis entitled **"DNA fingerprinting of selected cocoa (*Theobroma cacao* L.) varieties of Kerala Agricultural University"** is a record of research work done independently by **Megha Totaganti (2017-11-007)** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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Errors and omissions are entirely unintentional

Megha Totaganti

Dedicated to my beloved

Parents and Teachers

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ABBREVIATIONS

%	Percentage
>	Greater than
B	Beta
µg	Microgram
µl	Microliter
AFLP	Amplified Fragment Length Polymorphism
bp	Base pair
CCRP	KAU- Cadbury Co-operative Cocoa Research Project
CRC	Cocoa Research Centre
cm	Centimeter
CPBMB	Centre for Plant Biotechnology and Molecular Biology
CTAB	Cetyl Trimethyl Ammonium Bromide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTPs	Deoxyribo Nucleoside Triphosphate
EDTA	Ethylene Diamine Tetra Acetic acid
EST	Expressed Sequence Tags
gm	Gram
IBPGR	International Board of Plant Genetic Resources
ICTG	International Cocoa Gene bank
IPR	Intellectual Property Rights
ISSR	Inter Simple Sequence Repeat
KAU	Kerala Agricultural University
kb	Kilo base pairs

KOAC	Potassium acetate
L	Litre
M	Molar
MAS	Marker
mg	Milligram
ml	Millilitre
NaOAC	Sodium acetate
mM	Milli Molar
ng	Nano gram
^{o}C	Degree Celsius
OD	Optical Density
PBR	Plant Breeders Right
PCR	Polymerase Chain Reaction
pH	Hydrogen ion concentration
PIC	Polymorphic Information Content
PVP	Poly Vinyl Pyrrolidone
QTL	Quantitative Trait Loci
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
SDS	Sodium dodecyl sulfate
SSR	Simple Sequence Repeat
TAE	Tris Acetate EDTA
TE	Tris EDTA
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
UV	Ultra Violet
V	Volts

1. INTRODUCTION

Cocoa (*Theobroma cacao* L.) is an important tropical tree-crop belonging to the family Malvaceae. South America is considered as the place of origin of cocoa (Wood and Lass, 1985). The crop was introduced into India way back in 1798, at Courtallam in Tirunelveli district of the old Madras state (Ratnam, 1961). Cocoa is extensively cultivated in South India and its cultivation is now slowly extending to North Eastern states. The cultivated cocoa is traditionally sub divided into three main groups Criollo, Forastero and Trinitario (Ji *et al*., 2012). *Theobroma cacao* produces the fruit which is used for chocolate making.

Cocoa is a shade-loving perennial tree crop. The cocoa growing regions are largely centered in important biodiversity hotspots (Guiltinan, 2007). Africa is the major cocoa producing continent. It nearly contributes to 70 percent of world's production (Bruno *et al*., 2008). The International Cocoa Organization (ICCO) estimated that the global production of cocoa crossed the four million metric tons in 2015/16. Worldwide, it is reported that, approximately 5 to 6 million small holder farmers grow 90 percent of the world's production. Switzerland is the highest consumer of cocoa products. The Americas ranked second in production (0.64 million tonnes) while Asia and Oceania (0.4 million tons) ranked third among the producer countries of cocoa beans (Wickramasuriya and Dunwell, 2018).

Cocoa plant is cross pollinated and also self-incompatible. It is a diploid tree species (2n = 20), (Aikpokpodion, 2012) with a small genome. Genome size is 0.43 pico gram (equivalent to 415000kb), (Figueira *et al.,* 1992).

In India cocoa is grown as mixed crop with coconut and arecanut mainly in the states of Kerala and Karnataka. It is inter-cropped under coconut and to some extend in oil palm gardens in Tamil Nadu and Andhra Pradesh (Alban *et al*.,2016).

In breeding programmes, characterization of available genetic resources is essential (Spooner *et al.,* 2005). In the earlier record of crop breeding, the characterization of genotypes depended mainly on morphological features. On the grounds of morphological markers, genetic diversity was evaluated. However, it is not

easy to classify accessions based solely on their morphological characteristics as they are highly influenced by environmental factors (Ganesan *et al*., 2014).

DNA markers, act as an efficient tool to categorize and distinguish accessions and cultivars which are related in morphological characteristics and with indefinite character. DNA finger printing has been successfully applied for cultivar identification, calculating seed purity of hybrids and checking the genetic similarity between varieties (Aikpokpodion *et al*., 2009) and thereby, protects plant varieties and to resolve the Intellectual Property Right issues.

In consideration of the above, the present study was envisaged with the following defined objective:

1. Development of DNA fingerprints profile of cocoa varieties using SSR and ISSR markers.

2. REVIEW OFLITERATURE

2.1 General background

Cocoa is one of the tropical crops belonging to the class *Theobroma* and family Malvaceae (Wickramasuriya and Dunwell, 2018). It originated from Andes in South America (Ratnam, 1961; Diczbalis, 2013). Africa is the major continent to produce cocoa, however it is also distributed in Central America, southern Mexico and the Caribbean region. Cocoa beans are the primary source of raw material for the chocolate preparation and associated confectioneries which run into a multibillion-dollar industry. The International Cocoa Organization (ICCO) estimated that annual world production of cocoa is more than 4.0 million metric tons. Worldwide it is reported that approximately 5 to 6 million smallholder farmers grow 90 per cent of the world's production (Wickramasuriya and Dunwell, 2018).

Till the 20th century cocoa production was not commercialized. However, for manufacturing milk chocolate cocoa cultivation was started by Swiss in West Africa and now cocoa occupies the seventh place among the traded food commodity in the world (Diczbalis, 2013). Cocoa is cross pollinated and self-incompatible in nature. It is a diploid tree species (2n = 20) (Aikpokpodion, 2012; Wickramasuriya and Dunwell, 2018) with a very small genome.

Gene bank forms the starting point for any breeding programme. The existing genetic material are being effectively used based on its characterization (Spooner *et al*., 2005). Genotyping, genome mapping and phylogenetic studies were done in cocoa by utilizing Polymerase Chain Reaction (PCR) based DNA markers system (Guiltinan, 2007). Molecular characterization was also conducted by using microsatellite marker (Zhang *et al*., 2009).

In India, the systematic introduction of germplasms from University of Reading, England, through Cocoa Research Centre (CRC), Kerala Agricultural University (KAU) has increased the genetic base of cocoa (Minimol and Prasannakumari, 2013). The Central Sub-Committee on Crop Standards, Notification and Release of Varieties for Agricultural Crops has made it mandatory to submit DNA fingerprinting

information of the varieties proposed for national release/notification. Hence, the present study was under taken with the objective of molecular characterization of the seven cocoa varieties released from KAU. This is highly essential to preserve their distinctiveness, protect Plant Breeder's Right and to resolve issues related to Intellectual Property Right.

DNA markers act as an efficient tool to identify and differentiate accessions and cultivars which are similar in morphological trait with identical characteristics. The relevant literatures are reviewed under this chapter.

2.2 Usefulness of genetic markers in plants

Genetic markers are the biological features that are transmitted from one generation to another and are determined by allelic forms of genetic loci. They can be used to study the relationship between inherited disease and its genetic cause. Hence, they are used as experimental probes or tags to keep track of an individual, a tissue, a cell, a nucleus, a chromosome or a gene. Hence genetic markers act as a powerful tool for plant breeding.

The genetic markers can be largely classified into three categories: morphological markers, cytological markers and biochemical markers. Different kinds of genetic markers are used for different purpose and with different characters, each having its own benefit and drawback.

2.3 Polymerase chain reaction (PCR)

The PCR was a technological advancement in genome analysis as it results in the amplification of specific sequence of DNA from the total genomic sequence. It consists of basic principle of a copying enzyme, called DNA polymerase. Mullis and Faloona, (1987) showed that the enzyme is able to synthesize copy of DNA molecule from a DNA template *invitro*. The extension product of the template DNA, when it is denatured, results in single strand of DNA where each strand can serve as a template for the other reaction and vice versa. The continuous duplications due to repeated chain reaction, gives an exponential increase in DNA product synthesis and its accumulation. The oligonucleotide primers with short stretches of DNA sequence of 18-25 bp which are complementary to the genome are used for amplification. The target DNA to be

amplified consists of primer annealing sites in which primer binds. By selecting specific sequences as primers, amplification can be done from any region of target DNA. However, for amplification of highly specific regions, sequence of flanking regions is required (Karp *et al.,* 1997).

Large protein fragment (Klenow fragment) of *Escherichia coli* which was initially used as DNA polymerase I to catalyze the extension of the annealed primers was found to be thermally instable. It was one of the drawbacks of PCR based method. Heating step (above 92^{o}C) was required for denaturation of template as well as newly synthesized strands. This issue has been fixed efficiently by replacing the *E. coli*. DNA polymerase I with thermo-stable DNA polymerase (Taq polymerase) purified from thermophilic bacteria, *Thermus aquaticus* that will survive prolonged incubation at 95 ^{o}C (Saiki *et al.,* 1988).

2.4 PCR based molecular marker technique

The DNA marker systems are of PCR based markers type and hybridization based (non PCR) markers type (Joshi *et al*., 1999). The PCR based DNA marker system and its efficiency is determined based on assessment speed of thermal cyclers, cost of processing and the simplicity of techniques etc. The marker developed should be adequately informative and must able to differentiate between the individuals at allelic level (Charters and Wilkinson, 2000).

Agarwal *et al*. (2008) subdivided PCR-based techniques into arbitrarily primed PCR-based techniques and sequence targeted PCR-based techniques. Prior sequence knowledge is not needed for arbitrarily primed PCR technique or sequence non-specific techniques to perform. RFLP and RAPD are extensively used sequence non-specific PCR techniques, however microsatellite-based marker technique and single nucleotide polymorphism (SNPs) markers are sequence specific and much informative. The characteristics of good molecular markers in clued its polymorphism and uniform distribution throughout the genome length. They should be able to differentiate minute changes in gene sequence with adequate resolution and primers should be independent and reliable, quick, simple, cost effective and able to detect in small quantity of DNA samples**.**

DNA fingerprinting is a PCR based molecular marker technique which is widely adopted for characterizing individual plants and to study genetic diversity within basal population of a crop (Saunders *et al*., 2004).

2.5 Molecular markers in plant characterization

Molecular markers are small regions of DNA sequence showing polymorphism (base deletion, insertion and substitution) between different species. Molecular markers are used to identify a particular sequence of DNA from a pool of unknown DNA. These markers correlate with phenotypic expression of a trait. DNA based marker techniques such as RFLP (Restriction Fragment Length Polymorphism), RAPD (Random Amplified Polymorphic DNA), AFLP (Amplified Fragment Length Polymorphism) and SSR (Simple Sequence Repeats) are used in analysis of evolutionary relationship among different individuals, taxonomical classification, phylogenetic analysis and molecular genetic analysis (Agarwal *et al.*, 2008).

Ganesan *et al*. (2014) noted that it is essential to study genetic diversity patterns, genetic makeup and agronomic parameters of a plant for effective breeding, crop improvement, domestication and large-scale cultivation. DNA marker technology, derived from molecular genetics and genomics studies, provides excellent promises to crop breeding. Due to genetic linkage, markers of DNA are able to recognize the existence of allelic variation in the genes that underlie these characteristics. Efficiency and accuracy of DNA markers play major role in plant breeding and used as an advanced tool in marker-assisted selection (Collard and Mackill, 2008).

Molecular markers were established in 90’s and they are more informative and stable than morphological and agronomic traits when used in evaluation of genetic diversity (Cohen *et al*., 1991). According to Smith and Smith (1989) the morphological analysis along with molecular marker analysis can be the best method to evaluate the varieties, rather than the use of morphological characters alone for analysis. Molecular markers provide better coverage of the genome, leading to better estimation of relationships or molecular level divergence.

Molecular markers were used for quantitative trait locus (QTL) analysis to observe complex traits in cereals (Hodges, 1991). Yoshima *et al*, (1995) identified tightly linked markers to resistant genes which effectively helped for marker assisted gene pyramiding in rice. Molecular markers are helpful in the study of cocoa genetic groups as these genomics approaches are efficient for fingerprinting, gene tagging, gene mapping and determining the genetic structure and relationships among cocoa genetic groups (Laurent *et al*., 1993; Motamayor *et al*., 2003). Molecular markers will be efficient flags for breeders as these are situated near to the target gene in the particular area of a chromosome. The markers are helpful in early identification of the most desirable genotypes from a diverse population (Figueira, 2004). Marker-assisted selection improves the efficiency of plant breeding (Janila and Sharma, 2004).Collard and Mackill (2008) stated that, DNA markers can be incorporated in place of conventional methods, and they will serve as an efficient tool to replace conventional phenotypic selection. Li *et al.*, (2010) has done the marker-assisted selection for tillering in wheat.

Several molecular tools and procedures help to establish DNA fingerprint profiles and each of these procedures have its strength and weakness. Hence, a combination of marker system is recommended for confirmatory results (Karp *et al.*, 1996). Saunders *et al.* (2001) reported that, in plants, to identify cultivars and accessions in a pool, DNA fingerprinting can be applied. According to Degani *et al*. (2001), DNA fingerprinting can be helpful in establishing the identity of individuals and make easy to study breeding populations based on its genetic diversity analysis. In addition, it is reported to be successful in testing of seed purity of hybrid and also to analyze the cultivars and its genetic similarity (Aikpokpodion *et al*., 2010).

2.5.1 Inter simple sequence repeats (ISSR) markers for genetic diversity analysis

Inter Simple Sequence Repeats (ISSRs) are defined as fragments of DNA that range in the size of about 100-3000 bp particularly distributed in between adjacent, oppositely oriented microsatellite regions. ISSR marker technique requires only low quantity of template DNA (5-50 ng per reaction) and this is the major advantage of this marker technique (Spooner *et al*., 2005).

On the basis of several important unique features of ISSRs, the marker system emerged as an alternative system with the consistency and advantages of microsatellites (SSR) along with the broad taxonomic applicability of RAPDs. Studies conducted with ISSR markers accordingly indicated that**,** they are potentially useful for analysis of genetic diversity (Dongre and Parkhi, 2005; Behera *et al*., 2008; Luo *et al*., 2011; Singh *et al.,* 2012; Thul *et al*.,2012).

To find an efficient molecular marker system for genetic analysis of traditional and evolved basmati and non-basmati rice varieties, Nagaraju *et al*. (2001) used fluorescence based ISSR-PCR markers. Traditional basmati, evolved basmati and semi dwarf non-basmati rice groups were analyzed using 12 ISSR-PCR primers and total of 481 ISSR markers were developed in 24 varieties from the three groups. The lowest genetic diversity was noticed in traditional basmati varieties, were as highest genetic diversity was noticed in evolved basmati varieties.

A study was conducted by Dongre and Parkhi (2005) through the combination of PCR based RAPD, ISSR and microsatellite marker systems in order to identify cotton hybrids**.** Hybrid cotton H6 and its parents G.Cot.10 (male) and G.Cot.100 (female) were selected for the study. In the experiment twenty RAPD primers, nineteen ISSR primers and twenty-five reported polymorphic microsatellites were used**.** In the hybrid H6, ISSR primers IS4 and IS7 developed two female specific amplicons of size 500 bp and 1200 bp**.** The study concluded that for identification of cotton hybrids, utilization of all the three markers are reliable.

Luo *et al*., (2011) conducted genetic diversity analysis on 23 mango germplasm accessions from the Guangxi region of China by using 18 ISSR markers. The amplification resulted in 15 clear bands showing 55 per cent polymorphism with ISSR. Then based on this result the 23 cultivars were clustered in to three major groups.

According to the study conducted by Singh *et al.* (2012), RAPD and ISSR marker systems were useful in evaluating the genetic diversity in turmeric (*Curcuma longa L.*) A total number of 17 polymorphic primers which included 11 RAPDs and 6 ISSRs were used to analyze 60 genotypes. ISSR primers produced 66 bands and 52 of them were polymorphic with an average of 8.6 polymorphic fragments per primer. Number of bands per primer differed from one to 14 and amplicon size ranging from

200 to 2000 bp and normal percent of polymorphism was 95.40 per cent**.** By using these data, they developed dendrogram and analysis of dendrogram had shown low genetic diversity in between the accessions of turmeric.

A study was conducted by Thul *et al*., (2012) using 8 ISSR and 27 RAPD primers to check the genetic variability of 22 Capsicum accessions from *C. annuum, C. baccatum, C. chinense, C. eximium, C. frutescens*, and *C. luteum*. The results of genetic diversity analysis in comparison with morphological data indicated that accessions of *C. chinense* shared maximum similarity with *C. frutescens* for flower characters and found to be genotypically much closer. Similarly, the accessions of *C. luteum* shared maximum similarity with *C. baccatum* both morphologically and genotypically. Thus, the result indicated that ISSR and RAPD marker systems are helpful in identification of genetic stock of *Capsicum* species along with species specificity.

Baliyan *et al*. (2014) reported that ISSR markers along with morpho-agronomic characters are useful in the analysis of genetic diversity in *Chrysanthemum* species. Twenty-four chrysanthemum genotypes were subjected to genetic diversity analysis considering nine quantitative and five qualitative characters (morpho-agronomic) using 10 ISSR markers. Data pertaining ISSR after UPGMA analysis have shown that genotypes were markedly differentiated into several distinct groups.

Khalik and Osman (2017) conducted the genetic diversity analysis on *Plectranthus* spp. by using five ISSR and ten RAPD primers. It differentiated the characteristic of the closely related species after combined UPGMA cluster analysis.

2.5.1.1 ISSR markers for DNA fingerprinting in plants

According to a study conducted by Blair *et al*. (1999), ISSR was found to be useful in amplification of microsatellite motif and their frequency analysis and fingerprinting in rice**.** The study showed presence of different SSR motifs in 32 ISSR primers. These were subsequently utilized for amplification of 59 rice varieties**,** which represented the diversity of cultivated rice. The results exposed that, ISSR fingerprint was effectively applied to differentiate genotypes belonging to *Japonica* and *Indica*.

RAPD, ISSR, AFLP and SSR marker system was used by McGregor *et al.* (2000) and he conducted a comparative assessment of DNA fingerprinting using 39

tetraploid potato (*Solanum tuberosum* L.) germplasm. PCR based fingerprinting of germplasm was done by using 6 ISSRs primers, 20 RAPD, and 5 SSRs. These markers successfully differentiated 39 potato cultivars. The reproducibility of SSR and AFLP markers was found to be maximum *i.e.,* 100 and 99.6 per cent respectively, whereas reproducibility of ISSR was 87 percent.

Mogalayi (2011) undertook fingerprinting of black pepper varieties released by Kerala Agricultural University at Centre for Plant Biotechnology and Molecular Biology. Characterization of seven black pepper varieties was done by using 34 ISSR markers. Resolution capacity was evaluated and ranged from 5.42 to 12.28 and the value discovered for Polymorphism Information Content (PIC) ranged from 0.80 to 0.89 in ISSR. The high resolving power of the marker indicated its utility in identifying individual varieties and the observed PIC value indicated the variability among genotypes.

DNA fingerprinting of four KAU brinjal varieties along with three superior accessions and two wild relatives was conducted at CPBMB using 10 ISSR primers (Laxman, 2013). Resolving power (Rp) of marker system was calculated, according to the banding pattern on gel. Rp value of ISSR primers ranged from 9.90 to 28.44 and PIC value ranged from 0.83 to 0.96. Thus, the selected ISSR primers can differentiate the genotypes as well as can be used as the suitable primers for detecting polymorphism in brinjal.

Meena, (2014) conducted DNA fingerprinting of five cashew varieties released by Kerala Agricultural University by using eleven ISSR primers. She analyzed the resolving power of marker technique and for ISSRs, it ranged from 1.4 to 7.0 implied the potential of the selected markers to distinguish the varieties.

Ten ISSR primer sets were used for DNA fingerprinting of eight cocoa genotypes and 39 polymorphic amplicons were generated over eight genotypes. The bands were observed and consolidated into eight unique DNA fingerprint profiles. The result of ISSRs resolvingpowerwas14.75 indicated the efficient differentiating capacity of each marker system (Sujith, 2016).

2.5.2 Simple sequence repeats (SSR)

Microsatellite or simple sequence repeats (SSRs) are short (1-6 bp) tandemly repeated DNA motifs (mono to hexa-nucleotides) that generally occur abundantly in eukaryotic genomes (Beckman and Soller, 1986). They are viable to be used as different molecular markers by targeting the repeat region with unique flanking primer pairs (Asari *et al*., 2014). Improper binding of DNA polymerase or unequal crossover leads to tandem repeat insertion or deletion which creates variation in SSR length.

PCR can easily detect allelic differences in SSR loci using particular primers flanking the SSR motif. Among the various classes of molecular markers, SSRs have been widely used in many crop species for various plant genetic and breeding applications due to their simplicity, reproducibility, elevated polymorphism, codominant inheritance, relative abundance and excellent genome coverage (Powell *et al.*, 1996). In addition, because of the tiny quantity of DNA needed and their suitability for multiplexing on automated devices, SSRs are technically simple for analysis**.** SSR markers are commonly used in the fields of cultivar fingerprinting, genetic diversity evaluation, molecular mapping, QTL detection and marker choice. The facility to analyze genetic data and the capacity to detect heterozygotes using less complex scoring techniques rendered the SSR marker scheme more advantage over others.

2.5.2.1 SSR markers for genetic diversity analysis

Microsatellites (SSRs) are well-established marker system for assessing genetic diversity due to their high discriminatory power and polymorphic information content among individuals, which arises from their multi-allelic nature and co-dominant segregation of alleles (Powell *et al*. 1996). They have also been extensively used in many studies on genotype detection and genetic mapping (Ramsay *et al*., 2000) and linkage map construction (Pugh *et al*., 2004; Lu *et al*., 2005). Locus specificity is another important feature of genomic SSRs, which is an important advantage when working with species such as wheat (allopolyploid), in which only one of three homoeologous loci should ideally be tagged (Varshney *et al*., 2000). The assay conducted by Agarwal *et al*., (2008) showed wide-range of inter individual length polymorphisms during PCR analysis of unique loci using discriminatory primers sets. Lopez *et al*. (2004) reported 15 SSR markers were used in detection quantification of

in-vitro induced culturing chimerism of cocoa. To perform an assay, alternative SSR alleles which showed differential amplification at heterozygous loci was used. In addition, 233 somatic embryo regenerants of cocoa were subjected to capillary electrophoresis with calibrated reference data**.**

Zhang *et al*. (2006) suggested microsatellite markers for defining genetic uniqueness of varieties. Zhang *et al.* (2009) succeeded in characterization of international cocoa collection maintained in Costa Rica using several microsatellite markers**.** Twelve microsatellite markers, were used to analyze the genetic diversity between 574 cocoa accessions covering parental populations in West Africa gene bank and farmers' accessions from Nigeria (Aikpokpodion, 2012). The study showed that, significant genetic diversity was present in on-farm land gene bank collections of cocoa. SSR analysis have showed homozygous pattern, which revealed the impact of inbreeding in local parent population.

2.5.2.2 SSR markers for DNA fingerprinting in plants

Fifty-one SSRs were used in the DNA fingerprinting of 742 potato landraces. From this 24 most informative SSRs were identified. The microsatellite kit was extremely useful for discriminating 93.5 per cent of the 742 potato landraces (Ghislain *et al.*, 2009).

SSR-based fingerprinting was performed and pedigree analysis was carried out to determine relation between clonal cultivars of tea (Tan *et al*., 2015). The researchers evaluated 128 Chinese clonal cultivars using 30 SSR markers in order to identify the relationship between parents. Fingerprint data recognized 47 parent-related pairs out of which 33 clonal cultivars were comparable with known pedigree information and the remaining 14 were newly recognized.

2.5.2.3 SSR markers for DNA fingerprinting in cocoa

SSR marker-based DNA fingerprinting analysis was identified as the best molecular tool to detect the genetic diversity as it describes the international molecular norms for fingerprinting of cocoa DNA. Twenty-five SSRs were used for evaluation of around 690 cocoa accessions. Out of 15 SSRs were highly reproducible, reliable and able to differentiate divergent genotypes. Selected 15 SSR primers were used as

international molecular standards for DNA fingerprinting of cocoa (Saunders *et al*., 2004).

Fifteen microsatellite loci with high throughput genotyping system were used in microsatellite fingerprinting of 924 cocoa germplasm. In this study, fluorescent dye was used to label the forward primers and capillary electrophoresis was done on an ABI Prism 3730 genetic analyzer platform. In addition, software Gene Mapper 3.0 was used in DNA fingerprinting. The experimental bands were analyzed and condensed to 174 unique fingerprint profiles. Identification of mislabeled as well as duplicated accessions among the germplasm could be done by using thus, developed fingerprint (Irish *et al.*, 2010).

Eleven SSR primer sets were used in DNA fingerprint of eight genotypes and primers produced 34 polymorphic amplicons. The bands were observed and 24 unique DNA fingerprints profiles were established. The result of ISSRs resolving power is 2.50, indicating the effectiveness of selected primer for the development of DNA fingerprint (Sujith, 2016). Fingerprinting data was used for efficient exchange of quality breeding materials across the world from the international quarantine center.

3.MATERIALS AND METHODS

The study on DNA fingerprinting of seven selected cocoa (*Theobroma cacao* L.) varieties of KAU was conducted at the Center for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara, during 2017-2019. The materials used and methods followed during the study are given in this chapter.

3.1 Materials

3.1.1 Plant materials

The varieties selected for the study included one selection (CCRP 3) and six hybrids (CCRP 10, CCRP 11, CCRP 12, CCRP 13, CCRP 14, CCRP 15). Reddish coloured tender leaves from four month old grafted plants were used for DNA isolation and each variety was maintained under shad net at Cocoa Research Centre (CRC), Kerala Agricultural University (KAU) (Plate .1).

3.1.2 Laboratory equipments

Cocoa DNA fingerprinting was done at CPBMB, KAU by utilizing the available facilities and equipment. Eppendorf micropipettes were used for the study. KUBOTA 6500 was used for centrifugation of the samples. The estimation of quality and quantity of isolated DNA was done by using Spectrophotometer NanoDrop ® ND-1000. Polymerase chain reaction was conducted using thermal cycler Proflex, Life Technologies. Agarose gel electrophoresis was done using wide min-sub unit and power pack (BIO-RAD). Gel documentation was done using Gel Doc unit $^{TM}XR^{+}$ (BIO-RAD). Equipment used in this study is detailed in Annexure I.

3.2 Methods

3.2.1 Molecular analysis

Simple sequences repeats (SSR) and inter simple sequence repeats (ISSR) marker systems were used to achieve the molecular characterization and DNA fingerprinting of the seven cocoa varieties.

CCRP3

CCRP10

CCRP11

CCRP12

CCRP13

CCRP14

CCRP15

Plate 1. Cocoa varieties used in the study

3.2.1.1 Genomic DNA extraction

Reddish tender leaves were collected in the morning hours from the vigorously growing tips of the plant. Leaves collected were covered with aluminum foil and properly labeled. To avoid phenolic oxidation they were immediately stored in ice box containing ice. Samples were taken to the laboratory and the leaf surface was cleaned by washing with sterile distilled water followed by wiping with 70 per cent ethanol. The samples were weighed and stored at -20 ^{O}C until they were used for DNA extraction. Modified Delloporta method (Ileana, 2005) was used with slight modification for isolation of good quality DNA.

a. CTAB buffer:

- 5 per cent CTAB(W/V)
- 100 mM Tris base (pH8.0)
- 20 Mm EDTA(pH8.0)
- 1.4 M NaCl
- 1 per cent polyvinyl pyrrolidone (PVP)
- 0.2 per cent β- mercapto ethanol

b. 20 per cent SDS

c. 5M Potassium acetate (KOAC)

d. 3M Sodium acetate (NaOAC)

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

f. Chilled isopropanol

g. Ethanol 70 and 100 %

h. Sterile distilled water

i. RNase (1.0 %)

Leaf samples were washed with distilled water and dried using the sterile tissue paper. Preheated the freshly prepared CTAB (5 %) solution by keeping it in hot water bath at a temperature of 60 ^{o}C. About 0.08-0.10 g of tissue was weighed from the leaf sample after removing the midrib region. The weighed sample was kept in a mortar cleaned with ethanol. Then sample was powdered by adding liquid nitrogen to the mortar. A pinch of poly vinyl pyrrolidone (PVP) and 50 µl 2-β mercapto ethenol was added to the powdered sample to prevent poly phenol oxidation. Preheated CTAB (1 ml) was added to the powdered sample and immediately transferred to 2 ml Eppendorf tube containing 0.5 ml of CTAB and a pinch of PVP. 20 per cent sodium dodecyl sulfate (SDS) and one µl of RNase was added to the Eppendorf containing sample and the sample was incubated at 65 ^{o}C for 30 minutes with intermittent shaking after adding the RNase. To this 500 µl of 5M KOAC was added and mixed thoroughly by vigorous shaking and then incubated at 0 ^{o}C for 20 minutes. After that mixture was centrifuged (KUBATA 6500) at 13000 rpm for 15 minutes at 4 ^{o}C. The upper transparent solution was transferred to another 2 ml tube and equal volume of chloroform: isoamyl alcohol was added. The tube contents were properly mixed by moderate inversion and centrifuged at 13000 rpm for 15 minutes at 4^{o}C.

There were three distinct phases after centrifugation. Top aqueous phase contained DNA with small quantity of RNA and middle phase was formed by protein and other cell debris, bottom clear phase was formed by chloroform, isoamyl alcohol, some leaf pigments etc. The aqueous phase was carefully transferred to a new 1.5 ml Eppendorf tube and 500 µl of chilled isopropanol was added. This content inside the tubes were mixed by gentle inversion and incubated at -20 ^{o}C for 30 minutes. The tubes were centrifuged at 12000 rpm for 15 minutes at 4 ^{o}C for DNA pellet formation. Discarded the supernatant and carefully dried the pellet. Redissolved the dried DNA pellet in 700 µl extraction buffer and incubated at 4 ^{o}C for 20 minutes. Tubes were again centrifuged at 12000 rpm for 10 minutes. Transferred the supernatant into 1.5 ml tube and 75µl 3M NaOAC and 500µl isopropanol was added to the tube. The content was mixed properly by gentle inversion until DNA precipitated by forming thread like

structures. The tubes were kept at -20^{o}Cfor20minutesfor complete precipitation. Tubes were again centrifuged at 10000 rpm for 2 minutes for compression and pellet formation of DNA. The supernatant was carefully discarded without disturbing pellets in the tube. Washed the pellet by adding ethanol (70%) and centrifuged for 5 minutes at 10000 rpm. The ethanol was poured off and the pellets were air dried until ethanol was evaporated. Dissolved the air dried pellet in double distilled water and stored at -20 ^{o}C. Quality and quantity of isolated DNA was determined by using NanoDrop and by gel electrophoresis with using one per cent agarose gel.

3.2.1.2 DNA quantity assessment by gel electrophoresis

Isolated DNA samples were visualized and quantified under UV light by using agarose gel electrophoresis.

Reagent used

a. Agarose – 1.0 per cent (w/v)
b. 50X TAE buffer (pH8.0)
c. Running buffer (1XTAE)
d. 6X gel loading dye
e. Ethidium bromide (0.5µg/ml)

Procedure

Gel casting system was used for casting the gel. Ethanol (70%) was used for wiping gel casting tray and comb. Inside the gel casting system the tray was tightly set and the comb was properly placed. Agarose gel (1 %) was prepared by dissolving 1g agarose in 100 ml of 1X TAE buffer in a conical flask. Gel was melted by keeping in a microwave oven for 45 to 60 seconds to ensure the complete dissolution. 2-3µl diluted Ethidium bromide (0.5 µg/ml) was added to the molten gel and mixed gently then allowed to cool down to about 35 to 40 ^{o}C. The warm gel was poured in to the gel casting tray and kept undisturbed for 30 to 45 minutes at room temperature for solidification.

The comb was carefully removed from the gel after solidification. The gel casting tray along with the gel was placed in the electrophoresis unit containing running buffer (1X TAE). Six µl of samples (5 µl DNA and 1 µl 6X gel loading dye) were loaded in the wells. along with a suitable ladder of molecular weight (100-3000 bp). Electrophoresis (65 V) was carried out until the tracking dye reached 2/3rd of the gel. Electrophoresis gel was observed under UV exposure by carefully transferring to gel documentation unit (Gel Doc^{TM} XR+ System). Presence of DNA was indicated by thick bands near the wells.

3.2.1.3 DNA quantity assessment by spectrophotometer

The quality and quantity of the DNA was verified by using spectrophotometer (NanoDrop ND-1000). The maximum absorbance of protein was present at 280 nm and that of nucleic acid at 260 nm. For quantification, optical density at 260 and 280 nm was taken into consideration. UV absorption ratio at 260 and 280nm ($A^{260/280}$) if found to be in between 1.80 to 2.00 was indicative of good quality DNA. The value below 1.8 indicated that the sample is contaminated with protein and above 2.0 indicated RNA contamination.

Procedure

The computer in which the software ND-1000 is installed was connected to a spectrophotometer (NanoDrop-ND 1000). The nucleic acid option was chosen from the software window for initiating quantification. The sampling arm was opened and wiped with tissue paper and loaded with 1µl distilled water into the lower measurement pedestal. The sampling arm was then closed and the instrument measured automatically with the help of the software.

The distilled water (blank) was used to calibrate of instrument. The zero reading was set. After that, 1µl of each sample was loaded into the measurement pedestal and measured the OD value by choosing the option ‘measure’ in the software. Recorded the OD value along with quantity of DNA in nano gram per micro litter (ng/µl). The sampling arm was opened and both upper and lower pedestals were wiped using tissue paper after measuring each sample. The recorded DNA quantity and OD values (ng/µl) were used for dilution of the DNA.

3.3 DNA fingerprinting

ISSR (Inter Simple Sequence Repeats) and SSR (Simple Sequence Repeats) were the two different types of markers used in this study. DNA from all the seven selected varieties of cocoa were amplified with selected primers. The colour charts were used for the representation of amplification pattern of DNA for better explanation of the result.

3.3.1 Condition for DNA amplification

The standardized PCR conditions were used for amplification. The standardized reaction mixture contained template DNA, assay buffer A, dNTPs, primer, Taq DNA polymerase and autoclaved distilled water. The prepared master mix that were free from DNA template was transferred to 0.2ml PCR tubes and DNA template was added to this. Polymerase chain reaction was performed using thermal cycler (Model: Proflex, Make: Life). PCR conditions were standardized in such a way that, the temperature and time used (denaturation, annealing, elongation) and number of cycles were kept optimum for effective amplification.

3.3.2 Inter Simple Sequence Repeats (ISSR) analysis

For ISSR analysis good quality DNA, isolated from cocoa leaf samples were diluted to a concentration of 20 ng/µl. By repeating the PCR reaction for at least three times, reproducibility of selected ISSR and SSR primers under same PCR conditions and using same chemicals was assured. Screening was done by using standardized annealing temperature.

Reaction mixture (20 µl) taken in 0.2 ml PCR tubes were used to conduct the genomic DNA amplification and the reaction mixture comprises of the following chemicals.

a. Taq assay buffer A (10X) - 2.0 µl.
b. dNTP mix (10mM each) - 1.5 µl
c. Primer (10pM) - 1.5µl
d. Autoclaved distilled water - 12.6µl
e. Taq DNA polymerase (3U) - 0.4µl

f. Genomic DNA (10 ng) - 2.0µl

g. Total volume - 20.0µl

ISSR pattern was developed by following Standardized PCR conditions.

a. Initial denaturation (hot start) - 94 ^{o}C for 4minutes
b. Denaturation - 94 ^{o}C for 45seconds
c. Primer annealing - 43 to 55 ^{o}C for 1 minute
d. Primer elongation - 72 ^{o}C for 2minutes

(b–d: 36 cycles)

e. Final elongation - 72 ^{o}C for 8minutes
f. Incubation - 4 ^{o}C for infinity

3.3.2.1 Screening and analysis of ISSR primers

Thirty-five ISSR primers (Table 1) reported in the previous study were used for the study (Sujith, 2016). After the PCR, the amplified products were electrophoresed along with 1kb+ DNA ladder (Thermo Scientific) or 100-3000 bp ladder (GeNei^{TM}) on 1.5 per cent agarose gel stained with ethidium bromide. Gel documentation unit (Gel Doc^{TM} XR^{+}System) under UV exposure was used to visualize banding pattern. The image format gel pictures were saved for further scoring and recognition of polymorphism between the amplicons.

Table 1. Sequences of ISSR primer used in this study

SI. No.	Primer	Nucleotide Sequence
1.	HP 10	5’-GAGAGAGAGAGACC-3’
2.	HB12	5’-CACCACCACGC-3’
3.	Oligo ISSR 04	5’-ACACACACACACACACC-3’
4.	Oligo ISSR 05	5’-CTCTCTCTCTCTCTTG-3’
5.	Oligo ISSR 06	5’-GAGAGAGAGAGAGAGAC-3’
6.	Oligo ISSR 07	5’-CTCTCTCTCTCTCTTG-3’
7.	Oligo ISSR 08	5’-GAGAGAGAGAGAGAGAT-3’
8.	ISSR 2	5’-ATTATTATTATTATTCAT3-3’
9.	ISSR 3	5’-TTATTATTATTATTACTT-3
10.	ISSR 4	5’-ATTATTATTATTATTGTT-3’
11.	ISSR 5	5’-ATTATTGTTGTTGTTTTC-3’
12.	ISSR 6	5’-TTATTATTATTATTATAA-3’
13	ISSR 7	5’-ATTATTGTTGTTGTTGTA-3’
14	ISSR 8	5’-ATTATTATTATTATTGTA-3’
15	ISSR 9	5’-TTATTATTATTATTATTACT-3’
16	UBC 808	5’-AGAGAGAGAGAGAGAT-3’
17	UBC 810	5’-GAGAGAGAGAGAGAGAT-3’
18	UBC 811	5’-GAGAGAGAGAGAGAGAC-3’
19	UBC 815	5’-CTCTCTCTCTCTCTCTG-3’
20	UBC 818	5’-CACACACACACACACAG-3’
21	UBC 826	5’-ACACACACACACACACC-3’
22	UBC 827	5’-ACACACACACACACACG-3’
23	UBC 835	5’-AGAGAGAGAGAGAGAGYC-3’
24	UBC 841	5’-GAGAGAGAGAGAGAGAY-3’
25	UBC 844	5’-CTCTCTCTCTCTCTCTRC-3’
26	UBC 846	5’-CACACACACACACACART-3’
27	UBC 847	5’-CACACACACACACACARC-3’
28	UBC 848	5’-CACACACACACACACARG-3’
29	UBC 850	5’-GTGTGTGTGTGTGTGTYC-3’
30	UBC 854	5’-TCTCTCTCTCTCTCTCRG-3’
31	UBC 855	5’-ACACACACACACACACYT-3’
32	UBC 857	5’-ACACACACACACACACYG-3’
33	UBC 865	5’-CCGCCGCCGCCGCCGCCG-3’
34	UBC 866	5’-CTCCTCCATCCTCCTCCTC-3’
35	UBC 873	5’-GACAGACAGACAGACA-3’

3.3.3 Simple Sequence Repeats (SSR) analysis

For SSR analysis good quality DNA was isolated from cocoa leaf samples and diluted to a concentration of 20 ng/µl. screening was done by using standardized annealing temperature.

20 µl reaction mixture in 0.2 ml PCR tubes were used to conduct genomic DNA amplification and the mixture comprises of the following chemicals.

a. Taq assay buffer A (10X) - 2.0 µl.
b. dNTP mix (10mM each) - 1.5 µl
c. Forward primer (10pM) - 0.75µl
d. Reverse primer (10pM) - 0.75 µl
e. Autoclaved distilled water - 12.6µl
f. Taq DNA polymerase (3U) - 0.4µl
g. Genomic DNA (10 ng) - 2.0µl
h. Total volume - 20.0µl

SSR pattern was developed by following Standardized PCR conditions.

a. Initial denaturation (hot start) - 94 ^{o}C for 4minutes
b. Denaturation - 94 ^{o}C for 45seconds
c. Primer annealing - 46 to 64 ^{o}C for1 minute
d. Primer elongation - 72 ^{o}C for 2minutes

(b–d) 40cycles

e. Final elongation - 72 ^{o}C for 8minutes
f. Incubation - 4 ^{o}C for infinity

3.3.3.1 Screening and analysis of SSR primers

Thirty SSR primers were chosen for screening which included 30 markers used in previous study (Sujith, 2016). The chosen SSR primers for the analysis are listed in Table 2.

The amplified products of PCR were subjected to electrophoresis along with 100 bp - 3000 bp DNA ladder (Invitrogen) on 1.5 per cent agarose gel stained with Ethidium bromide. Gel documentation unit (Gel Doc^{TM} XR^{+}System) under UV exposure was used to visualize the banding pattern. The image format gel pictures were saved for further scoring and recognition of polymorphism between the amplicons.

3.4 DNA fingerprint development

Seven cocoa varieties were selected for the DNA fingerprint development by using selected 23 ISSR and 17 SSR primers (Tables 1 and 2). DNA fingerprint profile of the different varieties was developed by scoring visually different bands generated through electrophoresis based on molecular weight. Different colour codes were used for easy detection and interpretation of results. Polymorphic amplicons were identified by visual scoring of gel pictures of individual primers of all seven varieties. Amplicons which were shared by maximum of three varieties in ISSR and four varieties in SSR primers were selected for fingerprinting of individual genotypes.

Table 2. Sequences of SSR primer used in this study

SI.NO.	Primer	Nucleotide Sequence
1	mTcCIR8	F5’-CTAGTTTCCCATTTACCA-3’ R5’-TCCTCAGCATTTTCTTTC-3’
2	mTcCIR11	F 5’-TTTGGTGATTATTAGCAG-3’ R 5’-GATCGATTTGATGTGAG-3’
3	mTcCIR12	F5’-CAGCCGCCTCTTGTTAG-3’ R 5’-TATTTGGGATTCTTGATG-3’
4	mTcCIR18	F 5’-GATAGCTAAGGGGATTGAGGA-3’ R 5’-GGTAATTCAATCATTTGAGGATA-3’
5	mTcCIR24	F5’-TTTGGGGTGATTTCTTCTGA-3’ R5’-TCTGTCTCGTCTTTTGGTGA-3’
6	mTcCIR33	F 5’-TGGGTTGAAGATTTGGT-3’ R 5’-CAACAATGAAAATAGGCA-3’
7	mTcCIR40	F 5’-AATCCGACAGTCTTTAATC-3’ R 5’-CCTAGGCCAGAGAATTGA-3’
8	mTcCIR42	F 5’-TTGCTGAAGTATCTTTTGAC-3’ R 5’-GCTCCACCCCTATTTG-3’
9	SHRSTc49	F5’-ATCGCAGCAAACTCCCTCTC-3’ R5’-TTCTCTTCCCACCAAGTCCC-3’
10	SHRSTc51	F 5’-CTGTTTTTGCCTCCCTTGTTCT-3’ R 5’-ATTGCTGGTTGTTCTCCATCCT-3’
11	SHRSTc64	F 5’-TCCTACATTCCTGCACCC-3’ R 5’-TCGAGAGAAAAGCTCTTACACT-3’
12	mTcCIR7	F 5’-ATGCGAATGACAACTGGT-3’ R 5’-GCTTTCAGTCCTTTGCTT-3’
13	mTcCIR10	F5’-CCGAATTGACAGATGGCCTA-3’ R5’-CCCAAGCAAGCCTCATACTC-3’
14	mTcCIR15	F 5’-CAGCCGCCTCTTGTTAG-3’ R 5’-TATTTGGGATTCTTGATG-3’
15	mTcCIR22	F 5’-ATTCTCGCAAAAACTTAG-3’ R 5’-GATGGAAGGAGTGTAAATAG-3’
16	mTcCIR25	F 5’-CTTCGTAGTGAATGTAGGAG-3’ R 5-TTAGGTAGGTAGGGTTATCT-3’
17	mTcCIR26	F 5’-GCATTCATCAATACATTC-3’ R 5’-GCACTCAAAGTTCATACTAC-3’

Table 2. contd.

SI.NO.	Primer	Nucleotide Sequence
18	mTcCIR37	F5'-CTGGGTGCTGATAGATAA-3' R5'-AATACCCTCCACACAAAT-3'
19	mTcCIR60	F5'-CGCTACTAACAAACATCAAA-3' R5'-AGAGCAACCATCACTAATCA-3'
20	mTcCIR102	F5'-TTGTGAAAAGATTGCGA-3' R 5'-TTGCTTGTTATTGCTACAT-3'
21	mTcCIR121	F 5'-CATGTGCATTTAGGTGTC-3' R 5'TCTGGCTTCTTAGTGATAC-3'
22	mTcCIR146	F 5'-GCAAGGTCTTTTTACGAT-3' R 5'-ATGGACACGTCTAAGTTG-3'
23	SHRSTc52	F 5'-TTTTAGAGCATCCACTTCCT-3' R 5'-CCATTCTTTCCACACTGAGAG-3'
24	SHRSTc53	F 5'-TTCCCTTTCTTTCTCTCTCTC-3' R 5'-AGTCGTTGCTACTGCTGG-3'
25	SSRKAU15	F 5'-TGTTGCTCGAACTCTCCAAA-3' R 5'-CATAGGAGAGGTAACCCGCA-3'
26	SSRKAU19	F5'-ATTGTACAAAGACCCGTGGC-3' R5'-GTTGCACACTGGATCAATGC-3'
27	SSRKAU20	F 5'-AGGGTCCTTCGTTTGGAACT-3' R 5'-GCATTCCACTTGTGAAGCAT-3'
28	SSRKAU21	F5'-GGTCCAGTTCAATCAACCGA-3' R5'-TGAAGTCGTCTCATGGTTCG-3'
29	SSRKAU22	F 5'-GCAGAGGATATTGCATTCGC-3' R 5'-CAAACCGAACTCATCAAGGG-3'
30	SSRKAU24	F 5'-CCGAGGCGAATCTTGAATAC-3' R 5'-GCACCATCTCTTGTGCCTCT-3'

4. RESULT AND DISCUSSION

Seven cocoa varieties released from Cocoa Research Center, KAU were used for the study. The study was conducted at Centre for Plant Biotechnology and Molecular Biology (CPBMB), KAU during 2017-2019.The results of the present investigation on 'DNA fingerprinting of selected cocoa (*Theobroma cacao* L.) varieties of Kerala Agricultural University' are presented below.

4.1 Molecular characterization

4.1.1 Isolation and quantification of DNA

For molecular characterization, good quality DNA forms the basic requirement. In cocoa young pale green leaves (second and third leaf from the shoot tip) was reported to be the desirable part for DNA isolation by many scientist (Perry *et al*., 1998; Behera *et al*., 2008; Sarma and Tanti, 2017). However in early studies conducted by Kerala Agricultural University, tender reddish colored leaves were found to yield good quality DNA (Chandrakant, 2014 and Sujith, 2016). Hence in the present study also, tender reddish leaves were collected during early morning hours from the growing tip of cocoa plant for DNA isolation.

The method developed by Dellaporta *et al*., (1983) was found to yield good quality DNA from the Malvaceae family were the mucilage content is high (Ileana, 2005; Tussell *et al*., 2005; Ahmed *et al*., 2013). However when DNA was extracted employing this method, RNA contamination was observed. Hence, slight modification was made in the protocol and this yielded good quality DNA (Plate 2). Leaf sample (1g) was homogenized with liquid nitrogen and the polyphenol content was reduced by adding anti-oxidant β- mercaptoethanol and PVP. Instead of extraction buffer 2, 5 per cent CTAB was used. Chloroform Isoamyl alcohol was also added after first centrifugation, the complete protocol used in the study is detailed under 3.2.1.1

Many studies conducted on different crops belonging to Malvaceae family have reported that the leaf samples contains high amount of mucilage, and even after centrifugation it is difficult to remove the mucilage from DNA without losing it (Bayer

et al., 1999; Ahmed *et al*., 2013). Many scientists conducted this study in cocoa belongs to Malvaceae family and reported that the leaf used for DNA isolation was with high amount of polysaccharides, polyphenols, tannins and other secondary metabolites, which interfere with DNA extractions and polymerase chain reaction (PCR) amplification (Machado *et al*., 2005; Ghosh *et al.*, 2009; Chandrakant, 2014).

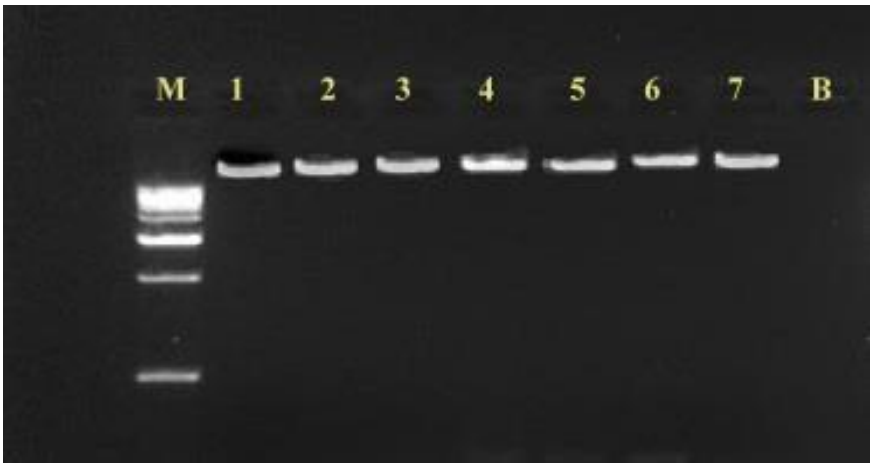
A protocol was developed for the isolation of good quality genomic DNA from *Abelmoscus esculentus*, belongs to Malvaceae family (Jose and Usha 2000). This method was a combination of Dellaporta *et al.* (1983) and CTAB method (Doyle and Doyle 1990). The commercial kits for DNA isolation from cocoa was also reported (Zhang and Stewart, 2000; Haymes *et al*., 2004; Gryson *et al*., 2007). Isolated DNA was quantified by different methods. Matrix Assisted Laser Desorption Ionisation-Time of Flight Mass Spectrometry (MALDITOF-MS) is a method to provide accurate molecular weight determination (Terzi *et al*., 2005 Gryson *et al*., 2007). Dolezel *et al*. (2007) measured the DNA content in plants by Flow cytometry (FCM) using DNA-selective fluorochromes.

In the present study, spectrophotometer analysis using nanodrop ND-1000® was employed for quantification, optical density at 260 and 280 nm was taken into consideration. OD ratio 260/280 was found in between 1.80 to 2.00 Table 3) which indicated good quality DNA (Meena, 2014; Laxman, 2013; Thakur *et al*., 2014; Agbagwa *et al.*, 2012; Sujith, 2016).

The dilution was made to a concentration of 20 ng/µl and further observed for good amplification. The same concentration (20ng/µl) was used for both ISSR and SSR analysis.

Table. 3 Quantification of DNA samples isolated from cocoa varieties

Varieties	**UV absorbance at 260nm(A_{260})**	**UV Absorbance at 280nm(A_{280})**	**Optical Density ($A_{260/280}$)**	**Quantity of DNA (ng/µl)**
CCRP3	6.20	3.22	1.92	189.4
CCRP10	7.30	3.66	1.99	511.20
CCRP11	16.60	8.25	2.01	280.30
CCRP12	9.53	4.76	2.00	917.30
CCRP13	13.19	6.59	2.00	566.90
CCRP14	11.69	5.90	1.98	748.30
CCRP15	9.60	4.82	1.99	725.10



M- Molecular weight marker (3kb), B- Blank, 1-CCRP 3, 2- CCRP 10, 3- CCRP 11, 4- CCRP 12, 5- CCRP 13, 6- CCRP 14, 7- CCRP 15

Plate 2. Genomic DNA isolated from seven cocoa varieties

4.2 Molecular marker analysis

DNA was isolated and screening was carried out with IISR and SSR primers to find out suitable ones with desirable banding pattern. Thirty-five ISSR and thirty SSR primers were used for the study.

4.2.1 Inter Simple Sequence Repeat (ISSR) analysis

4.2.1.1 Primer screening for ISSR assay

ISSR is a PCR based molecular marker system that gives the variation in microsatellite regions distributed throughout the genome in an individual (Zietkiewicz *et al.,* 1999). Arcade (2000) mentioned that ISSR markers are generated by microsatellite-repeat-anchored primers, amplifying regions between adjacent SSR loci. These primers, produce many polymorphic markers useful for genome mapping.

In the present study, thirty-five IISR primers were used for the analysis (Table 4) with reaction mixture composition and thermal conditions as detailed in materials and methods chapter 3.2.2. ISSR is a dominant marker system which is reported for DNA fingerprinting, genetic diversity and evolutionary study in different crops like of mango (Pandit *et al*., 2007), *Jatropha curcas* (Basha and Sujatha, 2007), *Glycyrrhiza uralensis* (Yao *et al*., 2008) faba bean (Razzak *et al*., 2019) cotton (Abdi *et al*., 20 12) *Aristolochia* (Sarma and Tanti, 2017), okra (Kumar and Sharma 2011), cocoa (Sujith, 2016) *etc*. High level reproducibility and successful establishment in various crops made ISSR markers as a potential tool for Marker Assisted Selection (Bornet and Branchard, 2001; Reddy *et al*., 2002).

Out of thirty-five, twenty-three ISSR primers were selected in this study based on the amplification pattern. Details of the selected primers and their annealing temperatures are specified is Table 5. ISSR markers have an advantage over other marker system since large number of polymorphic bands are produced, and have high transferability to other species. Moreover, it does not require any earlier information about DNA sequence and development cost is comparable (Arcade *et al.,* 2000; Barth *et al*., 2002; Archak*et al*., 2003; Brandao *et al*., 2011).

Table 4. Details of amplification pattern obtained with 35 ISSR primers

SI. No	Primers	Amplification pattern			
		No. of amplicon	Types of amplicons		Remarks
			Distinct	Faint	
1	HP 10	11	7	3	Selected
2	HB12	7	6	1	Selected
3	Oligo ISSR 04	6	6	0	Selected
4	Oligo ISSR 05	9	6	3	Selected
5	Oligo ISSR 06	0	0	0	Not selected
6	Oligo ISSR 07	4	4	0	Selected
7	Oligo ISSR 08	4	2	2	Selected
8	ISSR 2	0	0	0	Not selected
9	ISSR 3	9	9	0	Selected
10	ISSR 4	0	0	0	Not selected
11	ISSR 5	0	0	0	Not selected
12	ISSR 6	0	0	0	Not selected
13	ISSR 7	5	5	0	Selected
14	ISSR 8	0	0	0	Not selected
15	ISSR 9	0	0	0	Not selected
16	UBC 808	0	0	0	Not selected
17	UBC 810	12	12	0	Selected
18	UBC 811	8	8	0	Selected
19	UBC 815	5	4	1	Selected
20	UBC 818	9	8	1	Selected
21	UBC 826	10	9	1	Selected
22	UBC 827	8	8	0	Selected
23	UBC 835	7	6	1	Selected
24	UBC 841	8	7	1	Selected
25	UBC 844	16	14	2	Selected
26	UBC 846	9	6	3	Selected
27	UBC 847	6	5	1	Selected
28	UBC 848	0	0	0	Not selected
29	UBC 850	0	0	0	Not selected
30	UBC 854	7	7	0	Selected
31	UBC 855	9	8	1	Selected
32	UBC 857	10	10	0	Selected
33	UBC 865	0	0	0	Not selected
34	UBC 866	10	8	2	Selected
35	UBC 873	0	0	0	Not selected

Table 5. Sequences and annealing temperatures of the amplified ISSR primers

SI. No.	Primers	Nucleotide Sequence	Annealing temperature (°C)
1	HB10	5’-GAGAGAGAGAGACC-3’	38.0
2	HB12	5’-CACCACCACGC-3’	37.0
3	Oligo ISSR 04	5’-ACACACACACACACACC-3’	42.0
4	Oligo ISSR 05	5’-CTCTCTCTCTCTCTTG-3’	42.4
5	Oligo ISSR 07	5’-CTCTCTCTCTCTCTTG-3’	42.4
6	Oligo ISSR 08	5’-GAGAGAGAGAGAGAGAT-3’	42.9
7	Oligo ISSR 3	5’-TTATTATTATTATTACTT-3’	34.8
8	Oligo ISSR 7	5’-ATTATTGTTGTTGTTGTA-3’	45.0
9	UBC 810	5’- GAGAGAGAGAGAGAGAT-3’	50.5
10	UBC 811	5’-GAGAGAGAGAGAGAGAC-3’	43.3
11	UBC 815	5’-CTCTCTCTCTCTCTCTG-3’	44.9
12	UBC 818	5’-CACACACACACACACAG-3’	52.0
13	UBC 826	5’-ACACACACACACACACC-3’	53.3
14	UBC 827	5’-ACACACACACACACACG-3’	54.9
15	UBC 835	5’-AGAGAGAGAGAGAGAGYC-3’	45.6
16	UBC 841	5’-GAGAGAGAGAGAGAGAY-3’	46.0
17	UBC 844	5’-CTCTCTCTCTCTCTCTRC-3’	53.7
18	UBC 846	5’-CACACACACACACACART-3’	52.7
19	UBC 847	5’-CACACACACACACACARC-3’	53.1
20	UBC 854	5’-TCTCTCTCTCTCTCTCRG-3’	51.3
21	UBC 855	5’-ACACACACACACACACYT-3’	60.2
22	UBC 857	5’-ACAACACACCACACACYG-3’	57.3
23	UBC 866	5’-CTCCTCCTCCTCCTCCTC-3’	55.0

4.2.1.2 DNA fingerprinting with selected ISSR primers

Amplification of DNA samples from seven cocoa varieties using selected twenty-three ISSR primers (Table 5) were performed in order to develop ISSR fingerprint. The amplified pattern obtained with each primer was observed in detail to identify the polymorphic bands. The details of twenty-three amplified ISSR primers are given below.

4.2.1.2.1 HB10

HB 10 generated average of seven amplicons in all accession. The amplicon size varied from 300 bp to 1400 bp and pattern obtained is depicted in Plate 3. Polymorphic band were observed in CCRP 10, CCRP 11 and CCRP 14 at 1000 bp and in CCRP 14 and CCRP 15 at 600 bp length (Fig. 1). This indicated that the primer HB 10 can be used in final DNA fingerprinting.

4.2.1.2.2 HB12

The primer HB 12 had generated an average of six amplicons in each accession (Plate 4).The marker size had varied from 300 bp to 1250 bp. In CCRP 3 and CCRP 15 band was present at 1250 bp (Fig. 2). In CCRP 3, CCRP 10 and CCRP 12 polymorphic band was observed at 1000 bp, and CCRP 13, CCRP 14 had polymorphic band at 700 bp. Thus, primer HB 12 can be considered for final DNA fingerprinting.

4.2.1.2.3 Oligo ISSR04

Oligo ISSR 04 generated six clear amplicon in all seven genotypes of cocoa (Plate 5). The molecular size of the band differed from 450 bp to1500 bp. Polymorphic band was obtained in CCRP 10 and CCRP 15 at 1300 bp, CCRP 3 and CCRP 10 at 800 bp as well as at 450 bp (Fig. 3). The primer Oligo ISSR 04 can be considered an as ideal marker for identification of the hybrids CCRP 3, CCRP 10 and CCRP15.

4.2.1.2.4 Oligo ISSR05

Total nine amplicons were generated by Oligo ISSR 05 primer. Molecular size of the bands varied from 150 bp to1500 bp (Fig.4). Three unique polymorphic bands were obtained in the amplification pattern generated by Oligo ISSR05 primer (Plate 6). The unique polymorphic band was observed in hybrid CCRP 12 at 1400 bp and also at 300

bp and CCRP 15 had a band at 150 bp length. The primer Oligo ISSR 05 can be established as an ideal marker for the identification of CCRP 12 and CCRP15.

4.2.1.2.5 Oligo ISSR07

Primer Oligo ISSR 07 amplified an average of seven amplicons (Plate 6). The amplification pattern is shown in Fig.7. The molecular sizes of the amplicons generated ranged from the 550 bp to 1500 bp. Polymorphic bands were identified at 1250 bp in CCRP 10 and CCRP 12, at 1400 bp in CCRP 12 and CCRP 15. A unique band was also observed at 550 bp in CCRP 14. Thus, Oligo ISSR 07 is ideal for identification of CCRP 10, CCRP 12, CCRP 14 and CCRP 15.

4.2.1.2.6 Oligo ISSR08

Amplification pattern of Oligo ISSR 08 primer is shown in Plate 8 and the amplicons had 290 bp to 800 bp size. More than three varieties shared the similar amplicon pattern therefore this primer cannot be consided for the final DNA fingerprinting (Fig.6.)

4.2.1.2.7 Oligo ISSR3

Amplification pattern generated by the primer Oligo ISSR 3 in seven cocoa varieties were analyzed (Plate 9). Amplicons ranged from 400 bp to 1100 bp. This primer produced three polymorphic bands in CCRP 11, CCRP 12 and CCRP 14 at 850 bp. Similarly, polymorphic bands were observed in CCRP 12 and CCRP 15 at 700 bp (Fig. 7). A unique polymorphic band was observed at 400 bp in CCRP 12. This ISSR primer can be used in DNA fingerprinting.

4.2.1.2.8 Oligo ISSR 7

Primer Oligo ISSR 7 was used to generate amplification pattern in seven cocoa genotypes. Total four amplicons were generated in all the accession. The range of the amplicon varied from 600 bp to 1100 bp (Plate 10). Polymorphic bands were observed in CCRP 10, CCRP 11 and CCRP 13 at 900 bp length (Fig. 8). This primer can be selected for identification of hybrids CCRP 10, CCRP 11 and CCRP 13.

4.2.1.2.9 UBC 810

Two unique polymorphic amplicons were obtained when the primer UBC 810 was used for DNA amplification (Plate 11). Unique band was produced at 1400 bp in CCRP 11 and at 500 bp in CCRP 3. In total the polymorphic bands ranged from 350 bp to 1400 bp (Fig. 9). The primer UBC 810 can be used for fingerprinting of CCRP 11 and CCRP 3.

4.2.1.2.10 UBC 811

Average of six clear amplicons were present in all seven-samples using the primer UBC 811 (Plate 12). Bands of size 400 bp to 950 bp was observed. Amplification pattern gave distinct band at 950 bp in CCRP 3, CCRP 11 and CCRP 14(Fig. 10). CCRP 3, CCRP 14 and CCRP 15 had polymorphic band at 550 bp length. This primer can be used for DNA fingerprinting.

4.2.1.2.11 UBC815

The primer UBC 815 generated the distinct polymorphic bands at two different position (Plate 13). The molecular size of the band differed from 650 bp to 1500 bp. CCRP 11, CCRP 12 and CCRP 14 had polymorphic band at 700 bp and a unique distinct band observed at 650 bp in CCRP 12 (Fig. 11). The primer UBC 815 can be efficiently utilized in identification of CCRP 11, CCRP 12 and CCRP 14.

4.2.1.2.12 UBC 818

The primer UBC 818 generated seven amplicons in all the seven genotypes (Plate 14). Band size of generated amplicons ranged from 400 bp to 2500 bp as showninFig.12. This primer produced polymorphic band in CCRP10, CCRP11andCCRP 13 at 2500 bp and similarly in CCRP 3, CCRP 11 and CCRP 15 at 500 bp. Primer UBC 818 can be used for identification of CCRP 3, CCRP 11, CCRP 13, CCRP 14 and CCRP 15.

4.2.1.2.13 UBC 826

UBC 826 primer was used to develop the amplification pattern of seven cocoa genotypes and an average eight amplicons per accession were developed (Plate 15). Molecular size of band obtained ranged from 290 bp to 1000 bp length. The CCRP 11

had a unique polymorphic band at 310 bp (Fig. 13). The primer UBC 826 can be considered to be an ideal marker for identification of the hybrid CCRP11.

4.2.1.2.14 UBC 827

The amplification pattern on seven cocoa genotypes were developed, using the UBC 827 ISSR primer. Molecular size of the bands varied from 650 bp to 1500 bp length (Plate 16). Hybrid CCRP 12 had a unique polymorphic band at 1400 bp (Fig. 14). Hence primer UBC 827 can be selected for fingerprinting CCRP 12.

4.2.1.2.15 UBC 835

UBC 835 generated seven amplicons in each accession. The amplification pattern is shown in Plate 17. Molecular size of the band generated were from 250 bp to 1000 bp length (Fig. 15). At 400 bp a unique polymorphic band was observed in hybrid CCRP 10. Polymorphic amplicons were also observed at 350 bp in CCRP 10, CCRP 13 and CCRP 15. The primer UBC 835 can be consider as ideal primer for the identification of CCRP 10, CCRP 13 and CCRP 15.

4.2.1.2.16 UBC 841

UBC 841 had generated average eight clear amplicons in all seven genotypes. The molecular size varied from 290 bp to 1000 bp (Plate 18). A unique polymorphic band was found in hybrid CCRP 11 at 300 bp length (Fig. 16). Thus, primer could be used for further finger printing of CCRP 11.

4.2.1.2.17 UBC 844

The primer UBC 844was used in developing the amplification pattern and produced an average of fifteen band in all genotypes (Plate 19). Distinct bands were present at six different position in the seven genotypes. Molecular size of band generated varied from 290 bp to 2000 bp (Fig. 17). The hybrid CCRP 3 and CCRP 10 consisted of a polymorphic band at 400 bp. CCRP 11, CCRP 12 and CCRP 15 had polymorphic bands at 500 bp. At 750 bp, polymorphic bands were present in CCRP 3, CCRP 10 and CCRP 15. In hybrid CCRP 15 a unique band was observed at 590 bp and 300 bp. Polymorphic band at 1250 bp length was found in CCRP10, CCRP 11 and CCRP14. Thus, this IISR primer will be used in fingerprinting.

4.2.1.2.18 UBC 846

UBC 846 ISSR primer produced eight amplicons in all seven genotypes (Plate 20).They varied from 490 bp to 1300bp. Amplicons generated from this primer produced polymorphism at 1200 bp in CCRP 3, CCRP 10 and CCRP 13 and in CCRP 12 and CCRP15 at 850 bp (Fig. 18). UBC 846 primer can be utilized for further analysis.

4.2.1.2.19 UBC 847

Molecular size of the amplicons generated by UBC 847 ISSR varied from 700 bp to1850 bp (Plate 21). Polymorphism was observed in CCRP11, CCRP 12 and CCRP 13 at 800 bp (Fig. 19). Thus UBC 847 primer can be used in fingerprinting for these varieties.

4.2.1.2.20 UBC 854

The primer UBC 854 was used to amplify the DNA of seven genotypes. Molecular size of amplicons generated were from 400 bp to 1700 bp. Three distinct unique polymorphic bands were detected (Plate 22). Amplicons produced unique bands at 1300 bp, 1000 bp and 400 bp in CCRP 11, CCRP12, CCRP 13 respectively (Fig. 20).

4.2.1.2.21 UBC 855

Amplification was done in seven cocoa DNA samples by using the IISR primer UBC 855. The molecular size of amplicons varied from 250 bp to 1400 bp (Plate 23). Amplifications produced a unique band at 650 bp in CCRP 14. UBC 855 also produced polymorphic bands in CCRP 13 and CCRP 15 at 500 bp and also CCRP 11, CCRP 12 and CCRP 14 at 350 bp. Thus, primer UBC 855 can be an ideal primer for identification of CCRP 11, CCRP 12, CCRP13, CCRP 14 and CCRP 15 (Fig.21).

4.2.1.2.22 UBC 857

DNA samples amplified with the primer UBC 857, developed polymorphism. Molecular size varied from 500 bp to 1100 bp (Plate 24). A unique band in CCRP 10 at 750 bp was generated. CCRP 12 and CCRP15 hybrids had polymorphic band at 700 bp. A polymorphic band was found in CCRP 12 and CCRP 14 at 500 bp (Fig. 22).

Thus, primer UBC 857 can be utilized for identification of CCRP 10, CCRP12, CCRP14 and CCRP15.

4.2.1.2.23 UBC 866

The amplification pattern developed by UBC 866 primer in seven genotypes, is given in Plate 25. The range of molecular size of the generated bands was between 350 bp to 1600 bp (Fig. 23). UBC 866 primer produced the polymorphic bands at 1300 bp in CCRP 12 and CCRP 15 and at 700bp in CCRP10 and CCRP11. UBC 866 primer can be selected for final DNA fingerprinting.

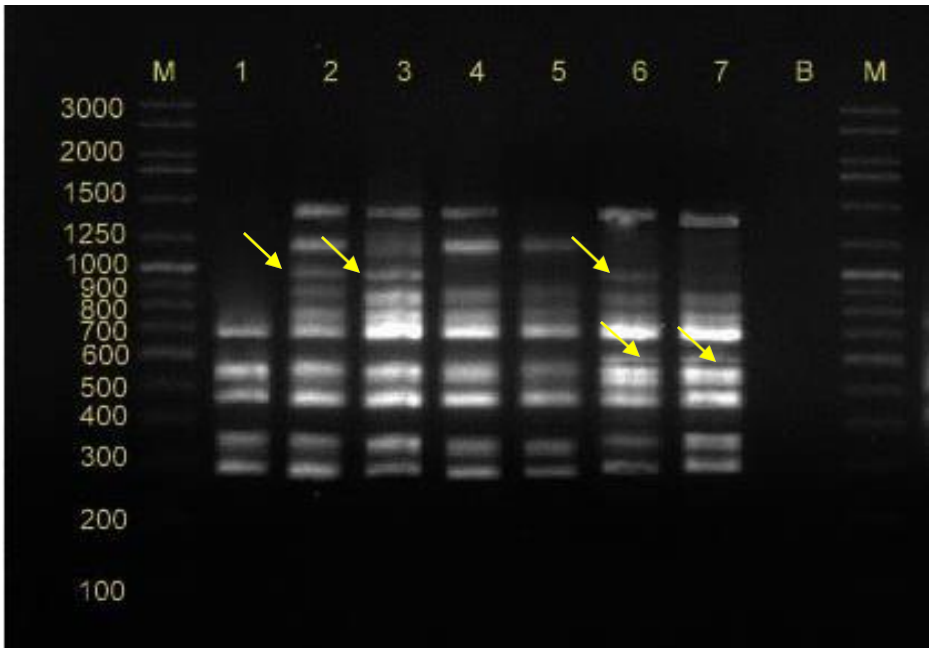
Thul *et al*., (2012) used 8 ISSR primers to check the genetic variability of 22 Capsicum accessions from *C. annuum, C. baccatum, C. chinense, C. eximium, C. frutescens*, and *C. luteum*. They found that IISR marker systems are helpful in identification of genetic stock of *Capsicum* species along with species specificity.

Khalik and Osman (2017) conducted the genetic diversity analysis on *Plectranthus* spp. by using five ISSR. It differentiated the characteristic of the closely related species after combined UPGMA cluster analysis.

Oligo ISSR 05 had three unique bands at 1400 bp and at 300 bp in CCRP12 and in CCRP15 at 150 bp length. Oligo ISSR 07 gave band at 550 bp in CCRP 14, Oligo ISSR 3 produced a unique band at 400bp in CCR12. USB 810 produced unique distinct band in selection CCRP 3 at 500 bp and hybrid CCRP 11 at 1400 bp. UBC 815 had polymorphism in CCRP 12 at 650, whereas UBC 826 gave band at 310 bp in CCRP 11, UBC 827 in CCRP 12 at 1400 bp. UBC 835 yielded the amplicon in CCRP 10 at 400 bp, UBC 841 in CRRP 11 at 300 bp. Primer UBC 844 generated highest (thirteen) number of polymorphic bands. The primer UBC 844 had two unique bands in CCRP 15 (590 bp and 300 bp). UBC 854 primer produced three unique bands in CCRP 11, CCRP 12 and CCRP 13 at 1300 bp, 1000 bp and at 400 bp respectively. UBC 855 yielded band at 800 bp in CCRP 14, UBC 857 in CCRP 10 at 750bp.

Among the seven cocoa genotypes, hybrid CCRP 12 had maximum number of unique bands generated by primer Oligo ISSR 05 (1400 bp and 300 bp), Oligo ISSR 3 (400bp), UBC 815 (650bp), UBC827 (1400bp) and UBC854 (1000 bp). All seven cocoa varieties registered unique polymorphic band with selected primers.

Thirteen ISSR primers were used to evaluate the population structure and genetic diversity in natural population of *Theobroma subincanum* in the Brazilian Amazon (Rivas *et al.,* 2013). DNA fingerprinting of promising cocoa (*Theobroma cocoa L.*) varieties were done by using selected ten ISSR primers (Sujith, 2016).



M- Molecular weight marker (3kb), B- Blank, 1-CCRP 3, 2- CCRP 10, 3-CCRP 11, 4- CCRP 12, 5- CCRP 13, 6- CCRP 14, 7- CCRP 15

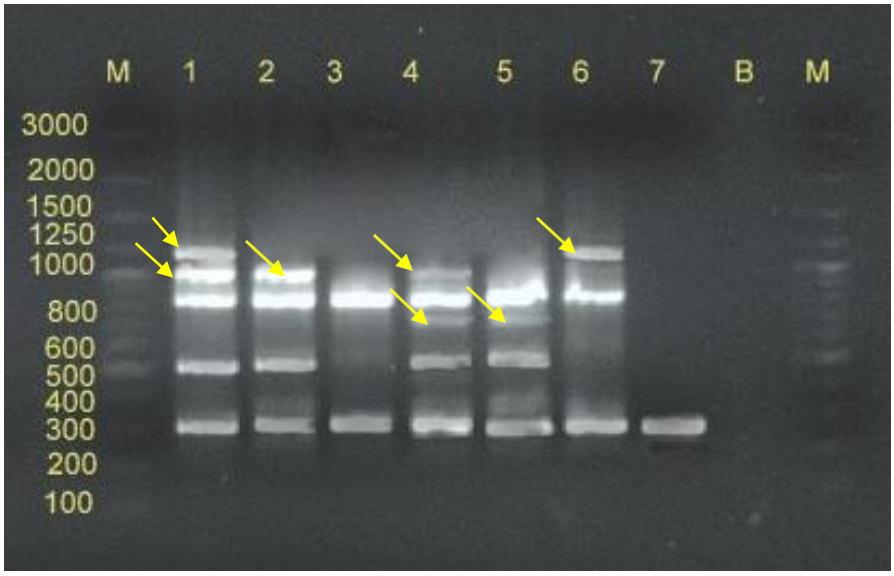
Plate 3. Amplification pattern generated with primer HB10

	1	2	3	4	5	6	7
Mol. Size (bp)/Varieties	CCRP 3	CCRP 10	CCRP 11	CCRP 12	CCRP 13	CCRP 14	CCRP 15
1400							
1250							
1000							
900							
800							
700							
600							
550							
450							
350							
300							

Colour code for sharing among varieties

1	2	3	4	5	6	7

Fig. 1 Colour chart developed using HB1



M- Molecular weight marker (3kb), B- Blank, 1-CCRP 3, 2- CCRP 10, 3- CCRP 11, 4- CCRP 12, 5- CCRP 13, 6- CCRP 14, 7- CCRP 15

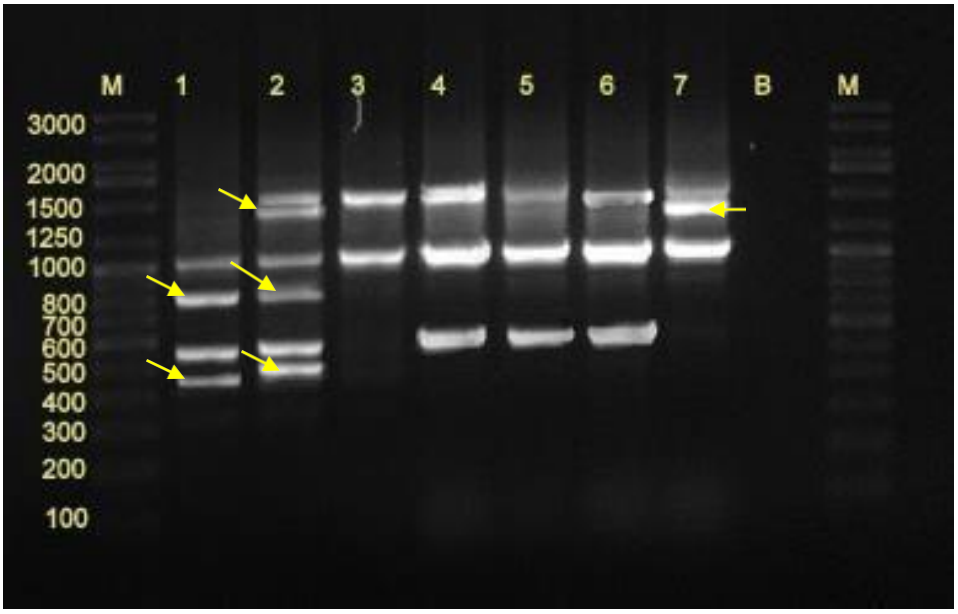
Plate 4. Amplification pattern generated with primer HB12

	1	2	3	4	5	6	7
Mol. Size (bp)/Varieties	CCRP 3	CCRP 10	CCRP 11	CCRP 12	CCRP 13	CCRP 14	CCRP 15
1250							
1000							
800							
700							
500							
300							

Colour code for sharing among varieties

1	2	3	4	5	6	7

Fig. 2 Colour chart developed using HB 12



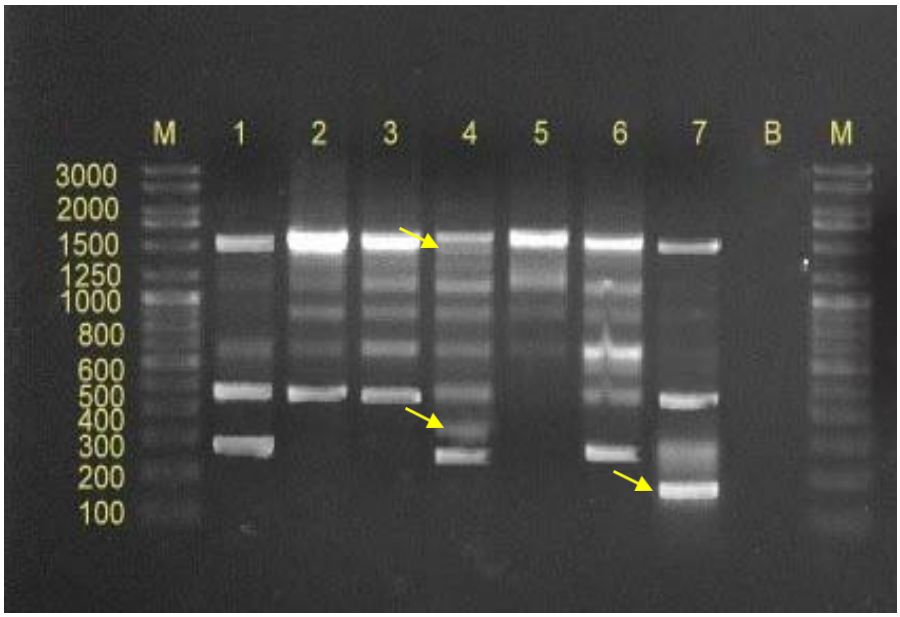
M- Molecular weight marker (3kb), B- Blank, 1-CCRP 3, 2- CCRP 10, 3- CCRP 11, 4- CCRP 12, 5- CCRP 13, 6- CCRP 14, 7- CCRP 15

Plate 5. Amplification pattern generated with primer Oligo ISSR04

	1	2	3	4	5	6	7
Mol. Size (bp)/Varieties	CCRP 3	CCRP 10	CCRP 11	CCRP 12	CCRP 13	CCRP 14	CCRP 15
1500							
1300							
1000							
800							
550							
450							

Colour code for sharing among varieties

Fig. 3 Colour chart developed using Oligo ISSR 04



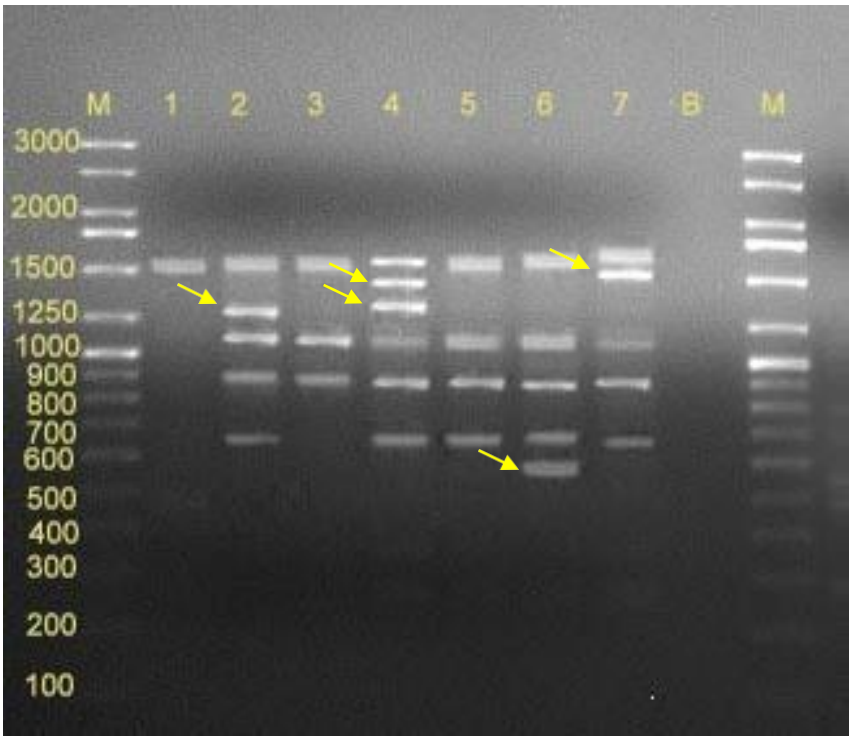
M- Molecular weight marker (3kb), B- Blank, 1-CCRP 3, 2- CCRP 10, 3- CCRP 11, 4- CCRP 12, 5- CCRP 13, 6- CCRP 14, 7- CCRP 15

Plate 6. Amplification pattern generated with primer Oligo ISSR 05

	1	2	3	4	5	6	7
Mol. Size (bp)/Varieties	CCRP 3	CCRP 10	CCRP 11	CCRP 12	CCRP 13	CCRP 14	CCRP 15
1500							
1400							
1100							
900							
650							
450							
300							
250							
150							

Colour code for sharing among varieties

Fig. 4 Colour chart developed using Oligo ISSR 05



M- Molecular weight marker (3kb), B- Blank, 1-CCRP 3, 2- CCRP 10, 3-CCRP 11, 4- CCRP 12, 5- CCRP 13, 6- CCRP 14, 7- CCRP 15

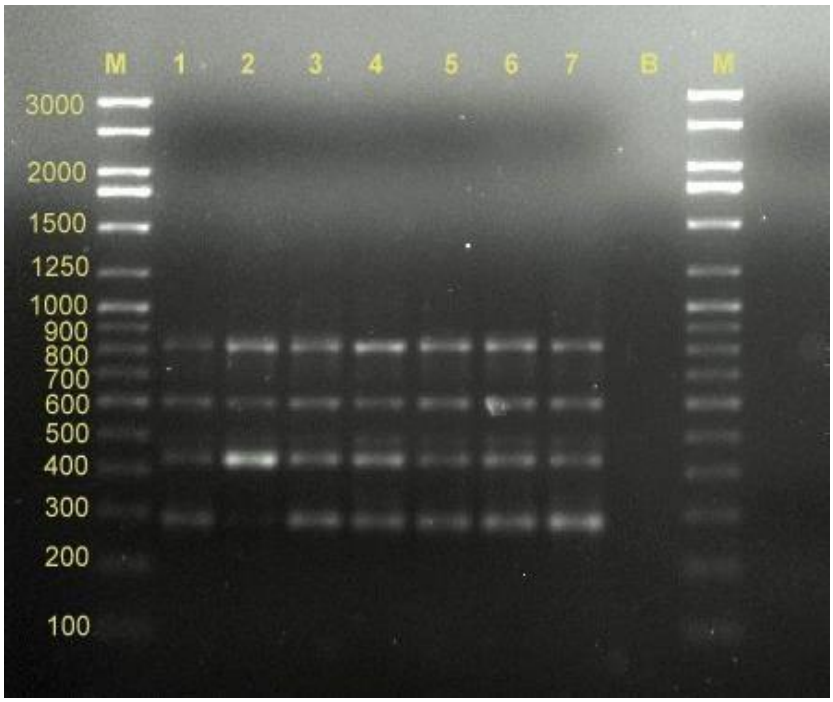
Plate 7. Amplification pattern generated with primer Oligo ISSR 07

	1	2	3	4	5	6	7
Mol. Size (bp)/Varieties	CCRP 3	CCRP 10	CCRP 11	CCRP 12	CCRP 13	CCRP 14	CCRP 15
1500							
1400							
1250							
1100							
900							
650							
550							

Colour code for sharing of bands among varieties

1	2	3	4	5	6	7

Fig. 5 Colour chart developed using Oligo ISSR 07



M- Molecular weight marker (3kb), B- Blank, 1-CCRP 3, 2- CCRP 10, 3-CCRP 11, 4- CCRP 12, 5- CCRP 13, 6- CCRP 14, 7- CCRP 15

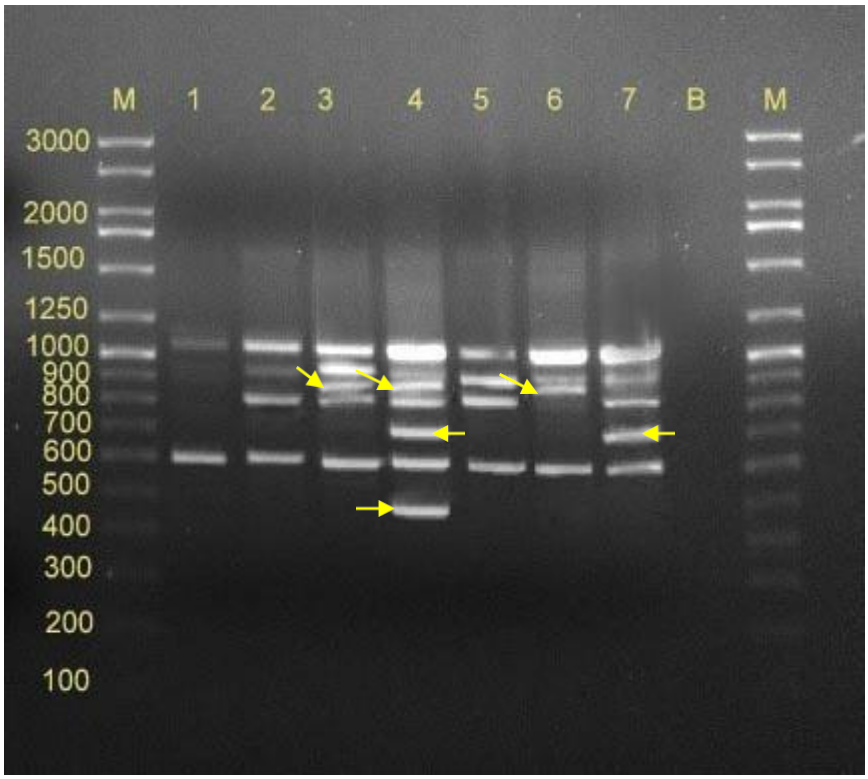
Plate 8. Amplification pattern generated with primer Oligo ISSR 08

	1	2	3	4	5	6	7
Mol. Size (bp)/Varieties	CCRP 3	CCRP 10	CCRP 11	CCRP 12	CCRP 13	CCRP 14	CCRP 15
800							
600							
420							
290							

Colour code for sharing of bands among varieties

1	2	3	4	5	6	7

Fig. 6 Colour chart developed using Oligo ISSR 08



M- Molecular weight marker (3kb), B- Blank, 1-CCRP 3, 2- CCRP 10, 3-CCRP 11, 4- CCRP 12, 5- CCRP 13, 6- CCRP 14, 7- CCRP 15

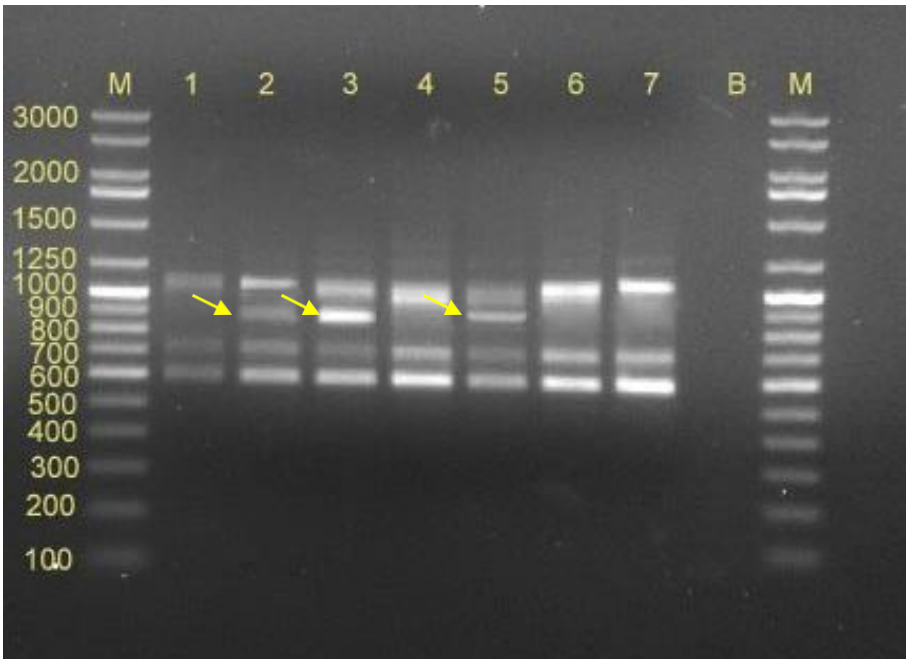
Plate 9. Amplification pattern generated with primer Oligo ISSR 3

	1	2	3	4	5	6	7
Mol. Size (bp)/Varieties	CCRP 3	CCRP 10	CCRP 11	CCRP 12	CCRP 13	CCRP 14	CCRP 15
1100							
900							
850							
800							
700							
600							
400							

Colour code for sharing of bands among varieties

1	2	3	4	5	6	7

Fig. 7 Colour chart developed using Oligo ISSR 3



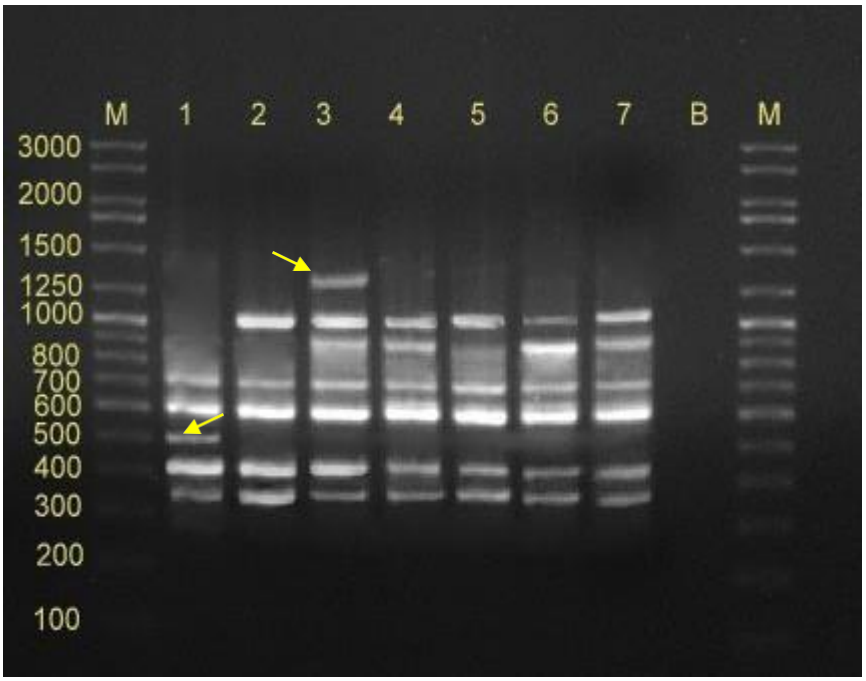
M- Molecular weight marker (3kb), B- Blank, 1-CCRP 3, 2- CCRP 10, 3-CCRP 11, 4- CCRP 12, 5- CCRP 13, 6- CCRP 14, 7- CCRP 15

Plate 10. Amplification pattern generated with primer ISSR 7

	1	2	3	4	5	6	7
Mol. Size (bp)/Varieties	CCRP 3	CCRP 10	CCRP 11	CCRP 12	CCRP 13	CCRP 14	CCRP 15
1100							
900							
700							
600							

Colour code for sharing of bands among varieties

Fig. 8 Colour chart developed using ISSR 7



M- Molecular weight marker (3kb), B- Blank, 1-CCRP 3, 2- CCRP 10, 3-CCRP 11, 4- CCRP 12, 5- CCRP 13, 6- CCRP 14, 7- CCRP 15

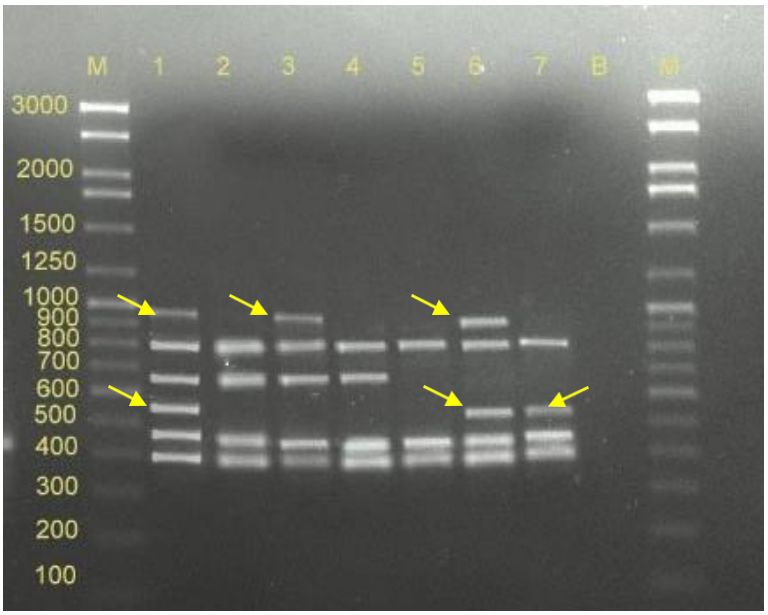
Plate 11. Amplification pattern generated with primer UBC 810

	1	2	3	4	5	6	7
Mol. Size (bp)/Varieties	CCR P3	CCRP 10	CCRP 11	CCRP 12	CCRP 13	CCRP 14	CCRP 15
1400							
1000							
900							
700							
600							
500							
400							
350							

Colour code for sharing of bands among varieties

1	2	3	4	5	6	7

Fig. 9 Colour chart developed using USB 810



M- Molecular weight marker (3kb), B- Blank, 1-CCRP 3, 2- CCRP 10, 3-CCRP 11, 4- CCRP 12, 5- CCRP 13, 6- CCRP 14, 7- CCRP 15

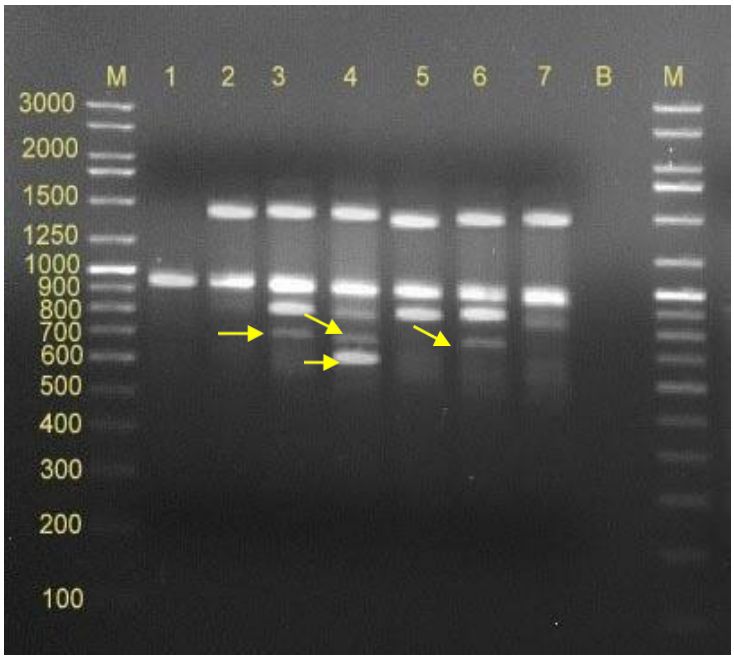
Plate 12. Amplification pattern generated with primer UBC811

	1	2	3	4	5	6	7
Mol. Size (bp)/Varieties	CCRP 3	CCRP 10	CCRP 11	CCRP 12	CCRP 13	CCRP 14	CCRP 15
950							
800							
650							
550							
450							
400							

Colour code for sharing of bands among varieties

1	2	3	4	5	6	7

Fig. 10 Colour chart developed using UBC811



M- Molecular weight marker (3kb), B- Blank, 1-CCRP 3, 2- CCRP 10, 3-CCRP 11, 4- CCRP 12, 5- CCRP 13, 6- CCRP 14, 7- CCRP 15

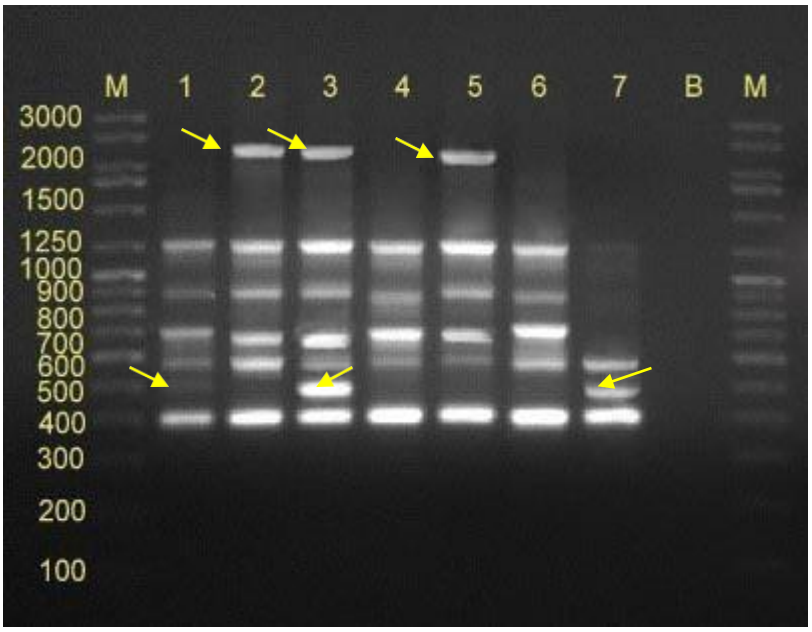
Plate 13. Amplification pattern generated with primer UBC815

	1	2	3	4	5	6	7
Mol. Size (bp)/Varieties	CCRP 3	CCRP 10	CCRP 11	CCRP 12	CCRP 13	CCRP 14	CCRP 15
1500							
1000							
800							
700							
650							

Colour code for sharing of bands among varieties

1	2	3	4	5	6	7

Fig. 11 Colour chart developed using UBC815



M- Molecular weight marker (3kb), B- Blank, 1-CCRP 3, 2- CCRP 10, 3-CCRP 11, 4- CCRP 12, 5- CCRP 13, 6- CCRP 14, 7- CCRP 15

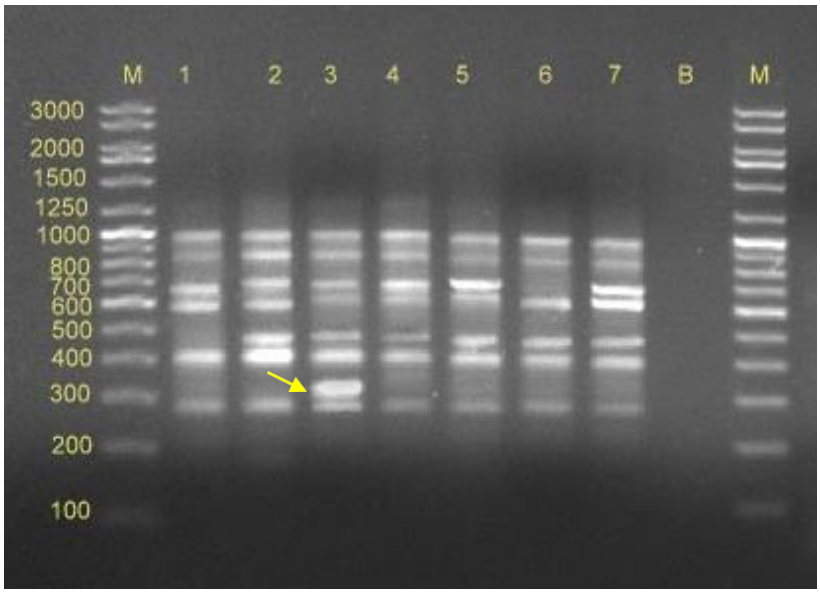
Plate 14. Amplification pattern generated with primer UBC 818

	1	2	3	4	5	6	7
Mol. Size (bp)/Varieties	CCRP 3	CCRP 10	CCRP 11	CCRP 12	CCRP 13	CCRP 14	CCRP 15
2500							
1250							
900							
700							
550							
500							
400							

Colour code for sharing of bands among varieties

1	2	3	4	5	6	7

Fig. 12 Colour chart developed using UBC 818



M- Molecular weight marker (3kb), B- Blank, 1-CCRP 3, 2- CCRP 10, 3-CCRP 11, 4- CCRP 12, 5- CCRP 13, 6- CCRP 14, 7- CCRP 15

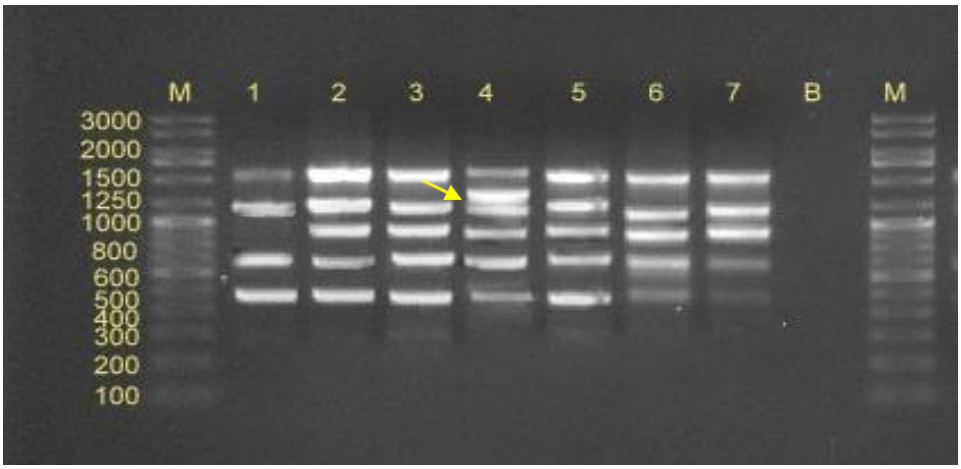
Plate 15. Amplification pattern generated with primer UBC 826

	1	2	3	4	5	6	7
Mol. Size (bp)/Varieties	CCRP 3	CCRP 10	CCRP 11	CCRP 12	CCRP 13	CCRP 14	CCRP 15
1000							
850							
650							
600							
450							
400							
310							
290							

Colour code for sharing of bands among varieties

1	2	3	4	5	6	7

Fig. 13 Colour chart developed using UBC 826



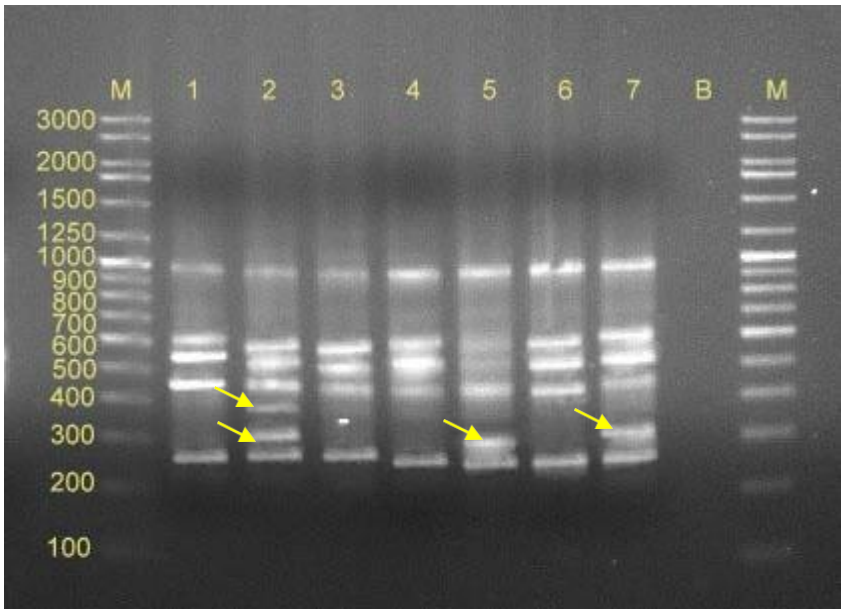
M- Molecular weight marker (3kb), B- Blank, 1-CCRP 3, 2- CCRP 10, 3-CCRP 11, 4- CCRP 12, 5- CCRP 13, 6- CCRP 14, 7- CCRP 15

Plate 16. Amplification pattern generated with primer UBC827

	1	2	3	4	5	6	7
Mol. Size (bp)/Varieties	CCRP 3	CCRP 10	CCRP 11	CCRP 12	CCRP 13	CCRP 14	CCRP 15
1500							
1400							
1250							
900							
700							
650							

Colour code for sharing of bands among varieties

Fig. 14 Colour chart developed using UBC827



M- Molecular weight marker (3kb), B- Blank, 1-CCRP 3, 2- CCRP 10, 3-CCRP 11, 4- CCRP 12, 5- CCRP 13, 6- CCRP 14, 7- CCRP 15

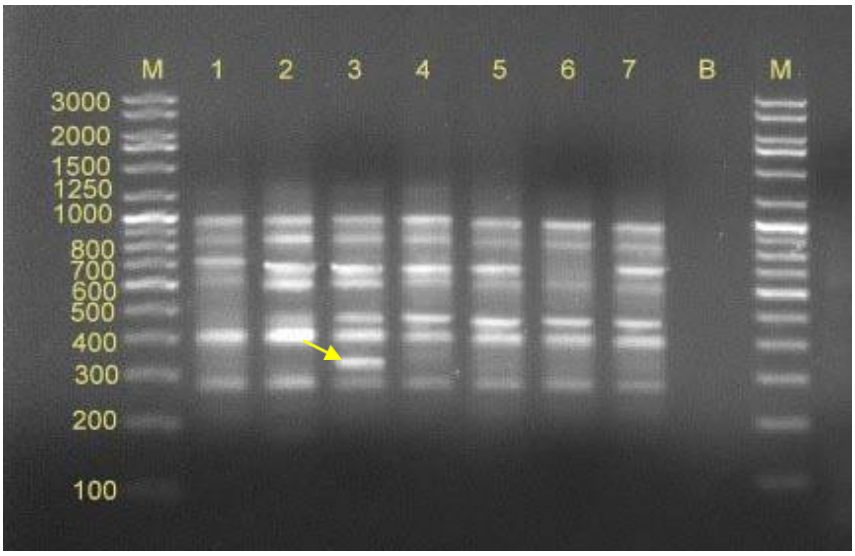
Plate 17. Amplification pattern generated with primer UBC 835

	1	2	3	4	5	6	7
Mol. Size (bp)/Varieties	CCRP 3	CCRP 10	CCRP 11	CCRP 12	CCRP 13	CCRP 14	CCRP 15
1000							
600							
550							
450							
400							
350							
250							

Colour code for sharing of bands among varieties

1	2	3	4	5	6	7

Fig. 15 Colour chart developed using UBC 835



M- Molecular weight marker (3kb), B- Blank, 1-CCRP 3, 2- CCRP 10, 3-CCRP 11, 4- CCRP 12, 5- CCRP 13, 6- CCRP 14, 7- CCRP 15

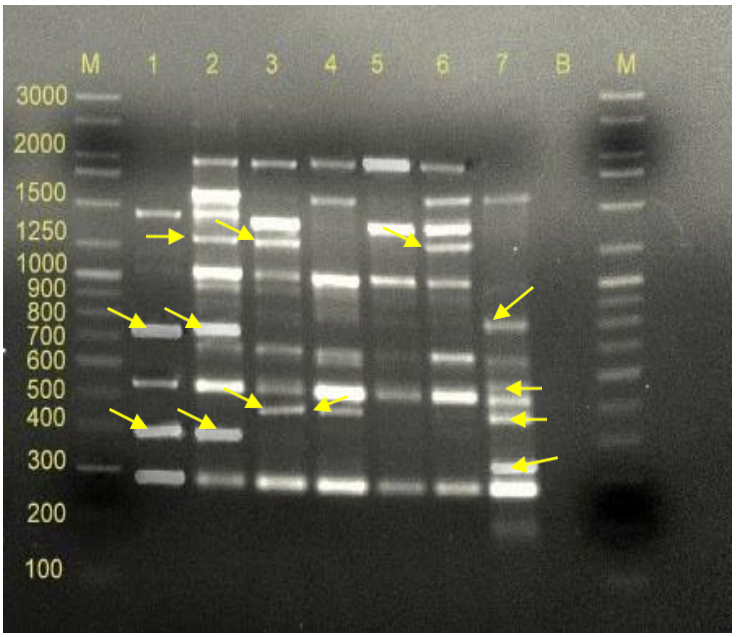
Plate 18. Amplification pattern generated with primer UBC 841

	1	2	3	4	5	6	7
Mol. Size (bp)/Varieties	CCRP 3	CCRP 10	CCRP 11	CCRP 12	CCRP 13	CCRP 14	CCRP 15
1000							
850							
700							
600							
450							
400							
300							
290							

Colour code for sharing of bands among varieties

1	2	3	4	5	6	7

Fig. 16 Colour chart developed using UBC 841



M- Molecular weight marker (3kb), B- Blank, 1-CCRP 3, 2- CCRP 10, 3-CCRP 11, 4- CCRP 12, 5- CCRP 13, 6- CCRP 14, 7- CCRP 15

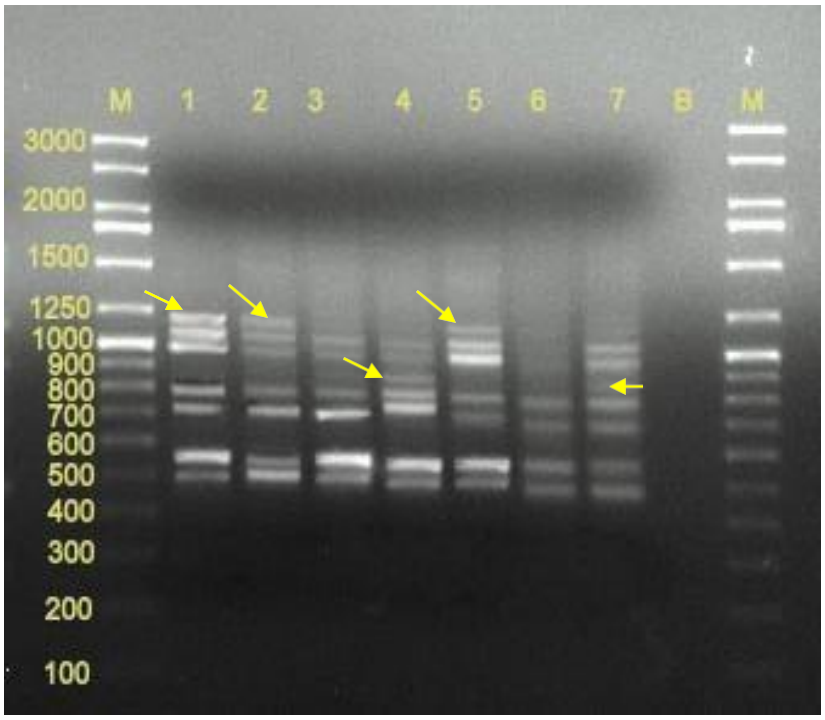
Plate 19. Amplification pattern generated with primer UBC 844

	1	2	3	4	5	6	7
Mol. Size (bp)/Varieties	CCRP 3	CCRP 10	CCRP 11	CCRP 12	CCRP 13	CCRP 14	CCRP 15
2000							
1550							
1480							
1250							
1000							
950							
800							
750							
650							
590							
550							
500							
400							
300							
290							

Colour code for sharing of bands among varieties

1	2	3	4	5	6	7

Fig. 17. Colour chart developed using UBC 844



M- Molecular weight marker (3kb), B- Blank, 1-CCRP 3, 2- CCRP 10, 3-CCRP 11, 4- CCRP 12, 5- CCRP 13, 6- CCRP 14, 7- CCRP 15

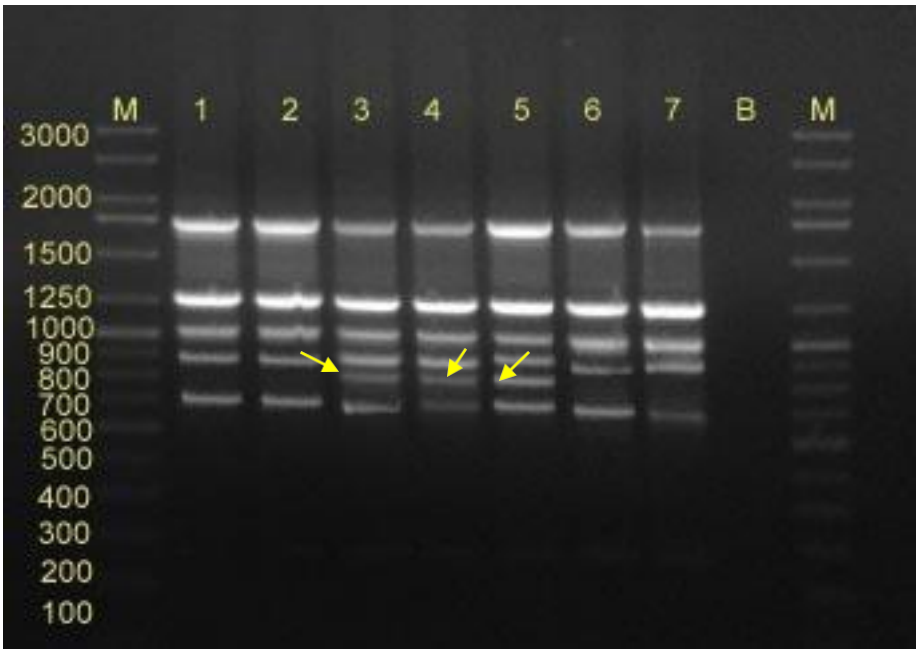
Plate 20. Amplification pattern generated with primer UBC 846

	1	2	3	4	5	6	7
Mol. Size (bp)/Varieties	CCRP 3	CCRP 10	CCRP 11	CCRP 12	CCRP 13	CCRP 14	CCRP 15
1300							
1100							
1000							
850							
800							
700							
550							
490							

Colour code for sharing of bands among varieties

1	2	3	4	5	6	7

Fig. 18 Colour chart developed using UBC 846



M- Molecular weight marker (3kb), B- Blank, 1-CCRP 3, 2- CCRP 10, 3-CCRP 11, 4- CCRP 12, 5- CCRP 13, 6- CCRP 14, 7- CCRP 15

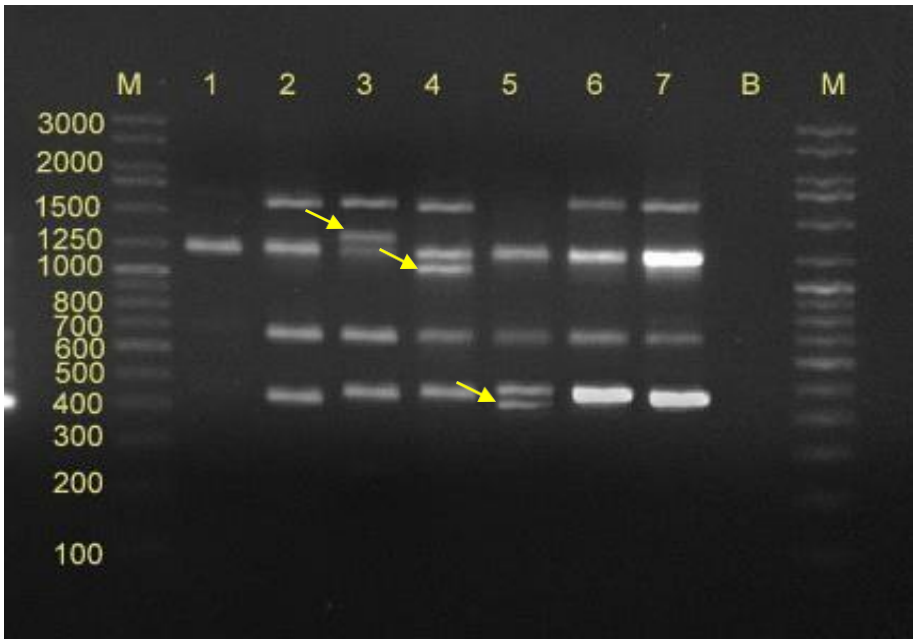
Plate 21. Amplification pattern generated with primer UBC 847

	1	2	3	4	5	6	7
Mol. Size (bp)/Varieties	CCRP 3	CCRP 10	CCRP 11	CCRP 12	CCRP 13	CCRP 14	CCRP 15
1850							
1250							
1000							
900							
800							
700							

Colour code for sharing of bands among varieties

1	2	3	4	5	6	7

Fig. 19 Colour chart developed using UBC 847



M- Molecular weight marker (3kb), B- Blank, 1-CCRP 3, 2- CCRP 10, 3-CCRP 11, 4- CCRP 12, 5- CCRP 13, 6- CCRP 14, 7- CCRP 15

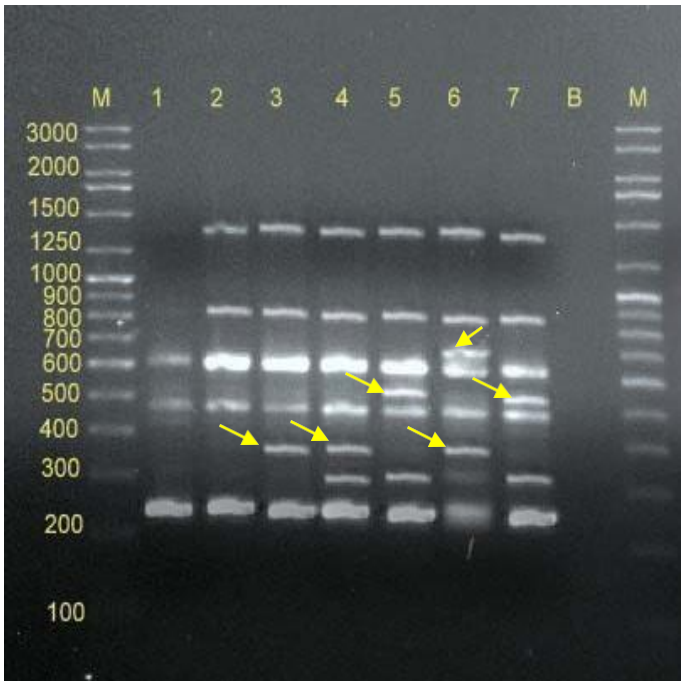
Plate 22. Amplification pattern generated with primer UBC 854

	1	2	3	4	5	6	7
Mol. Size (bp)/Varieties	CCRP 3	CCRP 10	CCRP 11	CCRP 12	CCRP 13	CCRP 14	CCRP 15
1700							
1300							
1250							
1000							
900							
450							
400							

Colour code for sharing of bands among varieties

1	2	3	4	5	6	7

Fig. 20 Colour chart developed using UBC 854



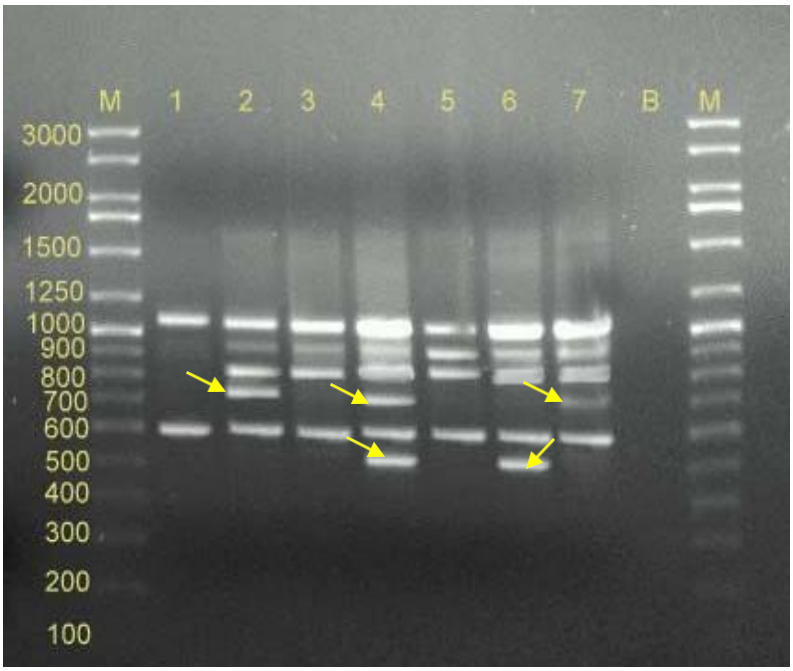
M- Molecular weight marker (3kb), B- Blank, 1-CCRP 3, 2- CCRP 10, 3-CCRP 11, 4- CCRP 12, 5- CCRP 13, 6- CCRP 14, 7- CCRP 15

Plate 23. Amplification pattern generated with primer UBC 855

	1	2	3	4	5	6	7
Mol. Size (bp)/Varieties	CCRP 3	CCRP 10	CCRP 11	CCRP 12	CCRP 13	CCRP 14	CCRP 15
1400							
800							
650							
600							
500							
490							
350							
300							
250							

Colour code for sharing of bands among varieties

Fig. 21. Colour chart developed using UBC 855



M- Molecular weight marker (3kb), B- Blank, 1-CCRP 3, 2- CCRP 10, 3-CCRP 11, 4- CCRP 12, 5- CCRP 13, 6- CCRP 14, 7- CCRP 15

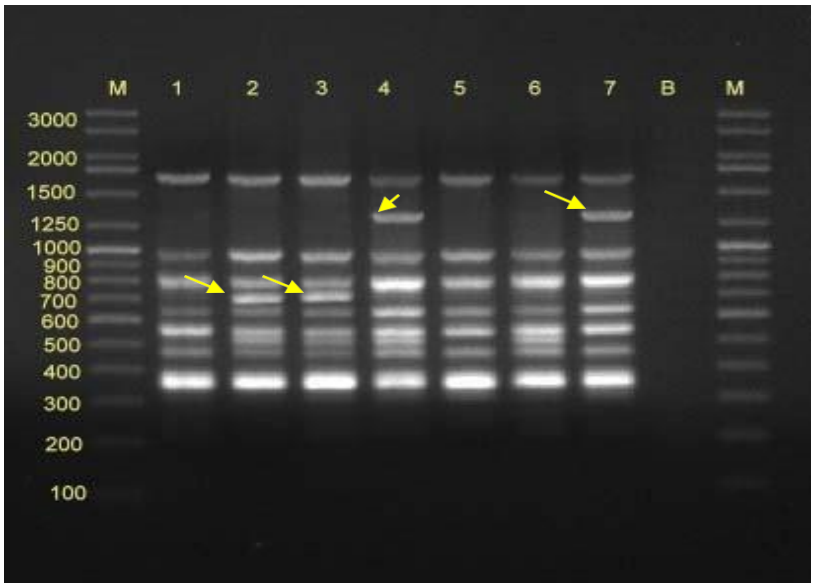
Plate 24. Amplification pattern generated with primer UBC 857

	1	2	3	4	5	6	7
Mol. Size (bp)/Varieties	CCRP 3	CCRP 10	CCRP 11	CCRP 12	CCRP 13	CCRP 14	CCRP 15
1100							
900							
800							
750							
700							
600							
500							

Colour code for sharing of bands among varieties

1	2	3	4	5	6	7

Fig. 22. Colour chart developed using UBC 857



M- Molecular weight marker (3kb), B- Blank, 1-CCRP 3, 2- CCRP 10, 3-CCRP 11, 4- CCRP 12, 5- CCRP 13, 6- CCRP 14, 7- CCRP 15

Plate 25. Amplification pattern generated with primer UBC 866

	1	2	3	4	5	6	7
Mol. Size (bp)/Varieties	CCR P 3	CCRP 10	CCRP 11	CCRP 12	CCRP 13	CCRP 14	CCRP 15
1600							
1300							
1000							
800							
700							
650							
550							
500							
450							
350							

Colour code for sharing of bands among varieties

Fig. 23. Colour chart developed using UBC 866

4. 2.2 Simple Sequence Repeat (SSR) analysis

SSR markers are developed by using sequence informations (Zalapa *et al*., 2012). SSR markers are co-dominant, multi-allelic and relatively abundant on the genome. (Varshney *et al*., 2008). Hence these markers are considered as the most important marker in plant genetics and breeding programs (Heinz and Tew 1987; Haq *et al*. 2016; Ali *et al*., 2017). Simple sequence repeat markers are mainly used in quantitative trait loci (QTL) mapping, development of linkage maps, genetic diversity studies, marker- assisted selection, cultivar fingerprinting, gene flow, parentage analysis and evolutionary studies (Cavagnaro *et al*., 2010; Zhu *et al*., 2011; Zalapa *et al*., 2012). SSR s have shown high level of molecular genetic diversity in a nucleus of cocoa genotype (Lins *et al*., 2016).

4.2.2.1 Primer screening for SSR assay

Based on the amplification pattern generated by 30 SSRs, 17 primers were selected for developing fingerprint (Table 6) The selected primer and their annealing temperature are detailed in Table 7.

SSR markers broadly used in marker analysis due to their high polymorphic information content, nature of high transferability, easy of genotyping through PCR, allelic nature and high discriminating capacity (Susilo *et al*., 2011; Bohra *et al*., 2017). Rafalski *et al.* (1996) reported that, only a small quantity of DNA is required for marker analysis, if SSR primers are used.

Table 6. Details of amplification pattern obtained with 30 SSR primers

SI. No.	Primers	Amplification pattern			
		No. of amplicon	Types of amplicons		Remarks
			Distinct	Faint	
1	mTcCIR8	2	2	0	Selected
2	mTcCIR11	4	2	0	Selected
3	mTcCIR12	2	1	1	Selected
4	mTcCIR18	3	3	0	Selected
5	mTcCIR24	2	2	0	Selected
6	mTcCIR33	2	2	0	Selected
7	mTcCIR40	3	3	0	Selected
8	mTcCIR42	2	2	0	Selected
9	SHRSTc49	2	1	1	Selected
10	SHRSTc51	1	1	0	Selected
11	SHRSTc64	2	2	0	Selected
12	mTcCIR7	0	0	0	Not selected
13	mTcCIR10	3	3	0	Selected
14	mTcCIR15	0	0	0	Not selected
15	mTcCIR22	2	2	0	Selected
16	mTcCIR25	0	0	0	Not selected
17	mTcCIR26	0	0	0	Not selected
18	mTcCIR37	2	2	0	Selected
19	mTcCIR60	2	2	0	Selected
20	mTcCIR102	0	0	0	Not selected
21	mTcCIR121	0	0	0	Not selected
22	mTcCIR121	0	0	0	Not selected
23	SHRSTc52	0	0	0	Not selected
24	SHRSTc53	2	1	1	Selected

Table 6. contd.

SI. No.	Primers	Amplification pattern			Remarks
		No. of amplicon	Types of amplicons		
			Distinct	Faint	
25	SSRKAU15	1	1	0	Selected
26	SSRKAU19	0	0	0	Not selected
27	SSRKAU20	0	0	0	Not selected
28	SSRKAU21	0	0	0	Not selected
29	SSRKAU22	0	0	0	Not selected
30	SSRKAU24	0	0	0	Not selected

Table 7. Details of amplified SSR primers

SI. No	Primers	Nucleotide Sequence	Annealing temperature (^{o}C)
1	mTcCIR8	F 5'-CTAGTTTCCCATTTACCA-3' R 5'-TCCTCAGCATTTTCTTTC-3'	53.7
2	mTcCIR10	F 5'-CCGAATTGACAGATGGCCTA-3' R 5'-CCCAAGCAAGCCTCATACTC-3'	53
3	mTcCIR11	F 5'-TTTGGTGATTATTAGCAG-3' R 5'-GATCGATTTGATGTGAG-3'	53.5
4	mTcCIR12	F 5'-CAGCCGCCTCTTGTTAG-3' R 5'-TATTTGGGATTCTTGATG-3'	53
5	mTcCIR18	F 5'-GATAGCTAAGGGGATTGAGGA-3' R5'-GGTAATTCAATCATTTGAGGATA-3'	58
6	mTcCIR22	F 5'-ATTCTCGCAAAAACTTAG-3' R 5'-GATGGAAGGAGTGTAAATAG-3'	55
7	mTcCIR24	F 5'-TTTGGGGTGATTTCTTCTGA-3' R 5'-TCTGTCTCGTCTTTTGGTGA-3'	53
8	mTcCIR33	F 5'-TGGGTTGAAGATTTGGT-3' R 5'-CAACAATGAAAATAGGCA-3'	55
9	mTcCIR37	F 5'-CTGGGTGCTGATAGATAA-3 R 5'-AATACCCTCCACACAAAT-3'	58
10	mTcCIR40	F 5'-AATCCGACAGTCTTTAATC-3' R 5'-CCTAGGCCAGAGAATTGA-3'	60
11	mTcCIR42	F 5'-TTGCTGAAGTATCTTTTGAC-3' R 5'-GCTCCACCCCTATTTG-3'	63.7
12	mTcCIR121	F 5'-CATGTGCATTTAGGTGTC-3' R 5'TCTGGCTTCTTAGTGATAC-3'	53.1
13	SHRSTc49	F5'-ATCGCAGCAAACTCCCTCTC-3' R5'-TTCTCTTCCCACCAAGTCCC-3'	50
14	SHRSTc 51	F 5'-CTGTTTTTGCCTCCCTTGTTCT-3' R 5'-ATTGCTGGTTGTTCTCCATCCT-3'	51
15	SHRSTc53	F 5'-TTCCCTTTCTTTCTCTCTCTC-3' R 5'-AGTCGTTGCTACTGCTGG-3'	52
16	SHRSTc564	F 5'-TCCTACATTCCTGCACCC-3' R 5'-TCGAGAGAAAAGCTCTTACACT-3'	57
17	SSRKAU15	F 5'-TGTTGCTCGAACTCTCCAAA-3' R 5'- CATAGGAGAGGTAACCCGCA-3'	63.7

4.2.2.2 DNA fingerprinting with selected SSR primers

Out of thirty SSR primers, only 17 primers were found amplified in seven genotypes (Table 7). The amplification pattern obtained with each primers was observed in detail to identify the polymorphic bands. The amplified SSR primers are detailed below.

4.2.2.2.1 mTcCIR 8

Amplification of DNA samples with primer mTcCIR 8 yielded polymorphic bands (Plate 26a). Molecular size of the bands ranged from 290 bp to 300 bp. A unique band was observed in the CCRP 11 hybrid at 300 bp. Hence this primer can be selected for final fingerprint of CCRP 11(Fig. 24a).

4.2.2.2.2 mTcCIR 10

Amplification pattern in seven genotypes through PCR with SSR primer mTcCIR 10 was performed (Plate 26 b). The amplicons obtained were polymorphic in CCRP 11 at 310 bp and CCRP 13 at 340 bp (Fig. 24b). Amplicon of molecular size 350 bp was shared by the remaining varieties. The amplicons generated at 310 and 350 bp can be further utilized for DNA fingerprinting.

4.2.2.2.3 mTcCIR 11

SSR analysis of the DNA samples using the primer mTcCIR 11 generated polymorphic amplicons (Plate 27a). The bands differed from 290 bp to 310 bp molecular size (Fig. 25a). At 290 bp in CCRP 11 and CCRP 13 at 310 bp unique polymorphic bands were generated. This information can help to generate final DNA fingerprint by using mTcCIR 11.

4.2.2.2.4 mTcCIR 12

Amplification pattern of seven genotypes with SSR primer mTcCIR 12 was analyzed (Plate 27b). Molecular size of the bands ranged from 250 bp to 270 bp. The amplicons generated with this primer established polymorphism. The amplicon obtained was at 270 bp length in CCRP 13. Hence, this primer is desirable for final DNA fingerprinting of CCRP 13 (Fig 25b).

4.2.2.2.5 mTcCIR 18

Amplification pattern of seven genotypes with primer mTcCIR 18 was analyzed (Plate 28a). Molecular size of amplicons varied from 345 bp to 355 bp. The amplicons obtained were polymorphic bands at 345 bp in CCRP 12 and 355 bp in CCRP15 (Fig. 26a). This particular amplicon information can be included in final DNA fingerprint.

4. 2.2.2.6 mTcCIR 22

Amplification of DNA samples with the primer mTcCIR 22 yielded bands of four different molecular size among the varieties (Plate 28b). A unique polymorphic band was observed in CCRP 13 at 200 bp. This can be used as a specific fingerprint for the variety (Fig. 26b). Therefore, such useful amplicon information will be utilized for development of final DNA fingerprint.

4. 2.2.2.7 mTcCIR 24

SSR analysis of the DNA samples using the primer mTcCIR 24 generated amplicons of two different lengths 150 bp to 200 bp (Plate 29a). The polymorphic amplicon in the variety CCRP 13 was at 150 bp and could be considered for the final DNA fingerprint (Fig. 27a).

4. 2.2.2.8 mTcCIR 33

Amplification pattern of seven genotypes with primer mTcCIR 33 was performed (Plate 29b). The amplicons obtained with this primer created polymorphic bands by generating a band in variety CCRP 13 at 300 bp (Fig. 27b). The amplicon obtained at 300 bp will be utilized for DNA fingerprinting.

4. 2.2.2.9 mTcCIR 37

The primer mTcCIR 37 had generated, amplicons at 150 bp and 200 bp (Plate 30a)**.** Polymorphic band was observed at 200 bp in CCRP 14 and in the other genotypes, amplicons were observed at 150 bp (Fig. 28a).

4. 2.2.2.10 mTcCIR 40

Three amplicons were generated by the primer mTcCIR 40 (Plate 30b). A polymorphic band was obtained at 200 bp in CCRP 12. A band at 250 bp was observed in the hybrid CCRP 3 (Fig. 28b). The other band obtained (240 bp) was shared between

five varieties. Useful amplicon patterns can be incorporated into final DNA fingerprint.

4. 2.2.2.11 mTcCIR 42

Amplification of cocoa genotypes with the primer mTcCIR42 yielded bands at 210 bp and 250 bp (Plate 31a). Polymorphic band was found in the variety CCRP 12 at 210 bp. Bands observed at 250 bp was shared by other six varieties. The primer can be selected for final DNA fingerprinting of CCRP 12 (Fig. 29a).

4. 2.2.2.12 mTcCIR 121

Amplification of cocoa genotypes with the primer SHRSTc 64 had yielded polymorphic bands (Plate 29b). A unique polymorphic band was observed at 140 bp in the variety CCRP 11. Another band at 150 bp was common in other genotypes (Fig. 29b). Therefore, the amplicon generated at 140 bp will be included for final DNA fingerprinting of CCRP 11.

4. 2.2. 2.13 SHRSTc 49

SSR primer SHRSTc 49 had generated (Plate 32a) polymorphic bands in CCRP 3, CCRP 13 and CCRP 15 at 195 bp. Other varieties consisted of a band at 300 bp (Fig 30a). Primer SHRSTc 49 can be selected for identification of CCRP 3, CCRP 13 and CCRP 15.

4. 2.2.2.14 SHRSTc 51

SHRSTc 51 primer were used in developing amplification pattern (Plate 32b). No polymorphism was observed. Hence the primer SHRSTc 51 cannot be selected for further analysis (Fig 30b). Molecular size of the amplicon was 200 bp.

4.2.2.2.15 SHRSTc 53

SHRSTc 53 primer was amplified in all seven varieties. Amplicon analysis produced unique polymorphic bands (Plate 33a). Bands were present in CCRP 10 at 230 bp and in CCRP 13 at 180 bp. Remaining hybrids constituted bands at 220 bp. Thus, primer can be selected for final DNA fingerprinting (Fig31a).

4.2.2.2.16 SHRSTc 64

Amplification pattern developed using SHRSTc 64 primer produced

polymorphism (Plate 33b). A distinct polymorphic band was present at 250 bp in hybrid CCRP14. An amplicon at 300 bp was present in other varieties. Hence, this SSR primer was selected for identification of CCRP 14 (Fig 31b).

4.2.2.2.17 SSRKAU 15

SSR analysis of the DNA samples using the primer SSRKAU 15 generated amplicons at single length (Plate 34). The obtained amplicons were found to be shared in all seven varieties and cannot be selected for the final DNA fingerprint (Fig. 32).

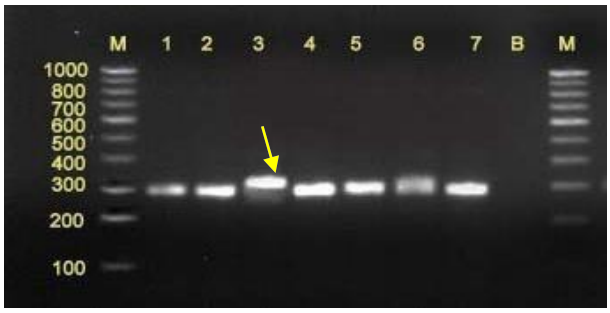
Thirty SSR primers were used in this study. Out of this, 17 primers produced amplicons and 15 primers gave polymorphic band which can be used to develop final DNA finger prints. Zhang *et al*., (2009) carried out molecular characterization of cacao genotypes by using 15 SSR primers, which provided high resolution with accessions. In Chinese sugarcane, genetic diversity analysis and molecular classification was done by using SSR markers (Ali *et al*., 2017). Bohra *et al*. (2017) screened pigeonpea genotypes by using 421 hyper variable (SSR) markers. Eleven selected SSR primers were used in DNA fingerprinting of promising cocoa (*Theobroma cocoa L.*) varieties (Sujith, 2016).

Primers which were selected for the development of final fingerprint included those that yielded unique polymorphic band. Primer mTcCIR 10, mTcCIR 11, mTcCIR 18 and mTcCIR40 generated two unique bands in different size in different varieties. Remaining primers produced only single unique band. The unique band developed by primermTcCIR 10in CCRP 11 at 310 bp and CCRP 13 at 340 bp. Primer mTcCIR 11 produced unique amplicon in CCRP11 at 290 bp and CCRP 13 at 310 bp. Other unique amplicons were observed in CCRP 12 at 345 bp and CCRP 15 at 355 bp (mTcCIR 18), CCRP 3 at 250 bp and CCRP 12 at 200 bp (mTcCIR 40). Hybrid CCRP 13 registered the highest number of unique band (seven) followed by CCRP 11(four).

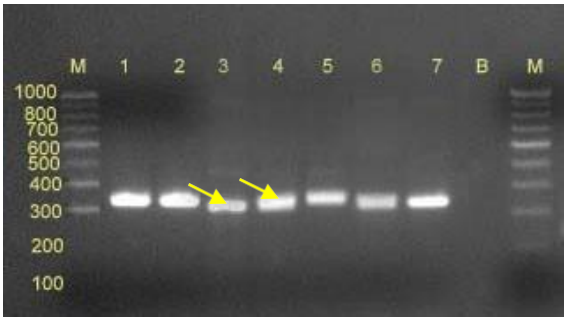
Many scientists used SSR primers in cocoa and reported the potentiality of SSR primers (Cryer *et al*., 2006; Aikpokpodion *et al.*, 2009; Zhang *et al*., 2009; Lins *et al*., 2016). Zhang *et al*. (2006) utilized the 15 SSR primers for cacao genotype identification, and consistent clone identification was obtained by less genotyping error. Opoku *et al*. (2008) reported 17 SSR primers for genetic diversity identification and

population differentiation in cocoa germplasm collection from Ghana.

SSR primers were used not only in cocoa also in various other crops. Seventy five SSR primers were used for DNA fingerprinting of rice, out of these, 71 primers were amplified. Zhang *et al.* (2015) had done fingerprinting on jute (58 accession) by using 28 SSR primer pairs and these primers produced total 134 polymorphic bands. It indicated that SSR are valuable molecular marker type for DNA fingerprinting and similarly genetic diversity analysis. Marjan *et al*. (2019) screened 50 SSR primers and selected eleven SSR primers for developing fingerprints of eight jack genotypes based on amplification pattern. Eleven SSR primers generated 28 useful distinctive polymorphic bands. Sochor *et al*. (2019) reported 23 SSR markers for phenotypic evaluation of lettuce. Out of 23 primers, 20 primers exhibited polymorphism. Simple Sequence Repeats (SSRs) have been functionally used in various crops for identification and confirmation of duplicate accessions.



a. Amplification with primer mTcCIR8



b. Amplification with primer mTcCIR10

M- Molecular weight marker (3kb), B- Blank, 1-CCRP 3, 2- CCRP 10, 3- CCRP 11, 4- CCRP 12,5- CCRP 13, 6- CCRP 14, 7- CCRP 15

Plate 26. Amplification pattern generated with primer mTcCIR8 and mTcCIR10

	1	2	3	4	5	6	7
Mol. Size (bp)/Varieties	CCRP 3	CCRP 10	CCRP 11	CCRP 12	CCRP 13	CCRP 14	CCRP 15
300							
290							

a. Colour chart - mTcCIR 8

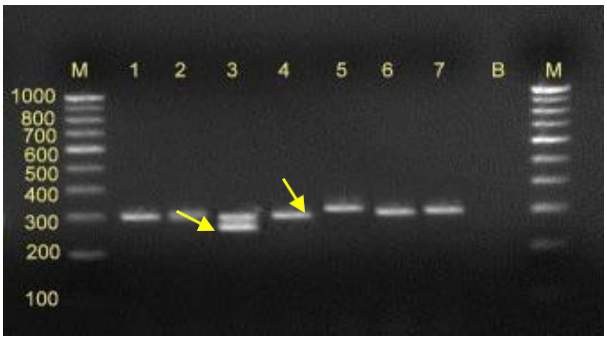
	1	2	3	4	5	6	7
Mol. Size (bp)/Varieties	CCRP 3	CCRP 10	CCRP 11	CCRP 12	CCRP 13	CCRP 14	CCRP 15
340							
330							
310							

b. Colour chart - mTcCIR 10

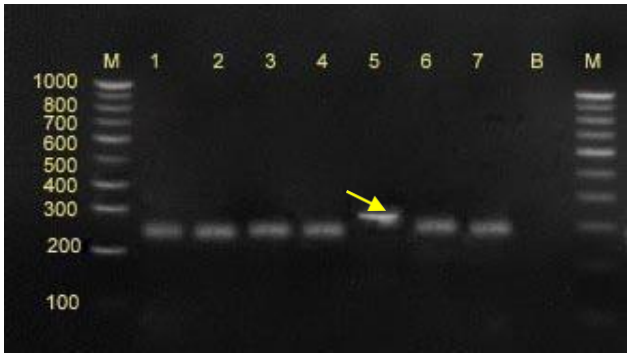
Colour code for sharing of bands among varieties

1	2	3	4	5	6	7

Fig. 24 Colour chart developed using mTcCIR 8 and mTcCIR 10



a. Amplification with primer mTcCIR11



b. Amplification with primer mTcCIR 12

M- Molecular weight marker (3kb), B- Blank, 1-CCRP 3, 2- CCRP 10, 3- CCRP 11, 4- CCRP 12,5- CCRP 13, 6- CCRP 14, 7- CCRP 15

Plate 27. Amplification pattern generated with primer mTcCIR 11 and mTcCIR 12

	1	2	3	4	5	6	7
Mol. Size (bp)/Varieties	CCRP 3	CCRP 10	CCRP 11	CCRP 12	CCRP 13	CCRP 14	CCRP 15
310							
300							
290							

a. Colour chart - mTcCIR11

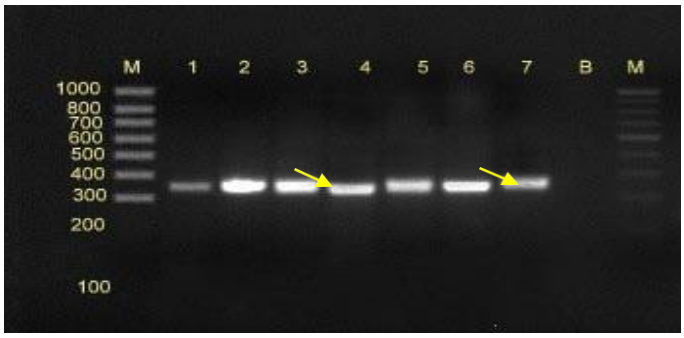
	1	2	3	4	5	6	7
Mol. Size (bp)/Varieties	CCRP 3	CCRP 10	CCRP 11	CCRP 12	CCRP 13	CCRP 14	CCRP 15
270							
250							

b. Colour chart mTcCIR 12

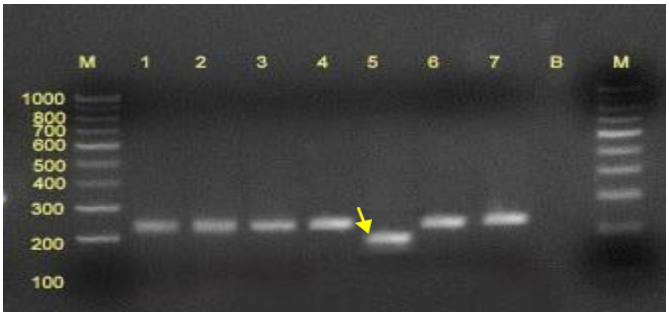
Colour code for sharing of bands among varieties

1	2	3	4	5	6	7

Fig. 25 Colour chart developed using mTcCIR 11 and mTcCIR 12



a. Amplification with primer mTcCIR18



c. Amplification with primer mTcCIR22

M- Molecular weight marker (3kb), B- Blank, 1-CCRP 3, 2- CCRP 10, 3- CCRP 11, 4- CCRP12, 5- CCRP 13, 6- CCRP 14, 7- CCRP 15

Plate 28. Amplification pattern generated with primer mTcCIR 18 and mTcCIR 22

	1	2	3	4	5	6	7
Mol. Size (bp)/Varieties	CCRP 3	CCRP 10	CCRP 11	CCRP 12	CCRP 13	CCRP 14	CCRP 15
355							
350							
345							

a. Colour chart mTcCIR18

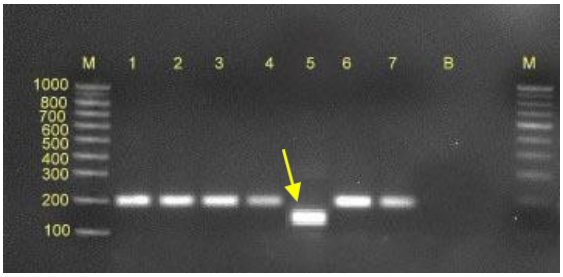
	1	2	3	4	5	6	7
Mol. Size (bp)/Varieties	CCRP 3	CCRP 10	CCRP 11	CCRP 12	CCRP 13	CCRP 14	CCRP 15
220							
200							

b. Colour chart mTcCIR 22

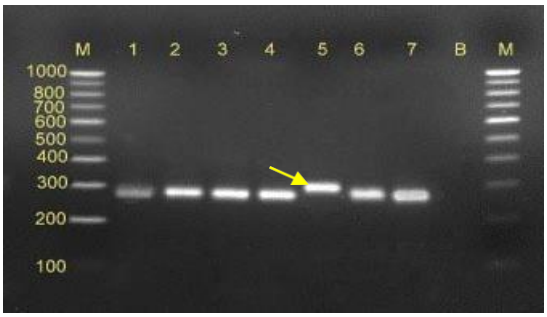
Colour code for sharing of bands among varieties

1	2	3	4	5	6	7

Fig. 26 Colour chart developed using mTcCIR 18 and mTcC 22



a. Amplification with primer mTcCIR24



b. Amplification with primer mTcCIR33

M- Molecular weight marker (3kb), B- Blank, 1-CCRP 3, 2- CCRP 10, 3- CCRP 11, 4- CCRP 12, 5- CCRP 13, 6- CCRP 14, 7- CCRP 15

Plate 29. Amplification pattern generated with primer mTcCIR 24 and mTcCIR 33

	1	2	3	4	5	6	7
Mol. Size (bp)/Varieties	CCRP 3	CCRP 10	CCRP 11	CCRP 12	CCRP 13	CCRP 14	CCRP 15
200							
150							

a. Colour chart mTcCIR24

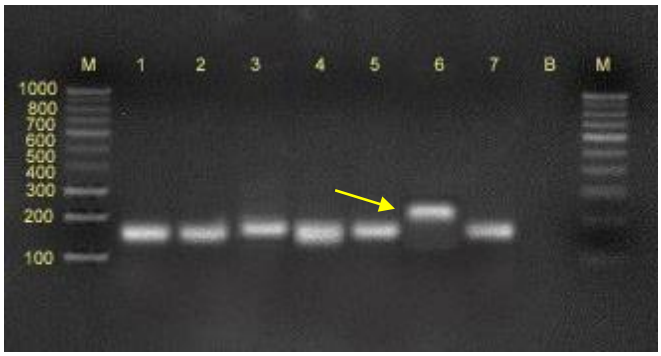
	1	2	3	4	5	6	7
Mol. Size (bp)/Varieties	CCRP 3	CCRP 10	CCRP 11	CCRP 12	CCRP 13	CCRP 14	CCRP 15
300							
290							

b. Colour chart mTcCIR33

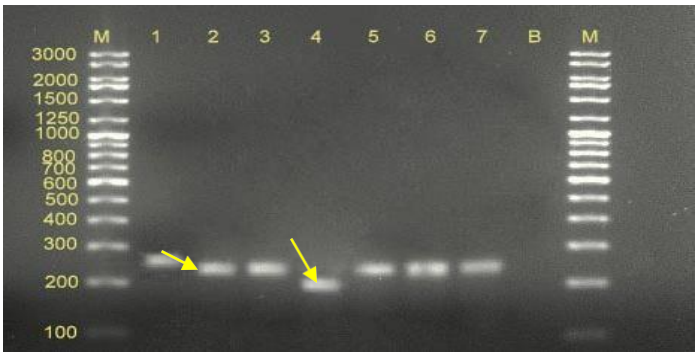
Colour code for sharing of bands among varieties

1	2	3	4	5	6	7

Fig. 27 Colour chart developed using mTcCIR 24 and mTcCIR 33



a. Amplification with primer mTcCIR37



b. Amplification with primer mTcCIR40

M- Molecular weight marker (3kb), B- Blank, 1-CCRP 3, 2- CCRP 10, 3- CCRP 11, 4- CCRP 12, 5- CCRP 13, 6- CCRP 14, 7- CCRP 15

Plate 30. Amplification pattern generated with primer mTcCI 37 and mTcCIR 40

	1	2	3	4	5	6	7
Mol. Size (bp)/Varieties	CCRP 3	CCRP 10	CCRP 11	CCRP 12	CCRP 13	CCRP 14	CCRP 15
200							
150							

a. Colour chart mTcCIR 37

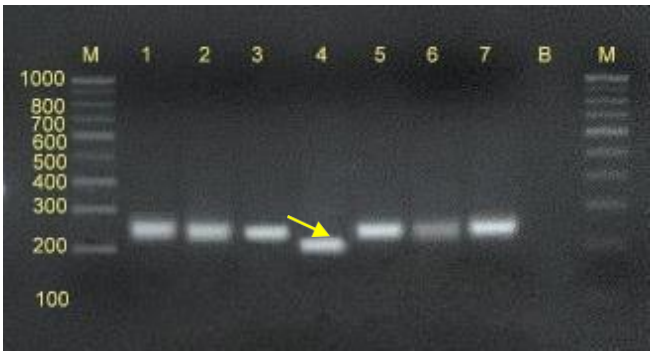
	1	2	3	4	5	6	7
Mol. Size (bp)/Varieties	CCRP 3	CCRP 10	CCRP 11	CCRP 12	CCRP 13	CCRP 14	CCRP 15
250							
240							
200							

b. Colour chart mTcCIR40

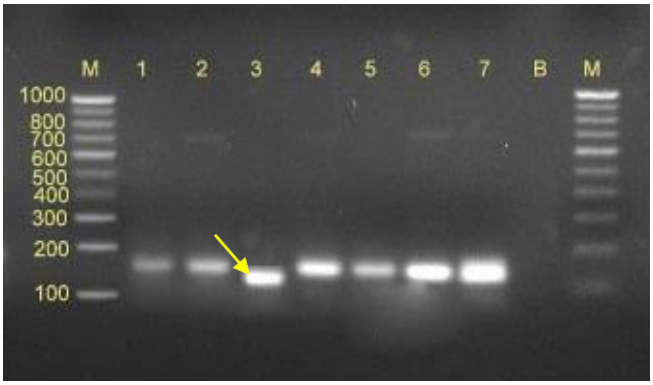
Colour code for sharing of bands among varieties

1	2	3	4	5	6	7

Fig 28 Colour chart developed using mTcCIR 37 and mTcCIR 40



a. Amplification with primer mTcCIR42



b. Amplification with primer mTcCIR 121

M- Molecular weight marker (3kb), B- Blank, 1-CCRP 3, 2- CCRP 10, 3- CCRP 11, 4- CCRP 12, 5- CCRP 13, 6- CCRP 14, 7- CCRP 15

Plate 31. Amplification pattern generated with primer mTcCIR 42 and mTcCIR 121

	1	2	3	4	5	6	7
Mol. Size (bp)/Varieties	CCRP 3	CCRP 10	CCRP 11	CCRP 12	CCRP 13	CCRP 14	CCRP 15
250							
210							

a. Colour chart mTcCIR42

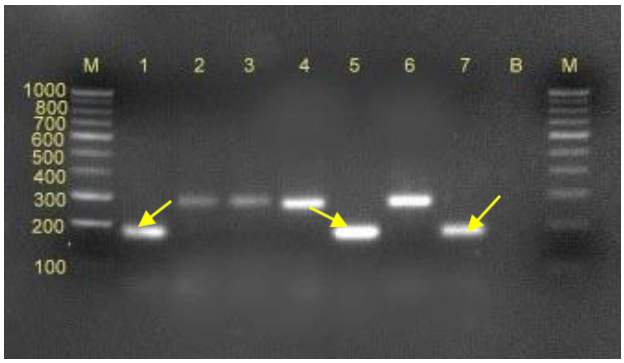
	1	2	3	4	5	6	7
Mol. Size (bp)/Varieties	CCRP 3	CCRP 10	CCRP 11	CCRP 12	CCRP 13	CCRP 14	CCRP 15
150							
140							

b. Colour chart mTcCIR121

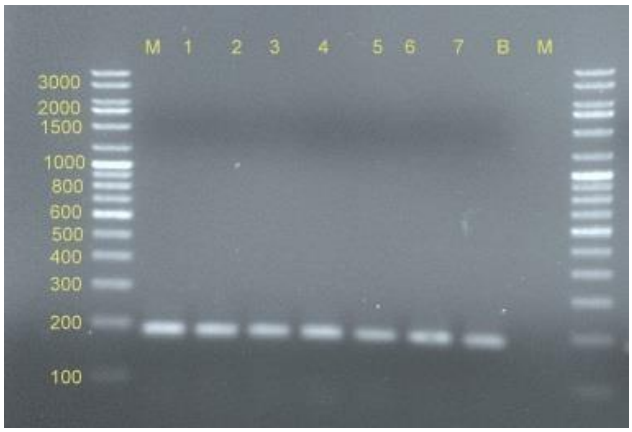
Colour code for sharing of bands among varieties

1	2	3	4	5	6	7

Fig. 29 Colour chart developed using mTcCIR 42 and mTcCIR1 121



a. Amplification with primer SHRSTc49



b. Amplification with primer SHRSTc51

M- Molecular weight marker (3kb), B- Blank, 1-CCRP 3, 2- CCRP 10, 3- CCRP 11, 4- CCRP 12, 5- CCRP 13, 6- CCRP 14, 7- CCRP 15

Plate 32. Amplification pattern generated with primer SHRSTc 49 and SHRSTc 51

	1	2	3	4	5	6	7
Mol. Size (bp)/Varieties	CCRP 3	CCRP 10	CCRP 11	CCRP 12	CCRP 13	CCRP 14	CCRP 15
300							
195							

a. Colour chart SHRSTc49

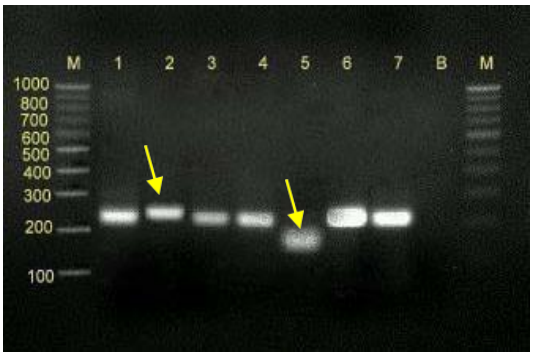
	1	2	3	4	5	6	7
Mol. Size (bp)/Varieties	CCRP 3	CCRP 10	CCRP 11	CCRP 12	CCRP 13	CCRP 14	CCRP 15
200							

b. Colour chart SHRSTc51

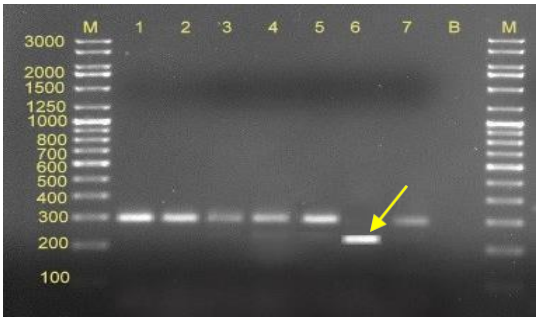
Colour code for sharing of bands among varieties

1	2	3	4	5	6	7

Fig. 30 Colour chart developed using SHRSTc 49 and SHRSTc 51



a. Amplification with primer SHRSTc53



b. Amplification with primer SHRSTc64

M- Molecular weight marker (3kb), B- Blank, 1-CCRP 3, 2- CCRP 10, 3- CCRP 11, 4- CCRP 12, 5- CCRP 13, 6- CCRP 14, 7- CCRP 15

Plate 33. Amplification pattern generated with primer SHRSTc 53 and SHRSTc 64

	1	2	3	4	5	6	7
Mol. Size (bp)/Varieties	CCRP 3	CCRP 10	CCRP 11	CCRP 12	CCRP 13	CCRP 14	CCRP 15
230							
220							
180							

a. Colour chart SHRSTc53

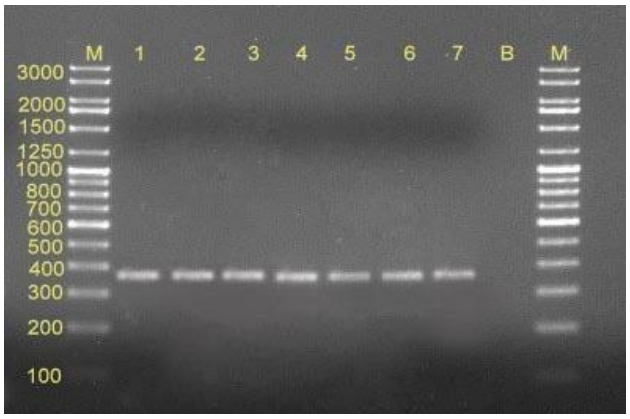
	1	2	3	4	5	6	7
Mol. Size (bp)/Varieties	CCRP 3	CCRP 10	CCRP 11	CCRP 12	CCRP 13	CCRP 14	CCRP 15
300							
25 0							

b. Colour chart SHRSTc 64

Colour code for sharing of bands among varieties

1	2	3	4	5	6	7

Fig. 31 Colour chart developed using SHRSTc 53 and SHRSTc 64



Amplification with primer SSRKAU 15

M-Molecular weight marker (3kb), B- Blank, 1-CCRP 3, 2- CCRP 10, 3- CCRP 11, 4- CCRP 12, 5-CCRP 13, 6- CCRP 14, 7- CCRP 15

Plate 34. Amplification pattern generated with primer SSRKAU 15

	1	2	3	4	5	6	7
Mol. Size (bp)/Varieties	CCRP 3	CCRP 10	CCRP 11	CCRP 12	CCRP 13	CCRP 14	CCRP 15
380							

Colour chart SSRKAU 15

Colour code for sharing of bands among varieties

1	2	3	4	5	6	7

Fig. 32 Colour chart developed using SSRKAU 15

4.3 DNA fingerprinting of individual cocoa varieties

The polymorphic colour chart of individual variety was developed by utilizing 23 selected ISSR primers. Similarly, 15 SSR primers were used for developing DNA fingerprint data and developed a colour chart. Amplicons which were shared by maximum of three varieties in case of ISSR s and four varieties in SSR primers were selected for fingerprinting of individual genotypes. DNA fingerprint of individual varieties are described below.

4.3.1 CCRP3

4.3.1.1 ISSR profiling

Out of 23 selected primers eight ISSR primers generated distinct polymorphic bands. The amplicons sizes obtained ranged from 250 bp to 1250 bp (Fig 33a). A total of 14 polymorphic loci were observed in hybrid CCRP 3. Primer HB12, Oligo 04, Oligo 05, UBC 810, UBC 811, UBC 818, UBC 844 and UBC 846 produced polymorphic bands of different lengths. Primer UBC 810 produced a unique band at 500 bp. Band at 500 bp produced by HB 10, 650 bp by UBC 811 and 250 bp by Oligo ISSR 05 were shared by more than three varieties, and hence, not utilized in generating the fingerprint. Remaining 11 bands were used for final DNA finger printing (Fig. 33).

4.3.1.2 SSR profiling

Out of 15 selected primers five primers yielded five distinct polymorphic band in hybrid CCRP 3. Amplicon size ranged from 195 bp to 350 bp (Fig. 33b). Two polymorphic bands among the five were selected for final fingerprint. Since the remaining three bands were shared by more than four varieties they were discarded. Primer mTcCIR 40 produced a unique polymorphic band at 250bp in CCRP 3.

4.3.2 CCRP 10

4.3.2.1 ISSR profiling

In CCRP 10 genotype, 22 selected ISSR primers were used for amplification. Among the selected primers, 12 yielded a total of 23 distinct polymorphic bands and these were used for developing colour chart. The amplicon size was in the range of 350

bp to 2500 bp (Fig. 34a). UBC 835 (400 bp) and UBC 857 (750 bp) produced unique bands. Polymorphic amplicons developed by primer HB 10, HB 12, Oligo ISSR 04, Oligo ISSR 07, ISSR 7, UBC 818, UBC 835, UBC 844, UBC 846, UBC 847 and UBC 866 were found to be useful and were utilized for the final fingerprint analysis.

4.3.2.2 SSR profiling

For the variety CCRP 10, SSR analysis was carried using selected 15 primers. Five polymorphic bands were obtained through five primers (Fig. 34b). Amplicons obtained varied in size from 230 bp to 350 bp. In hybrid CCRP 10, there was one polymorphic amplicon from primer SHRSTc 49 (300 bp) and a unique band from SHRSTc 53 (230 bp). These were selected for further analysis.

4.3.3 CCRP11

4.3.3.1 ISSR profiling

In genotype CCRP 11, 22 selected primers generated amplicons (Fig. 35a). Out of these, 14 primers yielded total of 22 polymorphic bands. The amplicons obtained were in a range between 300 bp and 2500 bp. The maximum number of polymorphic bands were generated by UBC 844.The primers HB10, ISSR 3, ISSR 7, UBC 810, UBC 811, UBC 815, UBC 818, UBC 826, UBC 841, UBC 844, UBC 847, UBC 854, UBC 855 and UBC 866 established polymorphism. The 14 polymorphic amplicons were chosen for the construction of final ISSR fingerprint of CCRP 10. Unique amplicons were generated by primers UBC 810 (1400 bp), UBC 826 (310 bp), UBC841 (300 bp) and UBC 854 (1300).

4.3.3.2. SSR profiling

SSR analysis of hybrid CCRP 11was done by using selected 15 SSR primers. It yielded amplicons of molecular size which ranged between 140 bp to 350 bp. Eight primers produced eight polymorphic bands (Fig. 35b). Primer mTcCIR 8 (300 bp), mTcCIR 10 (310 bp) mTcCIR 11(290 bp), mTcCIR 121 (140 bp) yielded unique bands. SHRSTc 49 generated another polymorphic band at 300 bp. SSR fingerprint of variety CCRP 11 was constructed using five useful amplicons.

4.3.4 CCRP12

4.3.4.1 ISSR profiling

In variety CCRP 12, amplicons with molecular weight ranging from 250 to 1550 bp were produced (Fig. 36a). Among 23 selected primers, 16 primers yielded 30 (highest number) polymorphic bands. Twenty polymorphic bands, shared only by a maximum of three varieties were utilized for further analysis. Primer Oligo ISSR 05 generated two unique bands at 1400 bp and 300 bp. ISSR 3 (400 bp), UBC 815 (650 bp), UBC 827 (1400 bp) and UBC 854 (1000 bp) primers also produced unique bands. Hence these were utilized to generate the DNA fingerprint of the variety CCRP12.

4.3.4.2 SSR profiling

In CCRP 12 genotype, out of selected 15 SSR primers, six primers yielded six polymorphic bands. Molecular size of the amplicons ranged between 200 bp and 345 bp. Among the polymorphic bands observed four were utilized for development of DNA fingerprinting (Fig. 36 b). Primers mTcCIR 18, mTcCIR 40, mTcCIR 42 registered unique bands at 345 bp, 200 bp, 210 bp respectively and SHRSTc 49 generated polymorphic band at 300 bp. The primers mTcCIR 10 (330 bp) and SHRSTc 53 (220 bp) yielded bands in more than four varieties and were not selected for further analysis.

4.3.5 CCRP13

4.3.5.1 ISSR profiling

DNA fingerprint of CCRP 13 was generated using 22 selected ISSR primers. Eleven polymorphic bands were observed from nine primers (Fig. 37a). Out of 11 polymorphic amplicons eight bands were considered for final fingerprint analysis which was shared only by three varieties. The amplicons generated by primers ranged from 300 bp to 2500 bp. A unique band was observed in UBC 854 (400 bp), while, primers HB 12, ISSR 7, UBC 818, UBC 835, UBC 846, UBC 847 and UBC 855 produced polymorphic bands. Primer HB 10 (1250 bp), HB 12 (500 bp) and UBC 855 (300 bp) were not included in the fingerprint as the polymorphic bands produced by them were expressed in more than three varieties.

4.3.5.2 SSR profiling

CCRP 13 was subjected to SSR analysis using 15 selected primers. Ten primers generated 10 polymorphic bands (Fig. 37b). The amplicons obtained were in the range of 150 bp to 350 bp. Out of 10 polymorphic amplicons, eight were chosen for development of final SSR fingerprint. Seven unique bands were produced by the primers mTcCIR 10 (340 bp), mTcCIR 11 (310 bp), mTcCIR 12 (270 bp), mTcCIR 22 (200 bp), mTcCIR 24 (150 bp), mTcCIR 33(300 bp) and SHRSTc 53 (180 bp). Polymorphic band was formed by SHRSTc 49 (195 bp). The remaining two amplicons were common in more than three varieties and hence the details were notutilized for the construction of final DNA fingerprint.

4.3.6 CCRP14

4.3.6.1 ISSR profiling

Twenty three selected ISSR primers were used for the analysis of hybrid CCRP 14. Among these eleven primers exhibited 17 polymorphic bands. Amplicons generated was in the range of 250 bp and 1550 bp (Fig. 38a). Polymorphic bands shared by a maximum of three varieties were produced by the primers HB 10 (1000 bp and 600 bp), Oligo ISSR 07 (550 bp), ISSR 3(850), BC 811 (950 bp and 550 bp), UBC 815 (700 bp), UBC 844 (1250 bp), UBC 855 (350 bp and 650 bp) and UBC 857 (500 bp). Unique polymorphic bands were produced by Oligo ISSR 05 (550 bp) and UBC 855 (650 bp). Thus, the primers enumerated above were utilized for final fingerprinting of CCRP 14.

4.3.6.2 SSR profiling

In CCRP 14, fifteen selected SSR primers were used for analysis. Seven primers produced seven polymorphic bands (Fig. 38b). The size of the amplicons ranged between 200bp and 350bp. Unique bands were found in the profile generated by primer mTcCIR 37 (200 bp) and SHRSTc 64 (250 bp). SHRSTc 49 (300 bp) also yielded polymorphic bands. Four bands were shared by more than four varieties and hence, not included to generated the DNA fingerprint of the variety.

4.3.7 CCRP15

4.3.7.1 ISSR profiling

Twenty-two selected primers generated amplicons in hybrid CCRP 15 (Fig.

39a). Fourteen primers exhibited 22 polymorphic bands in the genome of the hybrid CCRP 15. Molecular size of amplicons ranged from 150 bp to 1550 bp. Polymorphic amplicons were generated using primers HB10, HB 12, Oligo ISSR 04, Oligo ISSR 05, Oligo ISSR 07, ISSR 3, UBC 810,UBC 811, UBC 818, UBC 835, UBC 844, UBC 846, UBC 855, UBC 857 and UBC 866. Among these, primers Oligo ISSR 05 (250 bp), UBC 810 (900 bp), UBC 844 (1550 bp and 800 bp) and UBC 855 (300 bp) produced bands shared by more than three varieties. Hence these were not used to generate the ISSR DNA fingerprint of the variety. Unique band was observed in primer ISSR 05 (150 bp) and UBC 844 (590 bp and 300 bp).

4.3.7.2 SSR profiling

Genotype CCRP 15 was analyzed with 15 selected SSR primers (Fig. 39b). The molecular size of amplicons ranged from 195 bp to 355 bp. Distinct polymorphic band was generated by the primer SHRSTc 49 (195 bp) and an unique band by mTcCIR 18 (355 bp). These, two amplicons were further utilized for SSR fingerprint of CCRP 15. Remaining mTcCIR 10, mTcCIR 40 and SHRSTc 53 primers also gave polymorphic bands. However these were common in four varieties and hence not utilized further.

Mol Size(bp)/ Primers	HB 12	Oligo 04	Oligo 05	UBC 810	UBC 811	UBC 818	UBC 844	UBC 846
1250								
1200								
1000								
950								
800								
750								
650								
600								
550								
500								
450								
400								
250								

a. ISSR fingerprint

Mol Size(bp)/ Primers	mTcCIR 10	mTcCIR 18	mTcCIR 40	SHRSTc 49	SHRSTc 53
350					
330					
250					
220					
195					

b. SSR Fingerprint

Colour code for sharing of bands among genotype

1	2	3	4	5	6	7

Fig. 33 Fingerprint developed using ISSR and SSR primers – CCRP 3

Mol Size(bp)/ Primers	HB 10	HB 12	Oligo 04	Oligo 07	ISSR 7	UBC 811	UBC 818	UBC 835	UBC 844	UBC 846	UBC 857	UBC 866
2500												
1550												
1300												
1250												
1200												
1000												
950												
900												
800												
750												
700												
650												
500												
450												
400												
350												

a. ISSR Fingerprint

Mol Size(bp)/ Primers	mTcCIR 10	mTcCIR 18	mTcCIR 40	SHRSTc 49	SHRSTc 53
350					
330					
300					
240					
230					

b. SSR Fingerprint

Colour code for sharing of bands among genotype

1 2 3 4 5 6 7

Fig. 34 Fingerprint developed using selected ISSR and SSR primers –CCRP 10

Mol Size(bp)/ Primers	HB 10	ISSR 3	ISSR 7	UBC 810	UBC 811	UBC 815	UBC 818	UBC 826	UBC 841	UBC 844	UBC 847	UBC 854	UBC 855	UBC 866
2500														
1400														
1300														
1250														
1000														
950														
900														
850														
800														
700														
650														
500														
350														
310														
300														

a. ISSR Fingerprint

Mol Size(bp)/ Primers	mTcCIR 8	mTcCIR 10	mTcCIR 11	mTcCIR 18	mTcCIR 40	mTcCIR 121	SHRSTc 49	SHRSTc 53
350								
310								
300								
290								
240								
220								
140								

b. SSR Fingerprint

Colour code for sharing of bands among genotype

1	2	3	4	5	6	7

Fig. 35 Fingerprint developed using selected ISSR and SSR primers – CCRP 11

Mol Size(bp)/ Primers	HB 10	HB 12	Oligo 05	Oligo 07	ISSR 3	UBC 810	UBC 811	UBC 815	UBC 827	UBC 844	UBC 846	UBC 847	UBC 854	UBC 855	UBC 857	UBC 866
1550																
1400																
1300																
1250																
1000																
950																
900																
850																
800																
700																
650																
600																
550																
500																
400																
350																
300																
250																

a. ISSR Fingerprint

Mol Size(bp)/ Primers	mTcCIR 10	mTcCIR 18	mTcCIR 40	mTcCIR 42	SHRSTc 49	SHRSTc 53
345						
330						
300						
220						
210						
200						

b. SSR Fingerprint

Colour code for sharing of bands among genotype

1	2	3	4	5	6	7

Fig. 36 Fingerprint developed using selected ISSR and SSR primers – CCRP12

Mol Size(bp)/ Primers	HB 10	HB 12	ISSR 7	UBC 818	UBC 835	UBC 846	UBC 847	UBC 854	UBC 855
2500									
1250									
1200									
900									
800									
750									
700									
500									
400									
350									
300									

a. ISSR Fingerprint

Mol Size(bp)/ Primers	mTcCIR 10	mTcCIR 11	mTcCIR 12	MTcCIR 18	mTcCIR 22	mTcCIR 24	mTcCIR 33	MTcCIR 40	SHRSTc 49	SHRSTc 53
350										
340										
310										
300										
270										
240										
200										
195										
180										
150										

b. SSR Fingerprint

Colour code for sharing of bands among genotype

1	2	3	4	5	6	7

Fig. 37 Fingerprint developed using selected ISSR and SSR primers – CCRP 13

Mol Size (bp)/ Primers	HB 10	Oligo 05	Oligo 07	ISSR 3	UBC 810	UBC 811	UBC 815	UBC 844	UBC 855	UBC 857	UBC 866
1550											
1250											
1000											
950											
900											
850											
700											
650											
600											
550											
500											
350											
300											
250											

a. ISSR Fingerprint

Mol Size(bp)/ Primers	MTcCIR 10	MTcCIR 18	mTcCIR 37	MTcCIR 40	SHRSTc 49	SHRSTc 53	SHRSTc 64
350							
330							
300							
250							
240							
220							
200							

b. SSR Fingerprint

Colour code for sharing of bands among genotype

1	2	3	4	5	6	7

Fig. 38 Fingerprint developed using selected ISSR and SSR primers – CCRP 14

Mol Size(bp)/ Primers	HB 10	HB 12	Oligo 04	Oligo 05	Oligo 07	ISSR 3	UBC 810	UBC 811	UBC 818	UBC 835	UBC 844	UBC 846	UBC 855	UBC 857	UBC 866
1550															
1400															
1300															
1250															
900															
850															
800															
750															
700															
600															
590															
550															
500															
350															
300															
250															
150															

a. ISSR Fingerprint

Mol Size(bp)/ Primers	mTcCIR 10	mTcCIR 18	mTcCIR 40	SHRSTc 49	SHRSTc 53
355					
330					
240					
220					
195					

b. SSR Fingerprint

Colour code for sharing of bands among genotype

1	2	3	4	5	6	7

Fig. 39 Fingerprint developed using selected ISSR and SSR primers – CCRP 15

4.4 Overall DNA fingerprinting data

4.4.1 ISSR fingerprint

Twenty three ISSR primers were analyzed by using amplification pattern observed and colour chart was developed based on polymorphic data. All the 23 primers produced at least one unique functional polymorphic amplicon. Final ISSR fingerprint chart was developed by utilizing useful polymorphic amplicons established in all seven genotypes (Fig. 40). Molecular size of the ISSR fingerprints ranged from 150 bp to 2500 bp. In the seven genotypes studied, and a total of 99 polymorphic bands were obtained by 22primers.

CCRP 12 hybrid consisted of maximum number (20) of unique amplicons, whereas, CCRP 13 had only eight such amplicons. Among 92 polymorphic amplicons, 19 bands were found to be unique, 32 of them were shared by two varieties and remaining 48 polymorphic bands were shared in three varieties. CCRP 3 and CCRP 13 had only one unique band (500 bp and 400 bp respectively), while there were two in CCRP 10 (750 bp and 400 bp), four in CCRP 11(1400 bp, 1300 bp, 310 bp and 300bp), six in CCRP 12 (1400 bp, 1000 bp, 650 bp, 400 bp and 300 bp), two in CCRP 14 (650 bp and 550 bp) and three in CCRP 15 (590 bp, 300 bp and 150 bp. The result thus obtained is valuable for the detection of cocoa varieties studied.

4.4.2 SSR fingerprint

Seventeen selected SSR primers generated amplicons in all seven varieties and it was described in the form of colour chart (Fig. 41). Among the 17 selected primers, 15 primers produced at least one useful polymorphic amplicons in all seven varieties. A total 26 polymorphic amplicons were identified. The molecular size of DNA fingerprints thus generated, ranged between 140 bp to 355bp.

All the seven varieties had unique bands. A total of 19 unique amplicons were obtained in SSR profiles. Highest numbers of seven unique bands were observed in hybrid CCRP 13. Three polymorphic amplicons were shared by three varieties and four were shared by four varieties. The SSR profile thus obtained is of great utility for the identification of varieties studied.

Mol. Size (bp)/ Primers	CCRP 3	CCRP 10	CCRP 11	CCRP 12	CCRP 13	CCRP 14	CCRP 15
2500		UBC818	UBC818		UBC818		
1400			UBC 810	Oligo 05			Oligo 07
				Oligo07			
				UBC827			
1300		Oligo 04	UBC 854	UBC 866			Oligo 04
							UBC 866
1250	HB 12	UBC844	UBC844	0ligo 07		UBC844	HB 12
		Oligo 07					
1200	UBC 846	UBC 846			UBC 846		
1000	HB 12	HB 10	HB 10	HB12		HB 10	
		HB 12		UBC854			
950	UBC 811		UBC 811			UBC 811	
900		ISSR 7	ISSR 7		ISSR7		
850			ISSR 3	ISSR 3		ISSR 3	UBC846
				UBC 846			
800	Oligo 04	Oligo 04	UBC 847	UBC 847	UBC847		
750	UBC844	UBC844					UBC844
		UBC857					
700		UBC866	UBC 815	HB12	HB 12	UBC815	ISSR 3
				ISSR 3			
			UBC866	UBC 815			UBC857
				UBC857			
650				UBC815		UBC855	
600						HB 10	HB 10
590							UBC844
550	UBC 811					Oligo 07	UBC811
						UBC811	
500	UBC 810		UBC818	UBC844	UBC855	UBC857	UBC818
	UBC818		UBC844	UBC857			UBC844
							UBC855
450	Oligo 04	Oligo 04					
400	UBC 844	UBC835		ISSR 3	UBC854		
		UBC844					
350		UBC835	UBC855	UBC855	UBC835	UBC855	UBC835
310			UBC826				
300			UBC841	Oligo 05			UBC844
150							Oligo 05

Colour code for sharing among varieties

1	2	3	4	5	6	7

Fig. 40 Final fingerprinting of ISSR primers

	1	2	3	4	5	6	7
Mol. Size (bp)/ Primers	CCRP 3	CCRP 10	CCRP 11	CCRP 12	CCRP 13	CCRP 14	CCRP 15
355							**M 18**
345				**M 18**			
340					**M 10**		
310			**M 10**		**M 11**		
300		**S 49**	**S 49**	**S 49**	**M 33**	**S 49**	
290			**M 8** **M 11**				
270					**M 12**		
250	**M40**					**S 64**	
230		**S 53**					
210				**M 42**			
200				**M40**	**M 22**	**M 37**	
195	**S 49**				**S 49**		**S 49**
180					**S 53**		
150					**M 24**		
140			**M 121**				

Colour code for sharing among varieties

Fig. 41 Final finger printing of SSR primers

SUMMARY

5. SUMMARY

The study entitled 'DNA fingerprinting of selected cocoa (*Theobroma cacao* L.) varieties of Kerala Agricultural University' was conducted in the Department of Plant Biotechnology, College of Horticulture, Vellanikkara, during, 2017-2019. The objective of the study was to develop DNA fingerprint profile of cocoa varieties using SSR and ISSR markers. Seven cocoa varieties CCRP 3, CCRP 10, CCRP 11, CCRP 12, CCRP 13, CCRP 14, and CCRP 15, released from the Cocoa Research Centre, KAU were used for the study.

The results of the study are as follows

1. Dellaporta method (Ileana, 2005) with small modification was found to be suitable for DNA isolation from cocoa leaves.

2. Spectrophotometer NanoDrop® ND-1000 was used for the quantification of isolated DNA. The UV absorbance ratio ranged from 1.82-2.01, which indicated the presence of good quality DNA suitable for molecular characterization.

3. Based on amplification profile of 35 ISSR primers and 30 SSR primers, 23 ISSR and 17 SSR primers found to be polymorphic and were chosen for further analysis.

4. ISSR and SSR fingerprints were developed for individual variety. In ISSR assay the primer that produced amplicons which was shared by a maximum of three varieties and in SSR assay the primer that produced polymorphic bands in a maximum of four varieties were contemplated as beneficial polymorphism. DNA fingerprint profile was developed by including only such useful amplicon information.

5. The generated DNA fingerprint was adequate to differentiate between the varieties and all the selected varieties generated specific fingerprint.

6. Variety CCRP 3 registered unique amplicon at 510 bp on using primer UBC 81

7. Primer UBC 835 and UBC 857 produced unique amplicons at 400 bp and 750 bp respectively in variety CCRP 10.

8. Primer UBC 810 (1400 bp), UBC 826 (310 bp) and UBC 841 (300 bp) UBC 854 (1300bp) generated unique amplicons and formed specific DNA fingerprints of the hybrid CCRP 11.

9. Primer ISSR 3 (400 bp), UBC 815 (650 bp), UBC 827 (1400 bp), UBC 854 (1000bp) and Oligo O5 (1400 bp and 300 bp) generated unique bands for fingerprinting CCRP 12.

10. CCRP 13 registered unique band at 400 bp (UBC854).

11. In hybrid CCRP 14, unique amplicons were generated, by the primers UBC 855 (650 bp) and Oligo 07 (550 bp).

12. In CCRP15unique fingerprint was developed with the primer UBC 844 (590 bp and 300 bp) and Oligo 05(150bp).

13. SSR primer mTcCIR 40 generated unique amplicons at 250 bp in variety CCRP 3 andSHRSTc 53 at 230 bp in variety CCRP 10. These were the specific fingerprints of these varieties.

14. Unique fingerprint was found at 310 bp (mTcCIR 10), 300 bp (mTcCIR 8), 290 bp (mTcCIR 11) and 140 bp (mTcCIR 121) in CCRP 11.

15. DNA fingerprint with the primers mTcCIR 18 (345 bp), mTcCIR 40 (200 bp) and mTcCIR 42 (210 bps) were developed for CCRP 12.

16. Unique amplicons generated by primers mTcCIR 10 (340 bp), mTcCIR 11 (310 bp), mTcCIR 12 (270 bp), mTcCIR 22 (200 bp), mTcCIR 24(150 bp), mTcCIR 33 (300 bp) and SHRSTc 53 (180) formed the specific DNA fingerprint of CCRP13.

17. DNA fingerprint of CCRP14 comprised of amplicons generated at 250 bp (mTcIR 37) and 200 bp (SHRSTc 64).

18. The ISSR and SSR profile thus obtained is of great utility for the identification of the varieties studied.

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ANNEXURE I

List of laboratory equipment used for the study

Refrigerated centrifuge	:	Kubota, Japan
Horizontal electrophoresis system	:	BIO-RAD, USA
Thermal cycler	:	Proflex
Gel documentation system	:	Gel Doc^{TM} XR+BIORAD
Micropipettes	:	Eppendorf
Spectrophotometer	:	Nanodrop® ND-1000, USA

ANNEXURE II

Reagents required for DNA isolation

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

   CTAB : 2g

   (Cetyl trimethyl ammonium bromide)

   Trisbase : 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. **EB1 (Extraction Buffer 2) (100ml)**

3. 0.5M EDTA pH 8.0 : 2ml 1M

4. Tris pH: 8.0 : 5ml

3. 20 % SDS (do not autoclave) (100 ml)

SDS : 20 g

dH_2O : 100ml

4. 5M KOAC (Potassium acetate) (100 ml)

KOAC : 49.1 g

dH_2O : 100ml

5. **3M NaOAC (Sodium acetate) (100ml)**

NaOAC : 24.6 g

dH_2O : 100 ml

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

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

7. Chilled isopropanol

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

8. Ethanol (70 %)

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

9. TE buffer (pH 8, 100ml)

Tris HCl (10 mM) : 0.1576 g

EDTA (1 mM) : 0.0372g

(The solution was prepared, autoclaved and stored at room temperature)

ANNEXURE III

Composition of buffers and dyes used for gel electrophoresis

a. TAE Buffer50X

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

b. Loading dye6X

0.25 per cent bromophenol blue

0.25 per cent xylene cyanol 30 per cent glycerol in water

c. 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.

DNA FINGERPRINTING OF SELECTED COCOA (*Theobroma cocoa* L.) VARIETIES OF KERALA AGRICULTURAL UNIVERSITY

By

MEGHA TOTAGNATI

(2017-11-007)

ABSTRACT OF THE THESIS

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Faculty of Agriculture

Kerala Agricultural University, Thrissur

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ABSTRACT

Cocoa (*Theobroma cacao* L.), is an important tropical tree-crop belonging to the family Malvaceae. It has originated in South America (Wood and Lass, 1985) and was introduced to India, in 1798 at Courtallam in Tirunelveli district of the old Madras state (Ratnam, 1961). Now, cocoa is extensively cultivated in South Indian states and its cultivation is slowly extending to North Eastern states.

Central sub-committee on crop standards has made the DNA fingerprinting data mandatory for the national release and notification of varieties. DNA markers, act as active tool to categorize and distinguish accessions and cultivars which have similar morphological characteristics. DNA fingerprinting is effectively used for cultivar identification, assessing seed purity of hybrids and to compare genetic similarities between the cultivars. DNA fingerprints act as a powerful tool to protect Plant Breeder's Rights (PBR).

In the present study, seven cocoa varieties, CCRP 3 (selection), CCRP 10, CCRP 11, CCRP 12, CCRP 13, CCRP 14 and CCRP 15 (hybrids) were fingerprinted. Molecular characterization was performed with genomic DNA isolated using modified Delloporta method (Ileana, 2005). Thirty five ISSR (inter simple sequence repeats) primers and 30 SSR (simple sequence repeats) primer combinations were screened for marker polymorphism, of which 23 ISSR and 17 SSR primer combinations were selected for further study. ISSR and SSR amplification patterns differed among the varieties and thus the DNA fingerprints from each primer combination were developed.

ISSR primer UBC810 was found to distinguish CCRP 3 from other genotypes. Whereas, UBC835 and UBC857 produced unique amplicons in variety CCRP 10. Primer UBC810, UBC826, UBC841 and UBC854 generated unique amplicons and formed specific DNA fingerprints of the hybrid CCRP 11. Primer ISSR3, UBC815, UBC827, UBC854 and Oligo 05 gave highest (6) unique bands in CCRP12, whereas UBC 854 produced specific band in CCRP 13. Hybrid CCRP 14 generated unique amplicons with primers UBC855 and Oligo 07 which formed specific fingerprint of the hybrid. Similarly, CCRP15 generated three unique fingerprints with the primer UBC844 and Oligo05.

In SSR marker analysis, all the seven genotypes have at least one unique band. mTcCIR40 generated unique amplicons at 250bp length in CCRP 3 and SHRSTc53 at 230bp in CCRP 10. mTcCIR10, mTcCIR8, mTcCIR11 and mTcCIR121 generated distinct bands in CCRP 11. In CCRP 12 genotype, primer mTcCIR18 (345bp), mTcCIR40 (200bp) and mTcCIR42 (210bp) produced specific band. Unique bands were generated by primers mTcCIR10(340bp), mTcCIR11 (310bp), mTcCIR12(270bp), mTcCIR22(200bp), mTcCIR24 (150bp), mTcCIR33 (300bp) and SHRSTc53 for CCRP12. DNA fingerprint generated for CCRP 14 revealed that unique bands were developed at 250bp (mTcCIR37) and 200bp (SHRSTc64). Similarly, CCRP15 developed specific fingerprint at 355 bp (mTcCIR18).

ISSR amplicons shared by maximum of three varieties and SSR shared in four varieties were considered for developing final DNA fingerprint profile. The present study had facilitated to characterize the selected cocoa varieties of KAU and the data generated will be useful for varietal notification and in case of any third party litigations.