GENETIC DIVERSITY ANALYSIS OF RAMBUTAN (*Nephelium lappaceum* L.) ACCESSIONS USING MOLECULAR MARKERS

by

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THESIS

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DECLARATION

I, hereby declare that this thesis entitled **"GENETIC DIVERSITY ANALYSIS OF RAMBUTAN (*Nephelium lappaceum* L.) ACCESSIONS USING MOLECULAR MARKERS"** 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 this thesis entitled **"GENETIC DIVERSITY ANALYSIS OF RAMBUTAN (*Nephelium lappaceum* L.) ACCESSIONS USING MOLECULAR MARKERS"** is a record of research work done independently by **Mr. Gazel M Gaddafi (2019-12-051)** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to him.

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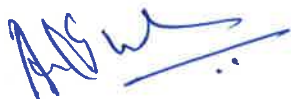

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LIST OF ABBREVIATIONS

%	Per cent
°C	Degree Celsius
B	Beta
µg	Microgram
µl	Microliter
AFLP	Amplified Fragment Length Polymorphism
Bp	Base pair
cm	Centimetre
CTAB	Cetyl Trimethyl Ammonium Bromide
°C	Degree Celsius
DNA	Deoxyribonucleic acid
DNTPs	Deoxyibo Nucleoside Triphosphate
EDTA	Ethylene Diamine Tetra Acetic acid
EST	Expressed Sequence Tags
g	Gram
ISSR	Inter Simple Sequence Repeat
KAU	Kerala Agricultural University
Kb	Kilo base pairs
L	Litre

M	Molar
Mg	Milligram
MI	Marker Index
mM	Milli Molar
ng	Nano gram
OD	Optical Density
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
rpm	Revolution per minute
RNA	Ribonucleic acid
RNase	Ribonuclease
SCAR	Sequence characterized amplified region
SNP	Single Nucleotide Polymorphism
SSR	Simple Sequence Repeat
STS	Sequence Tagged Sites
TAE	Tris Acetate EDTA
TE	Tris EDTA
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
UV	Ultra Violet
V	Volts

Dedicated to

My

Parents and Sister

Introduction

1. INTRODUCTION

Rambutan (*Nephelium lappaceum* L.) is a tropical fruit with a high potential for cultivation in Kerala. It is a member of the Sapindaceae family, which includes 125 genera and over 1000 species of trees and shrubs that are widely distributed in the tropics and warm regions, adapting to a variety of soil types ranging from low land-heavy soils to upland-hilly soil. It originated in South East Asia, specifically Indonesia and Malaysia. It is a medium-sized evergreen tree. Malaysia is currently the second-largest producer of rambutan in the world, after Thailand. The colour of rambutan fruits varies from red to yellow. They resemble litchi except for the long hair-like structures on the fruits. The aril, attached to the seed is its edible part. Carbohydrates, vitamin C, calcium, magnesium, and potassium are all abundant in rambutan fruits. The fruit is composed of 82% water, 0.9% protein, and 0.3% fat. The aril, the edible part of the fruit, makes up roughly 35 to 55 percent of its weight, while the skin and seed may make up 40 to 47 percent and 6 to 10 percent, respectively, of the fruit's overall weight.

The commercial cultivation of rambutan is confined to a few regions in Southern parts of India. With a total area of 1500 acres under cultivation in India, rambutan is primarily grown in Kerala and Karnataka. This crop is primarily planted as a courtyard crop or on the outskirts of residential gardens in Kerala. Rambutan has great potential for commercial cultivation in Kerala owing to the warm humid climate that prevails here. Currently, rambutan is grown commercially in the Pathanamthitta, Kottayam, Thrissur, and Ernakulam districts of Kerala. Consumers are attracted to the fruit's unique appearance, which enables farmers to get higher prices.

The species exhibits a lot of genetic variability in nature due to its widespread cross-pollination (Tindall *et al.,* 1994). Many commercial cultivars of rambutan are reported from Indonesia with specific fruit characters. These fruit

characters include skin colour of ripe fruit, flesh texture, flesh taste, and spintern density etc (Kuswandi *et al.,* 2014). The leaves of rambutan are much more diverse than the fruit characters and have the potential to be used as a cultivar marker. Several investigations had been conducted to find out specific characters for differentiating between rambutan cultivars. Several rambutan leaf characters had been evaluated as a specific marker, but the specific character has not yet been discovered (Barreto *et al.,* 2015).

Many of the tropical fruit species including rambutan are heterozygous in nature due to a high degree of outcrossing and require systematic morphological characterization supported by molecular characterization to study the extent of variability and utilization of existing germplasm. Morphological and biochemical markers are used commonly to detect genetic diversity in fruit crops. However, they are poor in detecting inter-varietal and intra-varietal polymorphisms on account of their environmental plasticity. Contrary to morphological characters, molecular characters have been extensively used as markers for differentiating cultivars and for identifying genetic variability in many species (Boczkowska and Tarczyk, 2013). DNA based molecular markers are the most advanced and reliable techniques to estimate genetic diversity and genetic relationship of horticulture plants. Random Amplified Polymorphic DNA (RAPD) technique was used to investigate the genetic diversity of rambutan accessions from Malaysia (Chew *et al.,* 2005). However, the RAPD technique lacks DNA fragment reproducibility due to its low annealing temperature. Meanwhile, Inter-Simple Sequence Repeat (ISSR) is a molecular marker that produces a large number of DNA loci by amplifying a DNA sequence region between two identical microsatellites. It produces a DNA result with high reproducibility, which can be used to identify genetic variation in lower taxa (Zietkiewicz *et al.,* 1994). Molecular markers such as SSR and ISSR markers are effective in assessing genetic diversity among cultivars because they provide unlimited potential markers to reveal differences at the molecular level.

A workshop held at the state level by the National Bank for Agriculture and Rural Development (NABARD) and the National Horticultural Board (NHB) of the Government of India found that the seedling progenies cultivated in Kerala have a lot of variation. Some of these genotypes were found to be significantly superior to established cultivars from Southeast Asian countries, demonstrating that it is necessary to select elite types (Sijimon, 2009). Muhamed (2016) studied the existing variability and genetic wealth available in the important rambutan growing tracts of Kerala. But studies on the characterization of rambutan genotypes using molecular markers are meagre.

In this context, the present study was taken up with the prime objective of characterizing rambutan genotypes using molecular markers to identify potential genotypes from locally available seedling population of rambutan for future development.

Review of Literature

2. REVIEW OF LITERATURE

Rambutan (*Nephelium lappaceum* L.) is an underutilized tropical crop widely cultivated in Southeast Asia. Rambutan has received relatively little attention in India, as most scholars are more interested in litchi. There exists greater variability in rambutan due to its natural cross-pollination behavior and sexual propagation. An attempt is made in this chapter to review the relevant literature that has been reported on the genetic variability in rambutan and other related fruit species.

2.1 Diversity in Rambutan

Rambutan, *Nephelium lappaceum L.,* is a medium-sized evergreen tree native to the warm, humid tropics. It belongs to the Sapindaceae family of berries, which also includes more than 1000 species of other trees and shrubs. These plants are adapted to a variety of soil types, from low, heavy soils to high, hilly soils. This tropical fruit has a high potential for commercial cultivation. It has evolved from Indonesia and Malaysia in South East Asia (Leenhouts, 1986). The seedling-originated rambutan trees can reach a height of up to 20 meters, but clonal trees are smaller, growing up to 4-7 meters tall and spreading. Leaves are alternating, paripinnate, ovate to obovate leaflets with a dimension of 5-28cm x 2-10.5cm, generally glabrous above and hairy beneath (Tindall,1994). The rambutan tree produces ovoid fruits with a dark crimson to yellow pericarp covered with soft spinterns on outside and mushy aril inside. The weight of the fruit varies between 20 to 60 gm, with 40-60% pericarp, 30-58% aril, and 4-9% seed. The aril is white or translucent, delicious, and juicy, and attached to the seed's testa (Sacramento *et al.,* 2013).

Rambutan is cross-pollinated and is assisted by insects in pollination and fruit development (Free, 1993; Zee, 1993). Bees (*Apis spp.* and *Trigona spp.*), butterflies (*Eristalis spp.* and *Lucilia spp.*), and flies (*Eristalis spp.* and *Lucilia spp.*) are all attracted to the fragrant rambutan blossoms (Chin and Phoon, 1982; Lim, 1984). The species has significant genetic diversity in nature owing to its cross-pollinated behavior (Tindall, 1994).

Several workers have studied the variability existing among rambutan genotypes using morphological and biochemical characters (Kuswandi *et al.,* 2014; Barreto *et al.,* 2015; Muhamed, 2016;).

Andrade *et al.,* (2009) characterized rambutan plants using morphological characters such as plant height, geometry, diameter, leaves and leaflet size, leaflet number, length of petiole, leaf area, leaflet color, and concluded that morphological descriptors can be effectively used in determining genetic diversity.

Foliar characteristics like leaf and leaflet size, leaflet area, number of leaflets, rachis length, and leaflet color were used by Barreto *et al*. (2015) to study the genetic variability in rambutan.

Magdalita and Valencia, (2009) evaluated 100 rambutan trees and released the first Philippine yellow rambutan variety, Amarillo, which has oblong-shaped fruits that weigh 18.38 g and are 52.65 mm long and 36.89 mm wide. It possesses thick and leathery skin and spines. The fruit is sweet with a TSS of 22.35° Brix and the flesh is white, juicy, silky, and easily separable from the seed. The oblong seed measures 25.40 mm long, 14.60 mm wide, and 5.70 mm thick and contain a reasonably substantial amount of edible components (60.7%).

Singh *et al*., 2017 identified two morphotypes in rambutan endemic to East Siang district of Arunachal Pradesh in a study on rambutan variability. Muhamed and Kurien (2019) studied the extent of variability existing in rambutan in Kerala and confirmed that there existed a high amount of variability among the seedling population which can be utilized for future crop improvement programmes.

Genetic variability among rambutan accessions from Malaysia had been studied using RAPD technique (Chew *et al.,* 2005). Andrade *et al.,* (2011) reported that both morphological and molecular markers were efficient in the distinction of varieties.

2.2 Characterisation of plants using Molecular markers

To characterise germplasm collections and analyse diversity, molecular markers had been utilised extensively (Zhao *et al.,* 2008). As a marker for cultivar differentiation and spotting genetic heterogeneity at numerous intra-specific levels, molecular character had been used widely in contrast to morphological character (Boczkowska and Tarczyk, 2013). Genetic markers make it simple to assess variation within or between populations caused by genetic and/or environmental factors. It had been discussed before nucleic acid and proteins were discovered (Park *et al.,* 2009).

There are various challenges in conducting the research of genetic diversity using only morphological characteristics, especially in perennial plants since morphological characters are influenced by the environment and observations take a long time. Undesirable features are passed down to offspring, which is another challenge (Azrai, 2005). By utilizing molecular primers, these issues can be efficiently solved.

Molecular Primers based on individual differences in DNA sequences detect more polymorphism than protein-based markers and morphological markers, so forming a new generation of genetic markers (Botstein *et al.,* 1980; Tanksley *et al.,*1989). As a result, DNA-based varietal characterization methods could potentially solve all of the constraints associated with morphological and biochemical data.

Compared to other marker technologies, DNA marker systems have many advantages. First of all, an infinite number of DNA markers can be produced; secondly, environmentally unaffected DNA marker profiles can be created; and thirdly, unlike isozyme markers, these DNA markers are not inhibited by tissue or developmental stage. These markers are excellent resources for studying genetic linkages, analysing genetic diversity, genome mapping, gene tagging, and plant variety rights (PVR) (Gopalsamy *et al.,* 2012). Moreover, microsatellites, one of the molecular marker types, are very helpful for a variety of genetic tasks, such as assessing diversity, cultivar identification through DNA fingerprinting, linkage analysis, and QTL analysis (Kalia *et al.,* 2011).

According to Samarai and Kazaz (2015), molecular markers are more accurate than morphological markers. But still, molecular primers are more expensive and demand more specialized knowledge from researchers.

DNA markers can be divided into two classes based on the method of analysis: hybridization-based markers and PCR-based markers. Restriction fragment length polymorphism (RFLP) is a hybridization-based marker, whereas PCR-based markers include cleaved amplified polymorphic sequence (CAPS), simple sequence repeat (SSR), inter simple sequence repeat (ISSR), single nucleotide polymorphism (SNP), random amplified polymorphic DNA (RAPD), Sequence characterised amplified region (SCAR), and sequence tagged sites (STS) (Datta *et al*., 2011).

Using the random amplified polymorphic DNA technology, Vanijajiva (2011) examined the genetic variability among Durian *Durio zibethinus Murr* cultivars from Nonthaburi province, Thailand. Gurijala *et al.,* (2015) used Random Amplified Polymorphic DNA (RAPD) markers to identify the genetic diversity of six *Mangifera indica* populations. Long *et al.,* (2015) conducted genetic analysis of litchi in Southern China and opined that improved RAPD technique combined with ISSR analysis can be used for the genetic diversity, germplasm resources preservation, molecular assisted breeding and genetic characterization. Priadi *et al.,* (2016) used phenotypic and genetic traits to investigate the relationship between eight carambola varieties grown at the Research Centre for Biotechnology-LIPI in Indonesia.

Wild relatives of rambutan found in Kalimantan had been examined using ISSR marker which showed high similarity between relatives (Napitu *et al.,* 2016). In addition, Manggabarani *et al.,* (2018) characterized 30 rambutan cultivars from Indonesia using ISSR markers. Tran *et al.,* (2019) demonstrated the usefulness of microsatellites for identification, genetic diversity analysis and germplasm conservation in lychee and related Sapindaceae forest species. Razak *et al*., (2020) generated 39 microsatellite markers using RAD sequencing technologies for the evaluation of genetic diversity of 22 rambutan varieties of which only 12 markers exhibited high quality call rates across the sample

assessed. Arias *et al.,* (2020) designed 36 SSR markers for *Nephelium lappaceum* L. which are highly polymorphic. Khang *et al.,* (2021) conducted diversity analysis in eight rambutan cultivars using Dna barcodes and ISSR markers.

2.2.1 Importance of ISSR Primers in Plants

ISSR is a new type of DNA Molecular Primer based on SSR sequence information in plant genomes created by Zietkiewicz *et al.,*(1994) and it produces a DNA result with high reproducibility, which can be used to identify genetic variation in lower taxa and it concluded that the Inter-Simple Sequence Repeat (ISSR) is a molecular marker that produces a large number of DNA than RAPD Markers loci by amplifying a DNA sequence region between two identical microsatellites. ISSR primers are Genomic DNA segments ranging in size from 100 to 3000 base pairs that are found between adjacent microsatellite sections that are orientated in opposite directions. PCR amplifies ISSRs by employing microsatellite core sequences as primers and a few specific nucleotides as anchors into non-repeat neighboring areas (16-18 bp). Approximately 10-60 fragments from numerous loci are created at the same time, separated by gel electrophoresis, and scored as the presence or absence of fragments of different sizes.

ISSR primers are made up of microsatellite sequences that are either unanchored (Gupta *et al.,* 1994; Wu *et al.,* 1994) or anchored by two or four arbitrary nucleotides at the 5' or 3' end. The insertion of a new base at the 5' or 3' end improves the specificity and reproducibility of their binding sites (Barth *et al.,* 2002).The sequence between two binding sites in opposing orientations within a reasonable distance is amplified, and binding site reduction or addition is recognised as band polymorphism (Yang *et al.*, 1996). ISSR markers out performed RAPDs in terms of effective multiplex ratio (12.5 for ISSR vs. 2.2 for RAPD analyses) and repeatability (Goulao *et al.,* 2001).

ISSR markers are faster and easier to employ than randomly amplified polymorphic DNAs (RAPD), and due to the increased primer length, they are more reliable than SSR markers. In comparison to SSRs, ISSRs have a lower developmental cost and do not require any prior knowledge of target sequences

(Aswathi *et al.,* 2004). Due to its ability to disclose more relevant bands from a single amplification, ISSR markers are considered the most effective markers (Farajpour *et al.,* 2011).

The ISSR Marker has been extensively used in plant genetics and breeding for variety identification, genetic diversity research, and drawing of plant genomes (Guilfeng *et al.,* 2017).

2.2.2 Molecular Characterization using ISSR Primers

Goulao *et al.,*(2001) employed 28 ISSR and 7 RAPD primers to characterize cultivated chestnuts and observed that the effective multiplex ratio(EMR) for ISSR markers were higher than that for RAPDs (12.5 for ISSR vs. 2.2 for RAPD analyses).

Zehdi *et al.,(*2004) examined the genetic variability in 49 Tunisian date-palm genotypes using 14 ISSR primers. The genetic linkages and estimated genetic distances between the accessions were estimated. Khouftimi and Boufagous genotypes showed a considerable degree of divergence, as indicated by the greatest distance value (0.7885).

Using the Inter Simple Sequence Repeat (ISSR) and Random Amplified Polymorphic DNA (RAPD) technique, the genetic diversity of 24 rambutan accessions from Malaysia was investigated by Chew *et al.*,(2005). Less polymorphism was observed for the accessions from MARDI Kemaman with ISSRs (87.10%) compared to RAPDs (97.33%). The mean Jaccard index of genetic similarity for RAPDs was 0.408, while that for ISSRs was 0.561.

The genetic diversity of 27 mulberry accessions (including 19 cultivated and 8 wild) was evaluated using 22 ISSR primers and 15 SSR primers (Weiguo *et al.,*2007). They found that wild species are genetically distinct from cultivated species and cluster analysis with both marker systems effectively analyzed the polymorphism and diversity levels in Mulberry.

The diversity of eight morphologically similar genotypes of longan was analysed using ISSR markers by Mariana *et al.,*(2008). The UPGMA and SAHN algorithms were used to create a dendrogram of the genotypes. The cluster displayed a similarity range between 0.34 and 0.86.

Noroozi *et al.,*(2009) used 6 inter simple sequence repeat (ISSR) primers to examine genetic connections among 31 cultivars of Iranian pistachio. The three primers amplified a total of 28 bands, 13 of which were polymorphic (46.42%), with an average of 9.3 bands per primer. The examined genotypes were clustered into 11 main groups in the unweighted pair group method with arithmetic averages (UPGMA) dendrogram.

Dje *et al.,*(2010) evaluated the genetic variability of 80 edible seeded accessions of *Citrullus lanatus* using the 20 ISSR primers. These primers produced 258 bands, of which 252 were polymorphic (97.67 percent). The accessions were grouped into three major clusters having small variances within each category. Between the 80 individuals, the pairwise genetic distance ranged from 0 to 0.61.

Luo *et al.,* (2011) employed ISSR and SCOT primers to compare 23 mango germplasm accessions obtained from China's Guangxi province. Eighteen ISSR primers amplified 156 bands, with 87 polymorphic bands (55.77%). The Xiang Ya Mango cultivars and their offspring showed a strong genetic resemblance.

Tian *et al.,*(2015) employed ISSR and RAPD markers for genetic diversity evaluations of 48 *Prunus mira L* samples and discovered that ISSR marker showed 77.80% polymorphism, which is more than RAPD (72.73%) polymorphism.

The study conducted by Napitu *et al.,* (2016) focused on exploring the genetic diversity of wild rambutan species in Sanggau Regency of West Kalimantan using the Inter-Simple Sequence Repeat (ISSR) marker. The results of the study indicated that the samples collected from Bonti exhibited the highest genetic diversity, as measured by Shannon's diversity index (He) value of 0.18. The analysis also revealed that there were many wild species of rambutan in the Sanggau Regency.

100 ISSR molecular markers were used to examine the genetic links between 24 ancient litchi types in a study by Guilfeng *et al.,*(2017). From these 13 primers with distinct bands and good repeatability were selected which developed 96 bands of which 43 were polymorphic with a polymorphism percentage of

44.79%. With an average relative genetic similarity coefficient of 0.61, the genetic similarity coefficients among the examined ancient litchi germplasm ranged from 0.43 to 1.00. The evaluated litchi genotypes were divided into three groups in UPGMA cluster analysis at the genetic similarity coefficient of 0.66.

Manggabarani *et al.,*(2020) employed 31 ISSR primers to assess genetic variability among 30 rambutan accessions collected from Cipaku Orchard and Mekar Sari Park in Indonesia. In this study, only 6 ISSR primers showed polymorphism with 58 polymorphic bands (87 percent). The UPGMA dndrogram clustered these accessions into three major groups. They concluded that the ISSR marker could be utilized as a reliable technique to characterize rambutan cultivars.

The genetic diversity and population relationships of 50 apricot (*Prunus armeniaca L.*) accessions were examined using four ISSR molecular markers by Sheikh *et al.,* (2021). The results showed that the number of alleles per locus ranged from 4 to 8, with a mean value of 6.75, and the mean effective number of alleles (Ne) per locus was 1.54. The polymorphic information content (PIC) values varied between 0.464 to 0.424, with 0.424 as the mean. With a mean value of 0.04, the marker index values ranged from 0.01 to 0.06.

Using ISSR markers, 24 different European cranberry bush (*Viburnum opulus L.*) genotypes were assessed by Yaman (2021). Among the twenty primers used 11 showed good polymorphism. In an ISSR investigation of *Prunus salicina L*, the 15 ISSR primers used resulted in 179 bands with sizes ranging from 300 to 2500 bp and polymorphism was 87.74% (Li *et al.,* 2022).

76 genotypes of *Selenicereus megalanthus* H. (yellow pitahaya) characterized using eight ISSR markers by Morillo *et al.,*(2022). In this study an average heterozygosity value of 0.34 and a genetic differentiation coefficient (Fst) of 0.26 were obtained, indicating a high level of genetic diversity.

Coronado *et al.,* (2023) conducted a study to characterize the genetic diversity of five *Passiflora spp.* species in Boyacá, Colombia, using ISSR markers. A total of 70 genotypes from 11 municipalities in Boyacá were analyzed. Eight ISSR primers generated 138 loci, resulting in a high percentage of polymorphic loci (>80%). The genetic similarity analysis grouped the genotypes

into two populations, primarily composed of individuals from the same species but with different geographic origins. The average heterozygosity values ranged from 0.29 to 0.36 for population I and II, respectively, while the polymorphic information content was relatively low. Moderate genetic differentiation (0.16) and high gene flow (3.35) were observed.

The diversity and population structure of 29 spring frost-tolerant genotypes of Persian walnut were studied using 14 ISSR markers by Holasou *et al.,* (2023). This study revealed that a moderate level of genetic diversity existed among the genotypes studied and the STRUCTURE analysis, UPGMA clustering, and principal components analysis consistently divided them into two major groups.

Ikten *et al.,*(2023) aimed to analyze the genetic relationship and develop molecular markers associated with sex expression in natural populations of *Ficus carica* (fig) trees. They utilized sequence-related amplified polymorphism (SRAP), male-specific marker-SRAP (MS-SRAP), and inter-simple sequence repeat (ISSR) primers to amplify DNA from 47 male and 49 female fig genotypes collected from various regions in Turkey (with two genotypes from the USA). A total of 62 primers produced 353 markers, with 149 being polymorphic. While the genotypes were successfully differentiated using unweighted pair group method with arithmetic mean (UPGMA) and principal component analyses (PCA), no strict clustering was observed based on gender or geographic origin. The average genetic similarity among the fig genotypes, combining data from ISSR, SRAP, and MS-SRAP, was 0.65.

2.2.3 Importance of SSR Primers in Plants

SSRs are di-, tri-, tetra-, and pentanucleotide tandem repeats (Hamada *et al.,*1982; Tautz, 1989; Weber and May, 1989) that may monitor high levels of variability at numerous loci and can serve as a substantial source of genetic diversity.

The term "microsatellite" or "simple sequence repeat" refers to tandemly repeated small nucleotide units in the genome that are between 1 to 5 bp in length

(Staub and Serquen, 1996; Powell *et al.,* 1996). Compared to other primers SSR Marker system requires only a small amount of DNA (Rafalski *et al.,*1996). It consists of 2 primers, which are complimentary to the adjacent region of repetitive sequences and assist to amplify them

SSR is a Polymerase chain reaction-based molecular approach (Staub and Serquen, 1996) that works on the idea of detecting polymorphisms caused by variations in the numbers of repeat units in different individuals. SSR is a good marker for mapping, diversification studies, fingerprinting, and population genetics because of its high amount of polymorphism (Jones *et al.,* 1997; Mohan *et al.,* 1997).

In a study by Garland *et al.,* (1999), microsatellites helped to create a database that can be used to identify varieties and develop molecular Primers for marker-assisted selection.

In genetic studies like population genetics, molecular breeding, and paternity testing microsatellites (also known as SSRs, or Simple Sequence Repeats) are one of the most frequently used molecular markers (Ellegren, 2004). SSR primers can be identified either by data mining of existing sequences (Sharma *et al.,*2007) or by creating and sequencing SSR-enriched libraries. SSR primers are numerous, co-dominant, multi-allelic, highly repeatable, and simple to use (Richard *et al.,* 2008).

SSR primers have a high degree of transferability between closely related species, which is one of their greater qualities (Ziya *et al.,*2016). SSR primers remain the most popular efficient, reliable, reproducible, and user-friendly technology to determine genotypes, even in the contemporary day when genotyping by sequencing (GBS) has become affordable (Hayano *et al.,*2017).

According to Aljumaili *et al.,* (2018), SSR is a useful marker for examining genetic variation. In many population genetic investigations of tropical plants, SSRs continue to be the marker of first choice (Martnez *et al.,* 2019)

2.2.4 Molecular Characterization using SSR Primers

Ravishankar *et al.,* (2011) developed a few SSR markers to analyze the genetic diversity of mango cultivars (*Mangifera indica*) and related species. On 30 different mango cultivars, 36 microsatellite loci were studied. The range of polymorphic information content values was 0.185 to 0.920. The research came to the conclusion that recently discovered SSRs would be transferrable to closely related *Mangifera* species like *M. odorata, M. anadamanica, M. zeylanica, M. camptosperma, and M. griffithii.*

Kumar *et al.,* (2013) investigated genetic variation of ten mango genotypes viz., Kalepad, Neelum, Swamarekha, Alphonso Rumani, Sendura, Banganapalli, Himayuddin, Mulgoa, and Bangalora. PCR amplification using 20 SSR primers yielded a total of 240 amplified products, of which 184 were polymorphic and 56 were monomorphic. The alleles found ranged in size from 120 to 369 bp. SSR markers were highly polymorphic with an average of 2.70 alleles per primer. The polymorphic information content (PIC) of SSRs was moderate, ranging from 0.320 to 0.774.

According to Madhou *et al.,* (2013), SSR markers were more accurate than other markers for revealing high levels of allele diversity and providing more data on codominant characters in lychees.

In a research on mulberry species, Mathithumilan *et al.,*(2013) developed several locus-specific genic and genomic SSR markers that can be utilized for molecular analysis. A total of 188 primers, including 51 genic SSR primers and 137 genomic primers, were designed. Of these 164 markers were polymorphic among mulberry species and 149 primers could be utilized for diversity analysis in other related species like Ficus, fig, and Jackfruit.

SSR molecular primers are employed in numerous conservation initiatives to protect tropical species. Nine polymorphic SSR primers were employed to interpret the genetic structure and diversity of *Annona cherimola Mill.* to maintain germplasm that could be a source of biotic and abiotic stress tolerance and to ensure food security for future generations (Larranaga *et al.,* 2017).

In the date palm, *Phoenix dactylifera*, 19 SSR primers were employed to ascertain the population structure of 195 accessions from Asia and Africa and to comprehend their susceptibility to diseases and insect pests (Chaluvadi *et al.,* 2018).

Using SSR molecular markers, Medrano *et al.* (2018) carried out an experiment to distinguish Mexican lemon hybrids. In the INIFAP Tecoman Experimental Field, a population of 203 hybrids from crosses made between 2009 and 2012 was utilized.

Using 12 simple sequence repeat (SSR) markers, the genetic diversity of 14 landraces of the Japanese plum *Prunus salicina Lindl*, which are adapted to an ecosystem with alternating wet and dry conditions, was identified by Acuna *et al.,* (2019). The experiment generated 66 different alleles and the levels of genetic diversity were moderate to high.

Hu *et al.,*(2019) developed novel EST-derived SSR markers based on the transcriptome sequencing data of the flesh of a new longan cultivar called "Xiangcui," and tested their applicability on 4 related plants (*Sapindus mukorossi, Rambutan, Litchi chinensis, and Dimocarpus confinis*) and studied their genetic relationships. The percentage of polymorphism ranged from 50% to 100%. The transfer rates of longan polymorphic primers to S. mukorossi Gaertn., *N. lappaceum Linn., L. chinensis Sonn*., and D. confines H.S.Lo were 38%, 38%, 68.09%, and 74.47%, respectively and UPGMA clustering analysis classified the 55 germplasms into 5 groups, including longan, longli, litchi, Sapindus, and Nephelium, with a genetic similarity coefficient of 0.561.

In order to eliminate duplications and for effective genetic characterization Patzak *et al.,* (2019) used 19 SSR molecular markers to characterize 123 local sweet cherry cultivars (*Prunus avium L)* and 115 polymorphic fragments were amplified. The hierarchical cluster analysis of genetic variation developed three primary clusters and 10 subgroups.

Tran *et al.,* (2019) conducted a genetic variability study on Litchi cultivars with 15 SSR primers which separated the 45 local Vietnamese lychee cultivars into three main groups using the average-based unweighted pair-group clustering

approach. Cluster 1 (Group A) consists of semi-natural lychees. while Cluster 2 (Group B) consists of developed cultivars ("intermediate") Cluster 3 (Group C) represents accessions of *X. noronhianum*.

Using 10 pairs of polymorphic SSR primers, the genetic diversity of 68 rambutan (*Nephelium lappaceum L.*) accessions was examined by Xing *et al.,*(2019) and the findings showed that all of the primers exhibited polymorphism; 20 polymorphic alleles were identified, and the average polymorphic information content (PIC) was 0.393. The genetic similarity among accessions ranged from 0.290 to 0.664.

The genetic diversity of mango cultivars from various regions was evaluated employing 46 polymorphic SSRs by Yamanaka *et al.,* (2019).

For the understudied *Nephelium lappaceum*, RAD sequencing technologies were used to create microsatellite markers by Razak *et al.,*(2020). A total of 1403 microsatellite markers including 853 di-, 525 tri-, 17 tetra-, 5 penta-, and 3 hexanucleotide microsatellite markers were successfully created. The genetic diversity of the 22 selected rambutan varieties was then assessed using a selection of 39 microsatellites. The diversity of the aforementioned rambutan varieties was evaluated using 12 microsatellites, which had high call rates in all samples. Twelve microsatellites were analysed, and the results showed an average of six alleles per locus and 72 total alleles. The largest degree of dissimilarity between R5 and R170 was revealed by the range of values for the pairwise genetic distance of shared alleles, which ranged from 0.046 (R134R170) to 0.818 (R5R170). These research results are particularly beneficial for varietal identification, proper management and protection of the genetic resources, and exploitation and utilisation in future breeding programmes.

Using capillary electrophoresis, 3870 SSR primer sets in total were produced by Arias *et al.,*(2020) five groups of tropical perennial plants with edible fruits and shoots and four to ten DNA samples from each plant group including Rambutan (Nephelium lappaceum L.), sapodilla (Manilkara zapota), Litchi (Litchi chinensis Sonn.), mangosteen (Garcinia mangostana Linn) and bamboo (Bambusa vulgaris) were tested. They selected 178 polymorphic SSRs (between 26 and 47

per group) that amplified all the samples, had robust fluorescence signals, displayed no stutters, and displayed the least amount of non-specific amplification or background fluorescence. 66,057 contig sequences generated in this study were uploaded to the GenBank database. These SSR Molecular primers can be effectively used for genetic diversity analysis and population genetic studies.

According to the findings of Barbosa *et al.,*(2021) 147 young leaf samples of *Passiflora setacea* were collected from 18 separate sites and subjected to SSR analysis. High levels of genetic divergence within populations were found, and the Mantel test revealed no link between genetic and geographical distances.

Using two SSR primers, a total of 10 polymorphic bands were formed among the 70 mulberry accessions, and the amplified loci showed high levels of polymorphism, Kadri *et al.,*(2021).

Haque *et al.,* (2022) conducted a genetic diversity analysis on 21Pummelo genotypes using 5 SSR molecular markers and the average PIC value was identified as 0.78 while the total PIC values ranged from 0.66 (CAC23) to 0.8787 (TAA41). The primer TAA41 displayed the largest number of alleles with the highest gene diversity value. The pummelo genotypes were grouped into three primary clusters in NJ cluster analysis as I, II, and III clusters and the average genetic distance was 0.8362.

A genetic diversity assessment of 46 economically significant mandarins grown in various countries was conducted with 110 SSR molecular primers. Among that 64 polymorphic markers amplified a total of 155 alleles and 20 SSR primers produced 20 unique alleles. The polymorphism information content values varied from 0.13 to 0.73, with an average of 0.45. The genotypes were divided into four primary clusters by the diversity analysis. The 'Nagpur' and 'Mudkhed' mandarins grouped in cluster I were closely connected as they displayed the highest genetic similarity with a similarity coefficient of 0.99 (Kaur *et al.,*2022).

The genetic evaluation of 179 persimmon germplasms from 16 distinct ecological populations in Zhejiang Province using 17 SSR molecular primers revealed a medium level of genetic variation (Xu *et al.,*2022). The study

discovered that significant gene exchange had taken place among the other populations, with the exception of the Tiantai Mountain and Xin'an River populations. A total of 228 polymorphic alleles with high polymorphism were identified and the average number of alleles per locus was 13.41, with a range of seven (MDP21) to 22 (DKMP13). The PIC values ranged from 0.8566 to 0.9437 with an average of 0.8965 per primer. The findings revealed a strong relationship between genetic and geographic distance.

Tamiru *et al.,* (2023) aimed to assess the genetic diversity and population structure of banana genotypes using 14 SSR markers. The study analyzed 96 banana genotypes and identified 187 alleles, with an average of 13.36 alleles per SSR marker. The polymorphic information content ranged from 0.52 to 0.93, indicating high diversity. Phylogenetic analysis, principal coordinate analysis, and structure analysis revealed mixed populations, suggesting that genotype grouping did not align perfectly with breeding history and genome composition. However, three major groups were confirmed through clustering and population structure analyses. Analysis of molecular variance indicated higher genetic variation within populations than between populations.

Uddin *et al.,* (2023) conducted a study on the genetic diversity of *Ziziphus nummularia* using morphological attributes and SSR markers. The results highlighted that the mean temperature was a significant factor limiting the distribution of *Z. nummularia*, with Swat being the current suitable growth region and Buner projected to be the future distribution. Phenotypic traits exhibited a significant variation meanwhile in SSR analysis a total of 120 alleles were amplified, with an average of 4.42 alleles per locus. The genetic analysis revealed moderate gene diversity and frequent gene flow among genotypes. Clustering and population structure analyses indicated migration routes and evolution in Swat and Buner regions. Importantly, the study demonstrated the prevalence of genetic variability and the presence of unique alleles in *Z. nummularia* across geographical boundaries.

Materials and methods

3. MATERIALS AND METHODS

The research entitled "Genetic diversity analysis of rambutan *(Nephelium lappaceum L.)* accessions using molecular markers" was conducted at the Regional Agricultural Research Station, Kumarakom, and Department of Fruit Science, College of Agriculture, Vellayani during the period 2020-2022. The materials and methods are given below.

3.1 Experimental materials

Twenty genotypes of rambutan were used as experimental material for this study.

3.1.1 Selection and Identification of superior genotype

The rambutan genotypes identified and classified as export, elite, and commercial types in a previous study by Muhamed (2016) were selected (Table 1).

3.1.2 Location

The samples for this study were collected from three districts of Kerala viz., Kottayam, Pathanamthitta, and Thrissur which are the main rambutan growing areas. Kottayam district lies at latitude 9°59 N, longitude 76°52 E, and elevation 3 m above mean sea level, Pathanamthitta at 9°26 N latitude, 76°78 E longitude, and 31m above mean sea level, and the Thrissur district is located at 10°53 N latitude, 76°2 E longitude, and 2.83 m above mean sea level.

3.1.4 Laboratory chemicals, glassware, and plastic wares

The study utilized laboratory chemicals sourced from Merk, Himedia, and Sigma. The primers used were supplied by IDT, USA. Plastic vessels were obtained from Tarsons Products Pvt. Ltd., while the glassware used was from Borosil. The master mixture was provided by SIGMA, and the ladder was from Invitrogen.

Table 1. Details of rambutan genotypes selected for the study

Si.no	Samples	Location
1	Col.19	Kurichi, Kottayam
2	Col.20	Kurichi, Kottayam
3	Col.01	Kurichi, Kottayam
4	Col.52	Chengalam, Kottayam
5	Col.53	Chengalam, Kottayam
6	Col.02	Akalakunnam, Kottayam
7	Col.03	Akalakunnam, Kottayam
8	Col.04	Akalakunnam, Kottayam
9	Col.05	Pallickathodu, Kottayam
10	Col.06	Pallickathodu, Kottayam
11	Col.48	Pampavalley,Pathanamthitta
12	Col.42	Pampavalley,Pathanamthitta
13	Col.15	Potta ashramam, Chalakudy, Thrissur
14	Col.61	Thekkemala, Kozhencherry, Pathanamthitta
15	Col.62	Thekkemala, Kozhencherry, Pathanamthitta
16	Col.81	Uthimood, Ranni, Pathanamthitta
17	Col.86	Kadayar junction, Ranni, Pathanamthitta
18	Col.87	Kadayar junction, Ranni, Pathanamthitta
19	Col.96	Attachakkal Junction, Konni, Pathanamthitta
20	Col.97	Attachakkal Junction, Konni, Pathanamthitta

3.1.5 Equipment

Regional Agricultural Research Station, Kumarakom, provided the necessary equipment for the work. The centrifuge 5430R (appendorf) was used for centrifugation, and the amplification of DNA was done with the Sure cycler 8800 PCR (Agilent Technologies). The quality and quantity of DNA were assessed using a Bio-Rad electrophoresis device and a Shimadzu UV visible Spectrophotometer. Bio-Rad gel documentation system was used to capture gel images.

3.2 Methods

3.2.1 Molecular analysis

Genetic diversity analysis of Rambutan accessions was performed using ISSR (Inter Simple Sequence Repeat) and SSR (Simple Sequence Repeat) markers.

3.2.2 Genomic DNA Extraction

The genomic DNA was extracted from the leaf samples collected from the selected accessions using the Doyle and Doyle method (1987), with minor modifications.

3.2.2.1 Sample preparation

Fully expanded, fresh, and tender leaves that are healthy were collected from different genotypes for DNA extraction in the early morning hours. The leaves were carried to the laboratory in an ice box after being covered in aluminum foil. The leaf surface was washed with distilled water, wiped with 70 % (v/v) ethanol, and then air dried. These leaves were sealed in polythene bags and stored at -80 ˚C until being used for DNA extraction. All the reagents used were autoclaved and stored at room temperature.

3.2.2.2 Plant genomic DNA isolation protocol

To isolate genomic DNA using the CTAB method, 2x CTAB isolation buffer was preheated to 60°C in a 50ml Oakridge centrifuge tube placed in a water bath. Fresh leaf material (2g) was ground to a fine powder in liquid nitrogen using a pre-chilled mortar and pestle. The powdered sample was transferred to a 2ml Eppendorf tube and 5ml of sorbitol buffer was added. The mixture was then spun at 10,000rpm for 5 minutes, and the aqueous phase was discarded. This sorbitol buffer pre-wash step was repeated 4 to 5 times. Next, the sample was incubated for 30 minutes at 60°C with occasional gentle swirling after adding newly prepared 1ml pre-heated CTAB extraction buffer along with a pinch of polyvinyl pyrrolidine (PVP) and 50µl of 2-β mercaptoethanol. A mixture of chloroform: isoamyl alcohol (24:1) was added in equal volume, and the tube was vigorously mixed by inversion. This step was repeated until the supernatant appeared clearer. The tube was then centrifuged at 10,000rpm for 15 minutes at room temperature and the content got separated into three distinct phases in that top aqueous layer was transferred to a sterile microcentrifuge tube. To precipitate the DNA, 2/3rd volume of cold isopropanol was added, and the tubes were gently inverted several times. The mixture was then incubated at -20°C for 30 minutes. It was then centrifuged at 4°C for 15 minutes at 12,000 rpm. The supernatant was carefully removed. The DNA pellets obtained were centrifuged at 1000 rpm for 5 minutes with 10–20 µl of wash buffer. The supernatant was cautiously discarded. With 70% ethanol, it was washed once more. Ethanol was decanted after the tubes were spun for 5 minutes at 1000 rpm. The remaining pellet was stored at -20°C after air drying and dissolving in 50 µl of TE buffer.

3.2.3 Purification of DNA

RNaseA was used to purify the DNA contaminated with RNA. 50 ml of the sample DNA was mixed with 1 µl of 10 mg/l RNaseA. The sample was then incubated for 1 hour at 37°C in a water bath. The tube was then filled with an equal volume of a 25:24:1 mixture of phenol, chloroform, and isoamyl alcohol.

It was gently mixed by inversion and centrifuged for 20 minutes at room temperature at 12000 rpm. Then the DNA precipitation steps described earlier under the DNA extraction procedure were repeated to obtain more pure DNA pellets. These were stored at -20°C after air drying and dissolving in 50 µl of TE buffer for further analysis.

3.2.4 Assessment of quality and quantity of DNA by Agarose gel electrophoresis

Agarose gel electrophoresis was used to determine the quality of isolated DNA. It is a technique for separating DNA molecules based on their size.

3.2.4.1 Procedure of agarose gel electrophoresis

Using 70% ethanol the comb, gel casting tray, and electrophoresis unit were cleaned. Dissolved 0.8 g of analytical grade (Ultra Pure DNA Grade) agarose in 100 ml of autoclaved 1x TAE by heating, then cooled to 60 ˚C. Added 1-2 µl of 10mg/ml ethidium bromide into it. This melted gel solution was poured into a casting tray after placing a comb, without forming any bubbles, and allowed to set for 40 minutes. After removing the comb, the gel was transferred to the gel tank of the electrophoresis unit with the wells directed towards the cathode and covered with 1x TAE buffer. DNA samples and ladder, mixed with tracking dye(1µl), were loaded into wells. The gel was run at 100V until the tracking dye reaches 2/3 of the gel. The gel was taken out and the DNA band was observed using a UV transilluminator and the gel documentation system. Images of gel that were captured using the gel documentation system and were saved. DNA purity, the presence of RNA and protein, the integrity of the DNA band, and its clarity were checked on the gel picture.

3.2.5 Assessing the quality and quantity of DNA by UV Spectrophotometer

Using a UV Visible spectrophotometer (Shimadzu UV 1800), the quantity and purity of DNA were evaluated. Protein has the maximum absorbance at 280 nm, while nucleic acid at 260 nm. Therefore, the OD 260/OD 280 ratio was measured

to determine the DNA's purity. The DNA is pure if the OD260/OD280 value is between 1.8 and 2, if the DNA is contaminated with proteins then the value will be less than 1.8 and if the value is greater than 2 then it is contaminated with RNA.

3.3 Genetic diversity analysis

SSR (Simple Sequence Repeat) and ISSR (Inter Simple Sequence Repeats) markers were employed in this research for genetic diversity analysis of rambutan

3.3.1 Inter Simple Sequence Repeat (ISSR) analysis.

The inter-simple sequence repeat region of rambutan's genomic DNA was amplified using repeat sequences as primers. For the ISSR analysis, the DNA was diluted to 40 ng/µl.

3.3.1.1 PCR components standardized for Amplification of ISSR Primers

The reaction mixture for PCR amplification was prepared to a volume of 20µl/ sample containing the following components as detailed in Table 2.

Table 2. Components of reaction mixture for ISSR analysis

SI No	ISSR PCR reaction Mixture	Concentration
1	Genomic DNA	40ng
2	$MgCl_2$	1.5mM
3	10X PCR Buffer	1x
4	dNTP mix (Promega)	200µm
5	Taq DNA polymerase (TakaRa)	0.5U
6	ISSR Primers	10 pM

PCR amplification was performed as per the programme detailed below:

Initial denaturation	: 94°C for 4 min
Denaturation	: 94°C for 30 Sec
Annealing	: 50°C to 55°C for 1min
Extension	: 72°C for 1 minute 50 Seconds
Final extension	: 72°C for 10 minutes
Infinite time storage	: 4°C
Number of cycles	: 35

Thirty ISSR primers were used for the preliminary screening for polymorphism in rambutan genotypes under study (Table 1). These ISSR primers were reported for rambutan by Manggabharani *et al.,* (2018).

Table 3. List of ISSR primers used for screening

Sl No	Primer Name	Sequence
1	UBC 807	(AG)8T
2	UBC 808	(AG)8C
3	UBC 813	(CT)8 T
4	UBC 817	(CA)8A
5	UBC 819	(GT)8 A
6	UBC 822	(TC)8A
7	UBC 825	(AC)8 T
8	UBC 828	(TG)8A
9	UBC 834	(AG)8YT
10	UBC 836	(AG)8YA
11	UBC 841	(GA)8YC
12	UBC 842	(GA)8YG
13	UBC 845	(CT)8RG
14	UBC 847	(CA)8RC
15	UBC 849	(GT)8YA
16	UBC 881	(GGGTG)3
17	PIET 10	(GT)9 T
14	N/ISSR-2	(AG)8 TC
15	ISSR-3	CTC CTCCTCCTC AC
16	ISSR 4	(GAG)5 AC
17	N/ISSR-4	(CT)8 GG
18	N/ISSR-5	(GA)8TT
19	ISSR-6	CTCTCTCTCTCTG
20	ISSR-8	ACA CAC ACA CAC ACA CTA
21	ISSR-9	CACACACACACAGT
22	ISSR-1	(AGG)5
23	ISSR-5	(GAG)5 AT
24	ISSR-10	(GA)6 CC
25	ISSR-15	(GTG)3 GC
26	ISSR-23	(GACA)3 CC
27	N/ISSR -1	(CA)6 AT
28	N/ISSR-2	(AG)8 TC
29	UBC-815	(CA)8 A
30	PIET 7	(GA)9 A

3.3.2 Simple Sequence Repeat (SSR) Analysis.

Genomic DNA rambutan was amplified using inter simple sequence repeat as a primer to amplify the simple sequence repeat region. The DNA was diluted to 40ng/µl for SSR analysis. The reaction mixture used for PCR amplification is described in Table 4. The reaction mixture prepared for each sample was 20µl.

Table: 4. Components of reaction mixture for SSR

Si no	Reaction Mixture	Concentration
1	Genomic DNA	40 ng
2	$MgCl_2$	1.5mM
3	dNTP mix (Promega)	200 µM
4	10X PCR Buffer	1x
5	SSR Primer Forward primer Reverse primer	 10 pM 10 pM
6	Taq DNA polymerase(TakaRa)	0.5u

PCR amplification procedure followed is detailed below:

Initial denaturation : 94°C for 4 min

Denaturation : 94°C for 30 Sec

Annealing : 50°C to 55°C for 1min

Extension : 72°C for 1 minute 50 Seconds

Final extension : 72°C for 10 minutes

Infinite time Storage : 4°C

Number of cycles : 35

The diversity study of genotypes was carried out using sixteen SSR primers which were reported previously by Arias *et al.,*(2020) for rambutan (Table 5).

Table 5. List of SSR primers used for preliminary screening

Sl No	Primer Name	Sequence	
		Forward	Reverse
1	NlaSSR21	CCGACTGCTAGTTTACTGCG	ACCAGGCCATCTTTATAGTCCC
2	NlaSSR23	GTTGGTGCAGTCAAGGCTC	GCTGACGACACCGCAGG
3	NlaSSR27	TCTGGTATTTCCCAATCAGCTAC	GGTTGGGTAGTGGAAACCG
4	NlaSSR31	TGTTCCTGCATGTTTATGGC	TCTGTACGTTGGCAGCTTG
5	NlaSSR32	TCGGTGACTCTAGCAAAGAGG	GGTTCAAACCTTCGTGGAC
6	NlaSSR36	ACCGGGAAATCAACAGAGG	AGTAGAAATGGTATCACATTGAGG
7	Stv-nel_08865_a	TTTCACAAAACACCTCTACAGTCCAG	GGACATCCTACAAAACCAGTGGAG
8	Stv-nel_05023_a	GAGAATTTGATGAAACTCACCGAG	AACAATTGCTTTGGTTTAAGATGG
9	Stv-nel_05532_a	TTTTCAAAGGGTTTTGTGAAATGG	AGTAGAGCTTTCACCGCATCAAAC
10	NlaSSR1	TGCAAGTGCATTTCAATAAGAGC	TGTGTCAAGATCAGTTGTAGGG
11	NlaSSR3	AGAAGTTGTGAGTCAACTGAGAC	AGATTTGGTACATACAGAGCAGG
12	NlaSSR 5	AGTCTACAATTTCGCCACAAAG	GTAACTCCAAGCAACCGCC
13	NlaSSR 7	ATTCAACAGGGCTCCTGCC	CAGTGCTCGTGTCTTCACC
14	NlaSSR 12	TCCAGTCTAAGAGCAGCAAATC	TGGTGGTGGCTGCAATCTG
15	NlaSSR 20	CCAGTTTGGATCCTGGACG	CTCCAACAAGGCCATGACAG
16	stv-nel_15792_a	TTCTCTCAGATGTCTTTGGACTTTAGC	TGTATATGGTGCCTTGGATCCTTC

3.4 Scoring of bands and data analysis

The gel image was captured using image lab software. The bands were given a score of 1 for their presence and a score of 0 for their absence. The band sizes were determined based on the size of the ladder. Based on these manually rated bands, genetic variability was assessed using NTSYS version 2.1 (Rohlf, 1992), and cluster analysis was performed using the Unweighted Pair Group Method (UPGMA) (Sneath and Sokal, 1973).

3.4.1 Polymorphism percentage

The percentage of polymorphism in the genotypes was calculated as follows. The total number of polymorphic bands / Total number of bands detected x 100= Polymorphism percentage.

3.4.2 Polymorphic information content (PIC) and Marker index (MI)

Weising *et al.,(*2005) proposed the polymorphic information content (PIC). Both PIC and MI confirm the primer's applicability; PIC indicates a marker's ability to detect polymorphism within a population, while MI aids in understanding a primer's ability to detect polymorphic locus among varieties.

Polymorphic information content in dominant marker (Roldan-Ruize *et al.,*2000),

$$PICi = 2fi(1-fi)$$

Where fi is the frequency of an amplified allele (band present) and $(1-fi)$ is the frequency of the null allele (band absent) of marker i.

Polymorphic information content in co-dominant marker (Milbourne *et al.,*1997)

$$PICi = 1-\sum pi2$$

Where pi is the frequency of i *th* allele.

Marker index (Powell *et al.,*1996) of both markers.

MI = PIC x No.of Polymorphic bands.

Results

4. RESULTS

The study on "Genetic diversity analysis of rambutan (*Nephelium lappaceum L.*) accessions using molecular markers" was carried out at the Regional Agricultural Research Station, Kumarakom, and Department of Fruit Science, College of Agriculture, Vellayani during the period 2020-2022. Twenty genotypes of rambutan collected from the Kottayam, Ernakulam, Pathanamthitta, and Thrissur districts of Kerala were used for the study. The results are described below.

4.1 DNA Extraction

The CTAB method explained by Doyle and Doyle (1987) was followed for DNA extraction with slight modifications. The modification suggested by Mir *et al.,* (2018) was incorporated into the protocol by adding polyvinylpyrrolidone (PVP) to improve DNA quality. The plant samples were subjected to sorbitol buffer pre-wash prior to DNA extraction with CTAB buffer to remove the interfering metabolites (Inglis *et al.,* 2018 and Manggabarani *et al.,*2018).

In order to isolate nick-free DNA, the plant material was powdered using liquid nitrogen (Blin and Stafford, 1976). To obtain a clearer solution, two cycles of centrifugation using a chloroform-isoamyl alcohol (24:1) mixture were performed. To thoroughly remove salt and detergents, 70% ethanol was used after washing the DNA pellet with the wash buffer. Additional purification steps were also taken to get DNA that was free of contaminants.

4.1.1 DNA Quantification

The UV spectrophotometer was used to quantify and evaluate the purity of the DNA. The nucleic acid has the highest absorbance at 260 nm, while protein has the highest at 280 nm. The OD 260/280 ratio was calculated to assess DNA purity. The OD ratio of DNA samples ranged between 1.81 and 1.95, indicating their purity. The DNA concentration of the samples varied between 220 to 386 ng/µl (Table 6).

Table 6. Quantification of purified DNA from Twenty rambutan genotypes

SI.No.	Genotypes	Optical density (A260/280)	Quantity(ng/µl)
1	Col.019	1.83	220
2	Col.020	1.80	278
3	Col.01	1.84	264
4	Col.052	1.92	317
5	Col.053	1.81	298
6	Col.02	1.90	377
7	Col.03	1.89	283
8	Col.04	1.95	347
9	Col.05	1.91	321
10	Col.06	1.88	265
11	Col.048	1.84	277
12	Col.042	1.89	386
13	Col.015	1.90	222
14	Col.061	1.86	293
15	Col.062	1.87	363
16	Col.081	1.83	319
17	Col.086	1.87	284
18	Col.087	1.83	322
19	Col.096	1.92	272
20	Col.097	1.80	329

Agarose gel electrophoresis was used to assess the quality and integrity of DNA (Sambrook *et al.,*1989).

M-DNA ladder (100bp), 1-Col.19, 2-Col.20, 3- Col.01,4-Col.02, 5-Col.03, 6-Col.04, 7-Col.05, 8-Col.52, 9-Col.53, 10-Col.06. M- DNA ladder (100bp), 11-Col.48, 12-Col.42, 13-Col.15,14-Col.61,15-Col.62, 16-Col.81, 17-Col.86, 18-Col.87, 19-Col.96, 20-Col.97.

Plate 1. DNA Isolated from twenty rambutan genotypes.

4.2 Molecular marker analysis

Molecular marker analysis of rambutan genotypes was carried out by using two different primer systems: Inter Simple Sequence Repeats (ISSR) and Simple Sequence Repeats (SSR). Preliminary screening of primers was carried out with thirty ISSR and sixteen SSR primers. The concentration of DNA used for PCR reactions was 40ng/µl. The results of the experiments are given below.

4.2.1 Preliminary screening of markers

4.2.1.1 ISSR Markers

Out of the thirty primers screened, eleven primers displayed good polymorphism and repeatability and they were utilized for further diversity analysis (Table 7 & 8).

Table 7. Details of DNA amplification pattern obtained with Thirty ISSR Primers

SI. No.	Primer	Amplification pattern			
		No.of amplicon	Types of amplicons		Remarks
			Distinct	Faint	
1	UBC 807	0	0	0	Rejected
2	UBC 808	3	2	1	Rejected
3	UBC 813	8	8	0	Selected
4	UBC 817	2	0	2	Rejected
5	UBC 819	9	7	2	Selected
6	UBC 822	8	0	8	Rejected
7	UBC 825	10	8	2	Selected
8	UBC 828	11	6	5	Selected
9	UBC 834	3	0	3	Rejected
10	UBC 836	5	1	4	Rejected
11	UBC 841	3	0	3	Rejected
12	UBC 842	5	3	2	Rejected
13	UBC 845	3	0	3	Rejected
14	UBC 847	6	1	5	Rejected
15	ISSR-3	2	2	0	Rejected
16	ISSR 4	4	0	4	Rejected
17	N/ISSR-4	9	0	0	Rejected
18	N/ISSR-5	7	4	3	Selected
19	ISSR-6	6	0	6	Rejected
20	ISSR-8	8	7	1	Selected
21	ISSR-9	2	0	2	Rejected
22	ISSR-1	9	7	2	Selected
23	ISSR-5	10	8	2	Selected
24	ISSR-10	9	4	5	Selected
25	ISSR-15	7	5	2	Selected
26	ISSR-23	8	7	1	Selected
27	N/ISSR -1	1	1	0	Rejected
28	UBC- 818	5	0	5	Rejected
29	UBC-815	0	0	0	Rejected
30	PIET 7	0	0	0	Rejected

Table 8. Details of Selected ISSR Primers

SI. No	Primer	Annealing temperature (°C)	Nucleotide sequence
1	ISSR-1	53	(AGG)5
2	ISSR-5	52	(GAG)5 AT
3	ISSR-10	50	(GA)6 CC
4	ISSR-15	50	(GTG)3 GC
5	ISSR-23	51	(GACA)3 CC
6	UBC 813	52.5	(CT)8 T
7	UBC 819	50	(GT)8 A
8	ISSR-8	54	ACA CAC ACA CAC ACA CTA
9	N/ISSR-5	52.5	(GA)8TT
10	UBC 825	50	(AC)8 T
11	UBC 828	51	$(TG)_8A$

4.2.1.2 Simple Sequence Repeat (SSR) markers

Sixteen SSR primer sets were screened for polymorphism and five SSR primer sets were selected for further analysis based on their amplification pattern (Table 9). The details of Selected SSR primers were given in Table 10.

Table 9. Details of DNA amplification pattern obtained with Sixteen SSR Primers

SI. No.	Primer	Amplification pattern			
		No.of amplicon	Types of amplicons		Remarks
			Distinct	Faint	
1	NlaSSR21	1	0	1	Rejected
2	NlaSSR23	3	2	1	Selected
3	NlaSSR27	0	0	0	Rejected
4	NlaSSR31	1	0	1	Rejected
5	NlaSSR32	1	0	1	Rejected
6	NlaSSR36	0	0	0	Rejected
7	Stv-nel_08865_a	0	0	0	Rejected
8	Stv-nel_05023_a	2	0	2	Rejected
9	Stv-nel_05532_a	0	0	0	Rejected
10	NlaSSR1	1	0	1	Rejected
11	NlaSSR3	4	4	0	Selected
12	NlaSSR 5	6	6	0	Selected
13	NlaSSR 7	3	2	1	Selected
14	NlaSSR 12	6	5	1	Selected
15	NlaSSR 20	0	0	0	Rejected
16	Stv-nel_15792_a	3	0	3	Rejected

Table.10 Details of Selected SSR Primers

SI. No.	Primer	Annealing temperature (°C)	Nucleotide sequence (5'-3')
1	NlaSSR 23	52	F: GTTGGTGCAGTCAAGGCTC R: GCTGACGACACCGCAGG
2	NlaSSR 3	55	F: AGAAGTTGTGAGTCAACTGAGAC R: AGATTTGGTACATACAGAGCAGG
3	NlaSSR 5	52	F: AGTCTACAATTTCGCCACAAAG R: GTAACTCCAAGCAACCGCC
4	NlaSSR 7	53	F: ATTCAACAGGGCTCCTGCC R: CAGTGCTCGTCTTCACC
5	NlaSSR 12	55	F: TCCAGTCTAAGAGCAGCAAATC R: TGGTGGTGGCTGCAATCTG

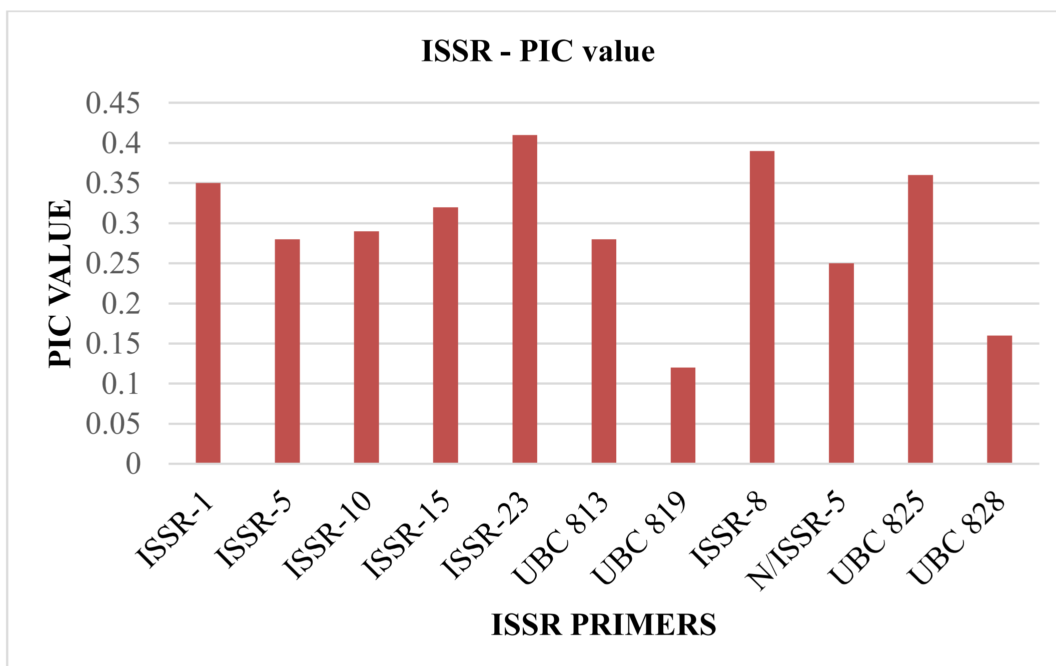
4.2.3 Polymorphic information content (PIC) of selected ISSR and SSR primers

The 11 ISSR primers developed a total of 83 loci among the 20 rambutan genotypes studied, with an average of 7.54 loci per primer (Table 11). The average number of polymorphic loci generated per primer was 6.0. The polymorphism percentage of ISSR primers ranged from 37.50% (UBC - 819) to 100% (ISSR-1 &UBC - 828). The Polymorphic Information Content (PIC) values of ISSR primers ranged from 0.12 (UBC-819) to 0.39 (ISSR-8) with an average value of 0.29 (Fig 1 a) which indicated the usefulness of these primers.

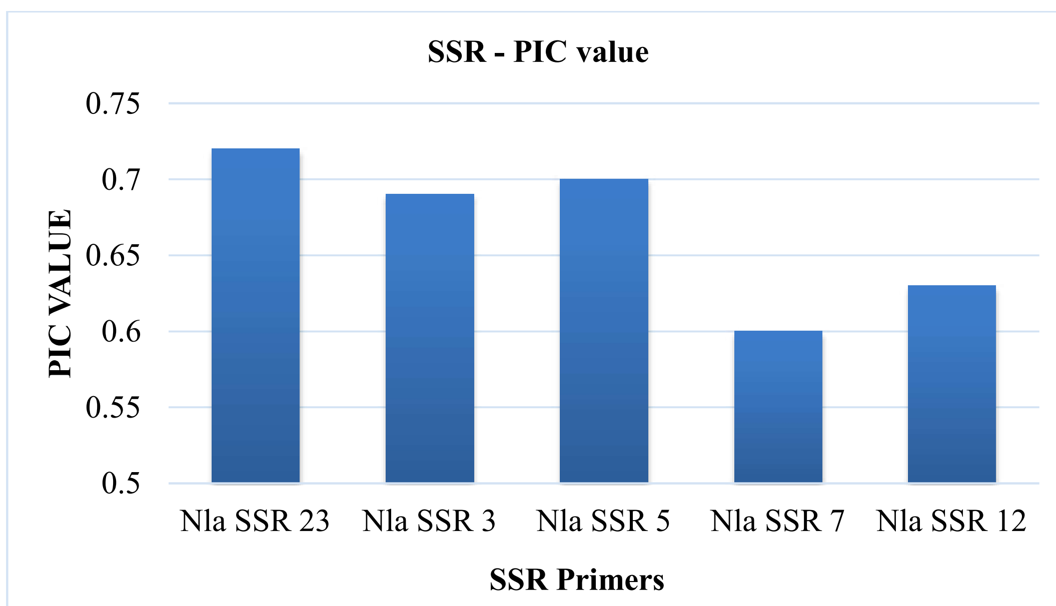
The SSR primers yielded a total of 18 amplicons with an average of 3.6 per primer (Table.12). The percentage of Polymorphism recorded was 100%. The PIC Value for the selected SSR primers ranged between 0.60 (NlaSSR 7) to 0.72(NlaSSR 23) with an average of 0.66 (Fig 1 b).

4.2.4 Marker index of selected ISSR and SSR primers.

Marker index (MI) was computed for selected ISSR (Table 11, Fig 2 a) and SSR (Table 12, Fig 2 b) primers. The MI values of ISSR Primers were within the range of 0.36 (UBC 819) to 2.88 (UBC 825) with an average of 1.70. In the case of SSR markers the MI varied between 1.80 (NlaSSR 7) to 2.88(NlaSSR 23) with an average value of 2.42.

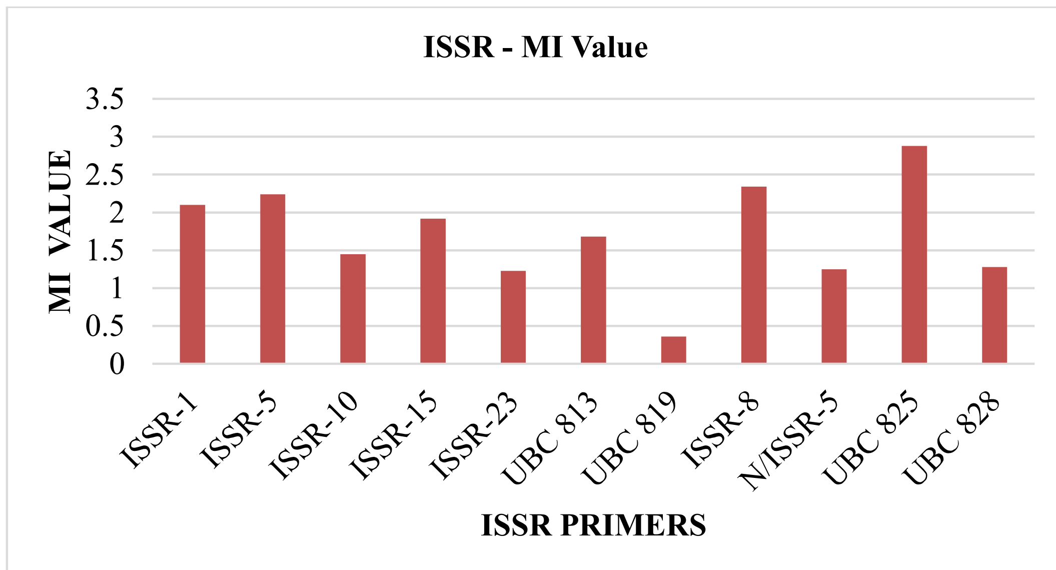


a. **PIC value of ISSR primers**

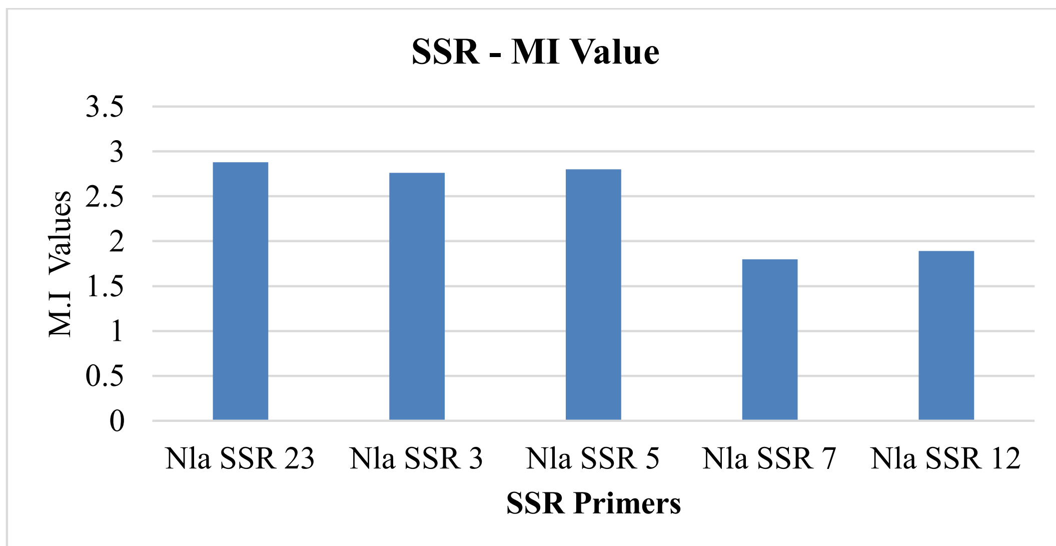


b. **PIC value of SSR primers**

Fig. 1 Polymorphic information content of selected ISSR and SSR Primers



a. MI value of ISSR primers



b. MI Value of SSR Primers

Fig.2 Marker index of selected ISSR and SSR primers

Table.11 Details of DNA amplification with selected 11 ISSR Primers

SI. No.	ISSR primer	Total no. of loci	No.of Polymorphic loci	Polymorphism (%)	Polymorphic Information Content (PIC)	Marker Index (M.I)
1	ISSR-1	6	6	100.00	0.35	2.10
2	ISSR-5	10	8	80.00	0.28	2.24
3	ISSR-10	9	5	55.56	0.29	1.45
4	ISSR-15	7	6	85.71	0.32	1.92
5	ISSR-23	6	5	83.33	0.41	1.23
6	UBC 813	7	6	85.71	0.28	1.68
7	UBC 819	8	3	37.50	0.12	0.36
8	ISSR-8	7	6	85.71	0.39	2.34
9	N/ISSR-5	7	5	71.43	0.25	1.25
10	UBC 825	9	8	88.89	0.36	2.88
11	UBC 828	8	8	100	0.16	1.28
Total		**83**	**66**	**873.84**	**3.21**	**18.73**
Average		**7.54**	**6.0**	**79.44**	**0.29**	**1.70**

Table.12 Details of DNA amplification with selected 5 SSR Primers

SI. No	SSR primer	Total no. of loci	No.of Polymorphic loci	Polymorphism (%)	Polymorphic Information Content (PIC)	Marker Index (M.I)
1	NlaSSR 23	4	4	100	0.72	2.88
2	NlaSSR 3	4	4	100	0.69	2.76
3	NlaSSR 5	4	4	100	0.70	2.80
4	NlaSSR 7	3	3	100	0.60	1.80
5	NlaSSR 12	3	3	100	0.63	1.89
Total		**18**	**18**	**500**	**3.34**	**12.13**
Average		**3.6**	**3.6**	**100**	**0.66**	**2.42**

4.2.5 Molecular analysis using the selected ISSR Primers

Molecular characterization of 20 promising rambutan genotypes was carried out with eleven selected ISSR primers and the details of amplification are described below:

4.2.5.1 ISSR – 1

PCR amplification using this primer resulted in six amplicons, all of which are polymorphic (Plate 2). The molecular weight of amplicons generated ranged from 480bp to 950bp. The polymorphism percentage was 100%.

4.2.5.2 ISSR – 5

This primer generated ten amplicons with sizes varying between 200bp to 1000bp. Among them, eight loci displayed polymorphism (Plate 3), resulting in a polymorphism percentage of 80%.

4.2.5.3 ISSR – 10

The amplification pattern with ISSR-10 revealed that nine amplicons were generated with sizes ranging from 180bp to 1000bp (Plate 4). Out of these five amplicons were polymorphic resulting in a 55.56% of polymorphism.

4.2.5.4 ISSR – 15

The ISSR analysis with the primer ISSR-15 developed seven amplicons (Plate 5), out of which six exhibited polymorphism. This resulted in a polymorphism percentage of 85.71%. The molecular weight of the amplified fragments ranged from 350bp to 1000bp.

4.2.5.5 ISSR-23

The primer ISSR-23 generated 6 bands on amplification between 300bp to 1000bp (Plate 6) and out of these, five bands exhibited polymorphism resulting in a polymorphism percentage of 83.33%.

4.2.5.6 UBC 813

The primer UBC 813, amplified seven bands (Plate 7) and six were found to be polymorphic. The polymorphism percentage was 85.71. The size of the amplicons varied between 450 to 1000bp. The polymorphic band at 650bp was shared between Col.04 and Col.05.

4.2.5.7 UBC 819

For the ISSR primer UBC 819 (Plate 8), 8 amplicons were generated of which only 3 were polymorphic resulting in a polymorphism percentage of 37.50%. The size of these amplicons was between 250 to 1000 bp.

4.2.5.8 ISSR-8

ISSR analysis using the ISSR-8 primer developed seven distinct bands of which six were polymorphic. This indicates a polymorphism percentage of 85.71%. The molecular size of the amplified fragments ranged from 400 to 1000bp.

4.2.5.9 N/ISSR-5

Using the N/ISSR-5 primer, DNA samples of twenty rambutan genotypes were subjected to ISSR analysis (Plate 10). Out of the seven loci amplified, five exhibited polymorphism, resulting in a polymorphism percentage of 71.43%. The amplified fragments ranged in molecular size from 200 to 1000bp.

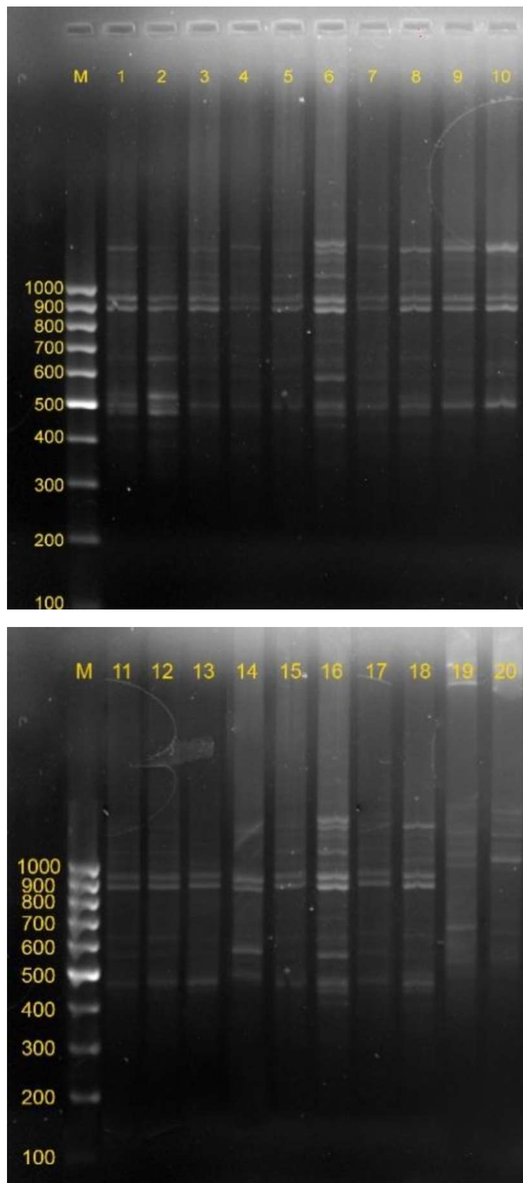
4.2.5.10 UBC 825

ISSR analysis with the primer UBC 825 amplified nine fragments (Plate 11). Out of these eight were polymorphic bands resulting in a polymorphism percentage of 88.89%. The molecular size of the amplified fragments ranged from 100 to 1000 bp. The polymorphic band at 180bp was amplified only in Col.62, Col.87, and Col.97.

4.2.5.11 UBC 828

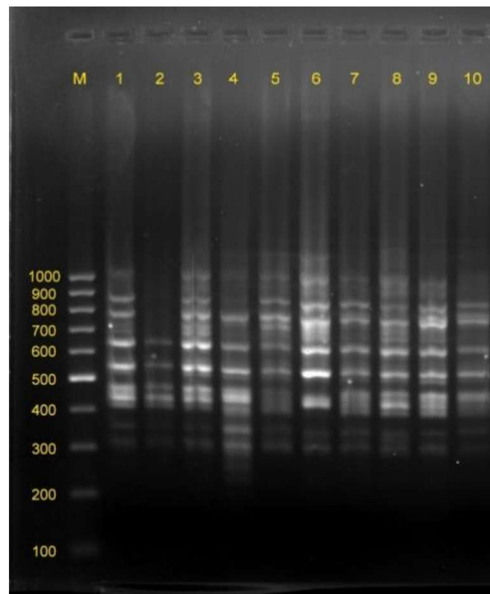
The UBC 828 primer exhibited an amplification pattern (Plate 12) with eight amplified loci which were polymorphic. The amplicons ranged in molecular size from

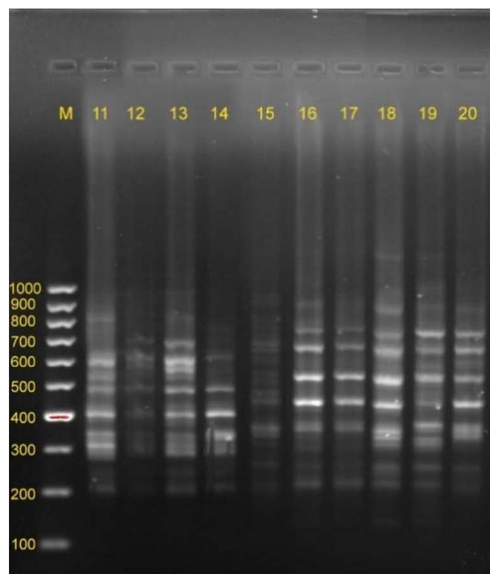
350 to 1000bp. The loci at 350bp and 700 bp were amplified only in the genotype Col.04 and hence may be used for identifying this genotype.



M-DNA marker (100bp), 1-Col.19, 2-Col.20, 3- Col.01, 4-Col.02, 5-Col.03, 6-Col.04, 7-Col.05, 8-Col.52, 9-Col.53,10-Col.06. M- DNA marker (100bp),11-Col.48, 12-Col.42, 13-Col.15,14-Col.61,15-Col.62,16-Col.81, 17-Col.86, 18-Col.87, 19-Col.96, 20-Col.97.

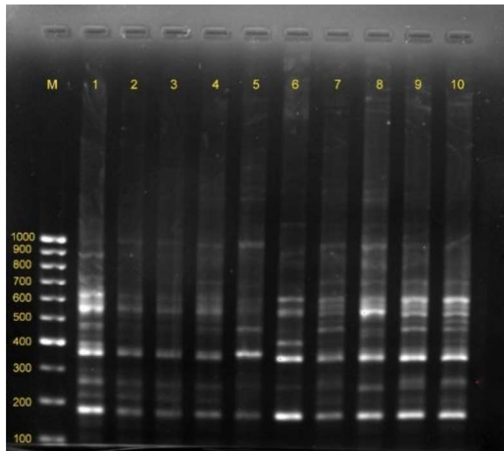
Plate 2. DNA amplification pattern generated with primer ISSR 1

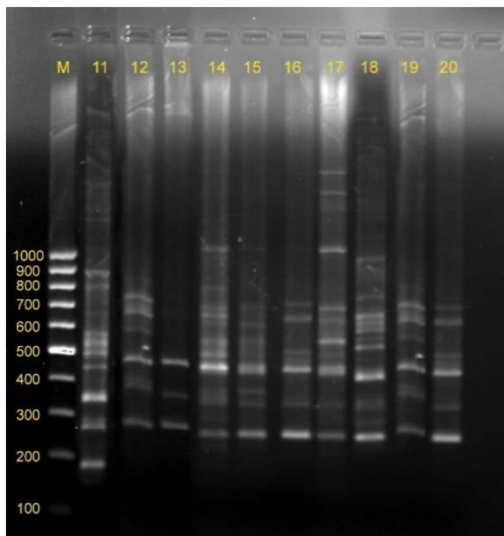




M-DNA marker (100bp), 1-Col.19, 2-Col.20, 3-Col.01, 4-Col.02, 5-Col.03, 6-Col.04, 7-Col.05, 8-Col.52, 9-Col.53,10-Col.06. M- DNA marker (100bp), 11-Col.48, 12-Col.42, 13-Col.15, 14-Col.61, 15-Col.62, 16-Col.81, 17-Col.86, 18-Col.87, 19-Col.96, 20-Col.97.

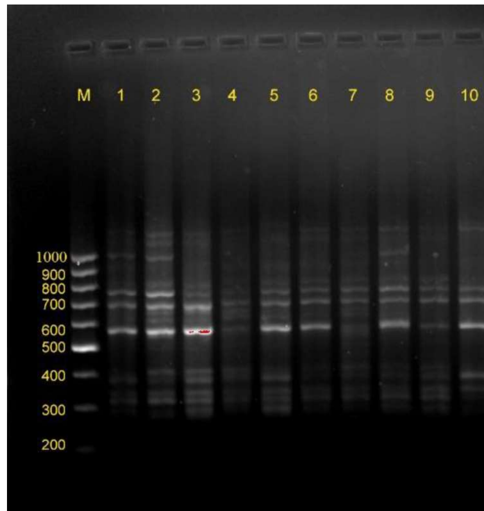
Plate 3. DNA amplification pattern generated with primer ISSR – 5

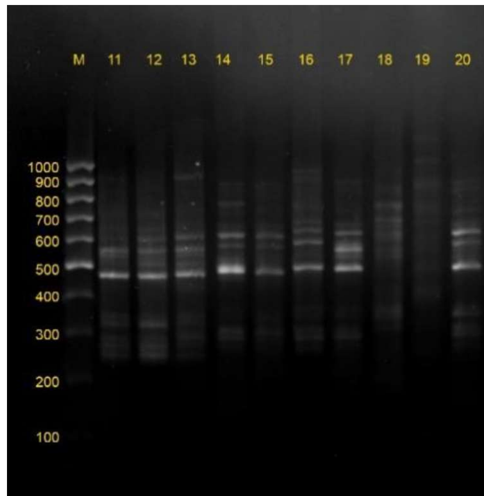




M-DNAmarker(100bp),1-Col.19, 2-Col.20, 3-Col.01, 4-Col.02, 5-Col.03, 6-Col.04, 7-Col.05,8-Col.52,9-Col.53,10-Col.06. M- DNA marker (100bp), 11-Col.48, 12-Col.42, 13-Col15, 14-Col.61, 15-Col.62, 16-Col.81, 17-Col.86, 18-Col.87, 19-Col.96, 20-Col.97.

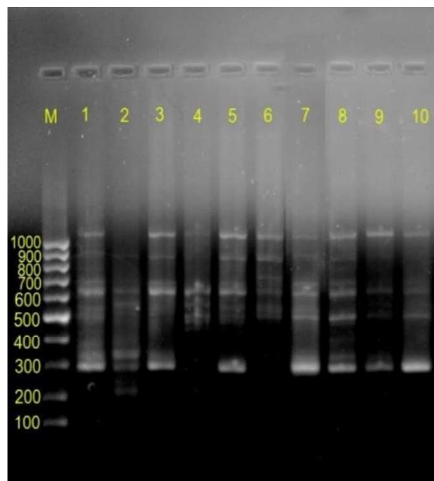
Plate 4. DNA amplification pattern generated with primer ISSR-10

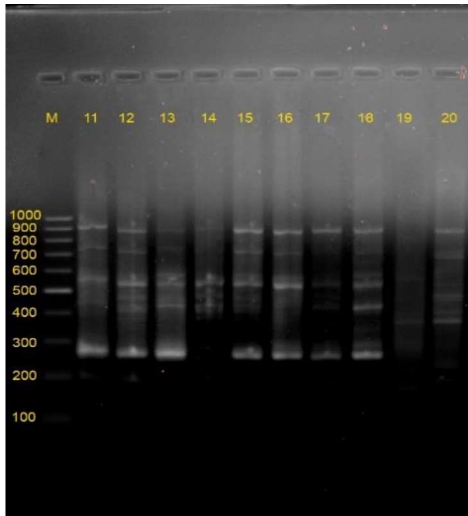




M-DNAmarker(100bp),1-Col.19,2-Col.20,3-Col.01,4-Col.02,5-Col.03,6-Col.04,7-Col.05,8-Col.52,9-Col.53,10-Col.06. M- DNA marker (100bp), 11-Col.48, 12-Col.42, 13-Col15,14-Col.61,15-Col.62,16-Col.81,17-Col.86, 18-Col.87, 19-Col.96, 20-Col.97.

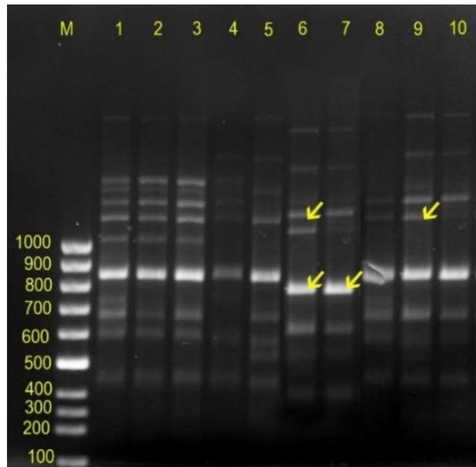
Plate 5. DNA amplification pattern generated with primer ISSR – 15

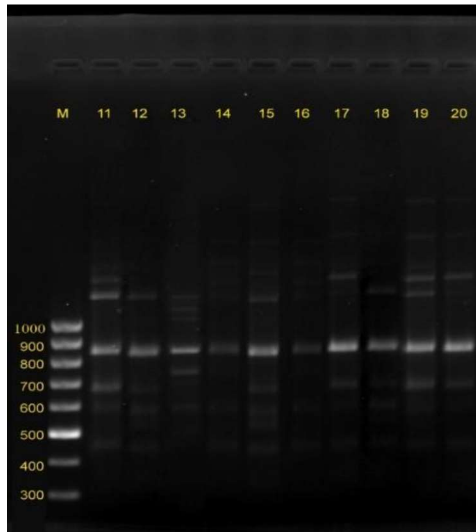




M-DNA ladder (100bp), 1-Col.19, 2-Col.20, 3- Col.01,4-Col.02, 5-Col.03, 6-Col.04, 7-Col.05, 8-Col.52, 9-Col.53, 10-Col.06. M- DNA ladder (100bp), 11-Col.48, 12-Col.42, 13-Col.15,14-Col.61,15-Col.62, 16-Col.81, 17-Col.86, 18-Col.87, 19-Col.96, 20-Col.97.

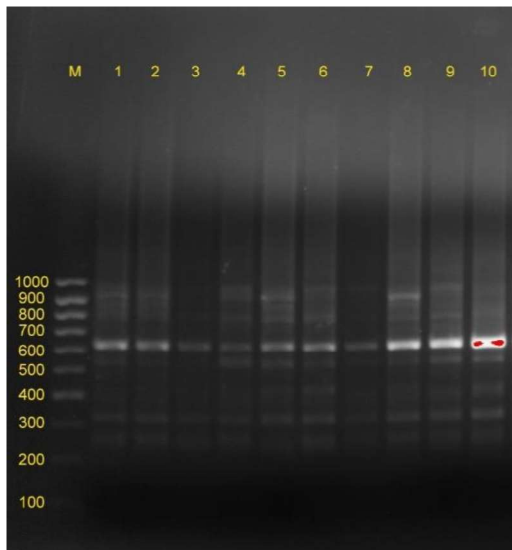
Plate 6. DNA amplification pattern generated with primer ISSR – 23

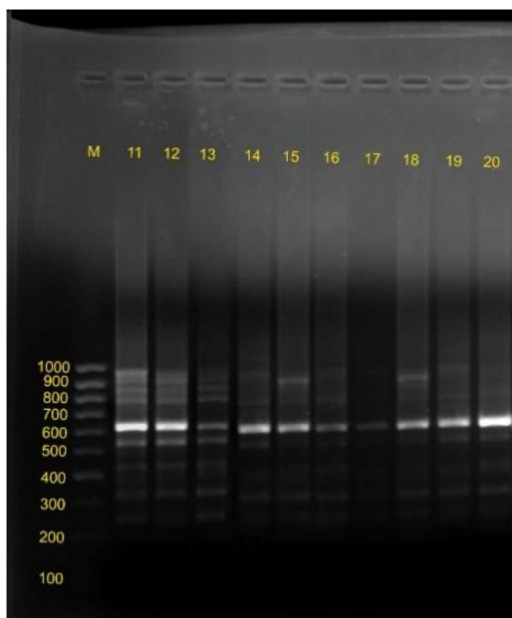




M-DNA marker (100bp), 1-Col.19, 2-Col.20, 3-Col.01, 4-Col.02, 5-Col.03, 6-Col.04, 7-Col.05, 8-Col.52, 9-Col.53, 10-Col.06. M- DNA marker 100bp), 11-Col.48,12-Col.42,13-Col15,14-Col.61,15-Col.62,16-Col.81,17-Col.86,18-Col.87,19-Col.96, 20-Col.97.

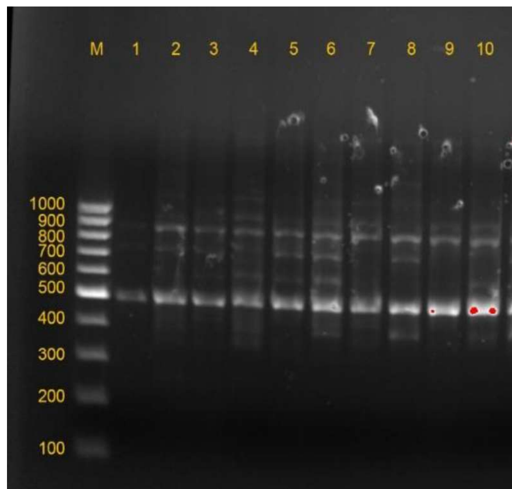
Plate 7. DNA amplification pattern generated with primer UBC 813

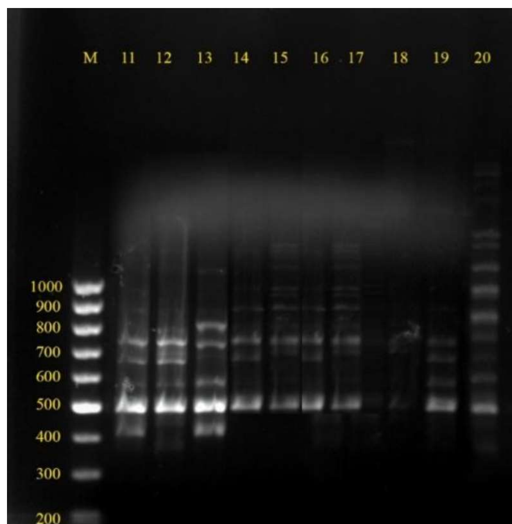




M-DNA marker (100bp), 1-Col.19, 2-Col.20, 3-Col.01, 4-Col.02, 5-Col.03, 6-Col.04, 7-Col.05, 8-Col.52, 9-Col.53,10-Col.06. M- DNA marker (100bp), 11-Col.48, 12-Col.42, 13-Col.15, 14-Col.61, 15-Col.62, 16-Col.81, 17-Col.86, 18-Col.87, 19-Col.96, 20-Col.97.

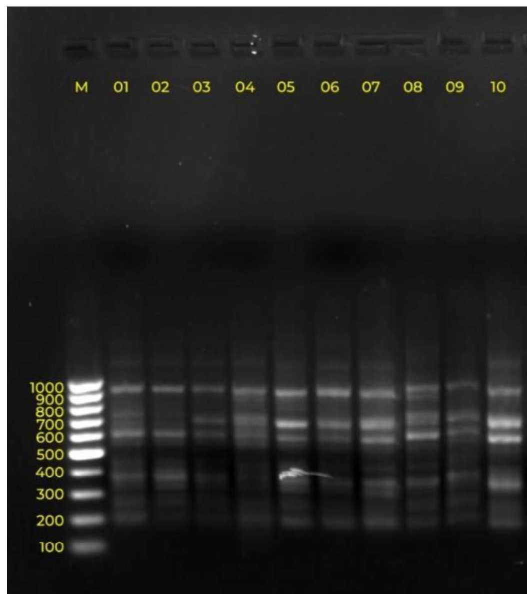
Plate 8. DNA amplification pattern generated with primer UBC 819

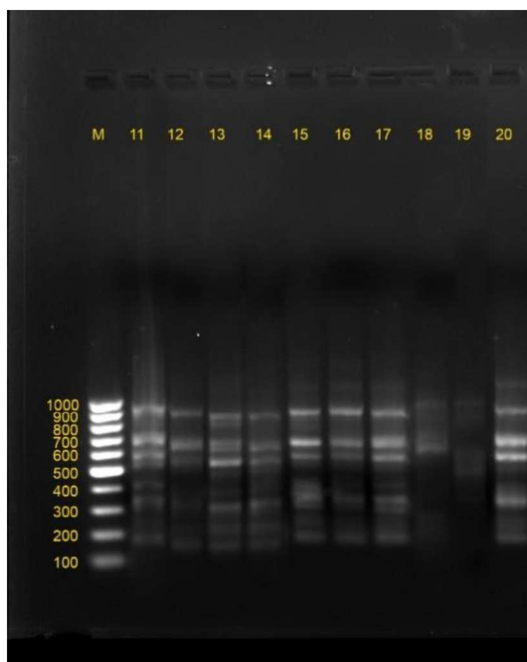




M-DNA marker (100bp), 1-Col.19, 2-Col.20, 3-Col.01, 4-Col.02, 5-Col.03, 6-Col.04, 7-Col.05, 8-Col.52, 9-Col.53, 10-Col.06. M- DNA marker 100bp), 11-Col.48, 12-Col.42, 13-Co.15,14-Col.61, 15-Col.62, 16-Col.81, 17-Col.86, 18-Col.87, 19-Col.96, 20-Col.97.

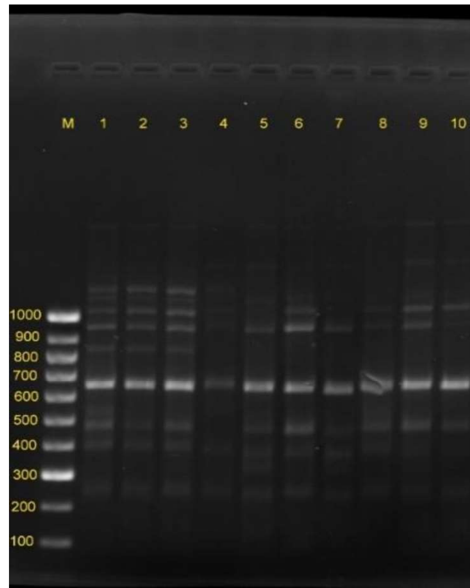
Plate 9. DNA amplification pattern generated with primer ISSR - 8

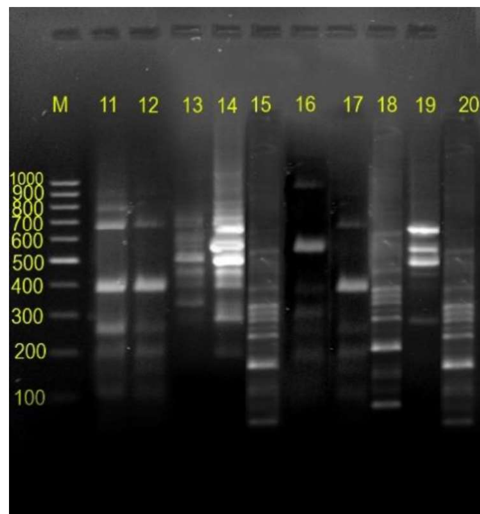




M-DNA marker (100bp), 1-Col.19, 2-Col.20, 3-Col.01, 4-Col.02, 5-Col.03, 6-Col.04, 7-Col.05, 8-Col.52, 9-Col.53,10-Col.06. M- DNA marker (100bp), 11-Col.48, 12-Col.42, 13-Col.15, 14-Col.61, 15-Col.62, 16-Col.81, 17-Col.86, 18-Col.87, 19-Col.96, 20-Col.97.

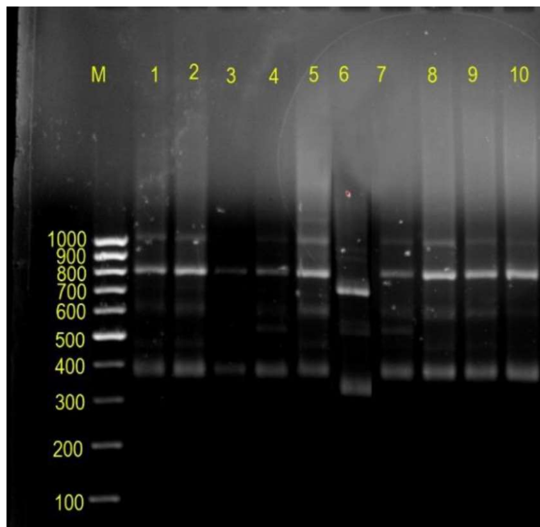
Plate 10. DNA amplification pattern generated with primer N/ISSR -5

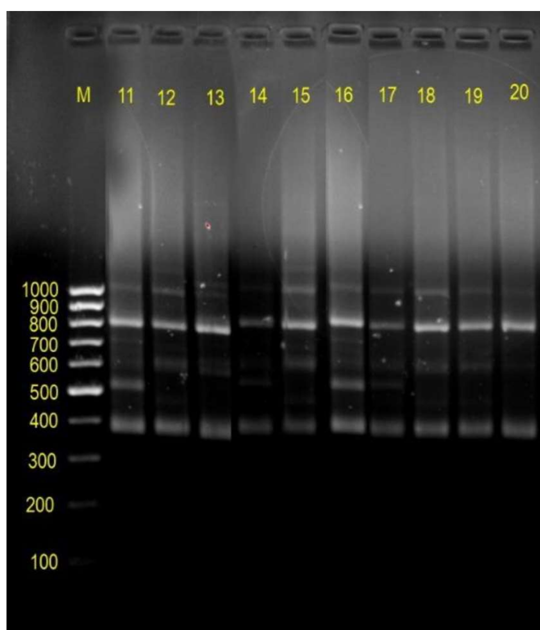




M-DNA marker (100bp), 1-Col.19, 2-Col.20, 3-Col.01, 4-Col.02, 5-Col.03, 6-Col.04, 7-Col.05, 8-Col.52, 9-Col.53,10-Col.06. M- DNA marker (100bp), 11-Col.48, 12-Col.42, 13-Col15,14-Col.61, 15-Col.62, 16-Col.81, 17-Col.86, 18-Col.87, 19-Col.96, 20-Col.97.

Plate 11. DNA amplification pattern generated with UBC 825





M-DNA marker (100bp), 1-Col.19, 2-Col.20, 3-Col.01,4-Col.02, 5-Col.03, 6-Col.04, 7-Col.05, 8-Col.52, 9-Col.53,10-Col.06. M- DNA marker (100bp), 11-Col.48, 12-Col.42, 13-Col.15, 14-Col.61, 15-Col.62, 16-Col.81, 17-Col.86, 18-Col.87, 19-Col.96, 20-Col.97.

Plate 12. DNA amplification pattern generated with UBC 828

4.2.6 Molecular analysis using selected SSR Primers

The DNA samples from twenty rambutan genotypes were amplified using five SSR primers selected based on preliminary screening (Table 14). The amplification pattern of each primer was further utilized for genetic diversity analysis. The details of amplification with selected primers are described below:

4.2.6.1 NlaSSR 23

The amplification pattern of twenty rambutan genotypes with SSR primer NlaSSR 23 is shown in Plate - 13. The molecular size of amplicons generated by this primer ranged from 220 to 450 bp. All four loci amplified were polymorphic. The polymorphic band at 450bp was shared by accessions Col.42, Col.61, Col.62, Col.81, and Col.97.

4.2.6.2 NlaSSR 3

The plate.14 displays the amplification pattern generated with primer NlaSSR3. This primer yielded four distinct and polymorphic bands resulting in a 100% polymorphism. The size of the amplicons varied from 180 to 300 bp. The genotypes Col.05, Col.53, Col.62, and Col.96 shared the polymorphic band at 300 bp.

4.2.6.3 NlaSSR 5

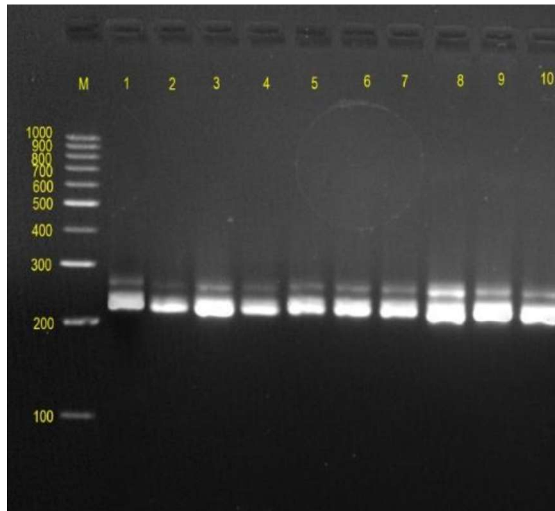
When the DNA samples of twenty rambutan genotypes were amplified with the SSR primer NlaSSR 5 (Plate 15), a total of five bands were amplified, and all of these exhibited polymorphisms. The polymorphism percentage was 100%. The polymorphic band at 280 bp was present in Col.03, Col.42, Col.48, and Col.53. A unique band was observed at 300 bp for Col.42.

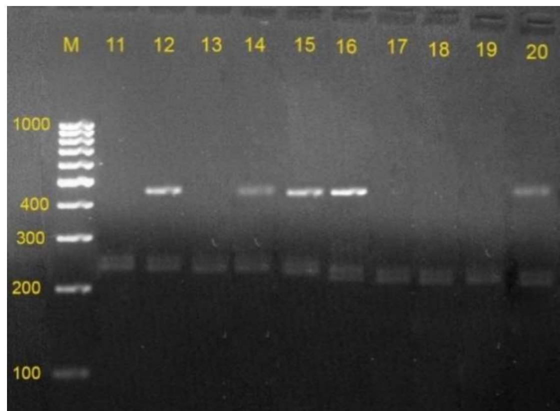
4.2.6.4 NlaSSR 7

SSR analysis of DNA samples using the NlaSSR 7 primer developed three polymorphic bands (Plate 16). The molecular size of these amplicons varied between 220 and 300bp. The percentage of polymorphism recorded was 100%. The genotypes Col.06, Col.09, Col.53, and Col.97 developed band at 300bp.

4.2.6.5 NlaSSR 12

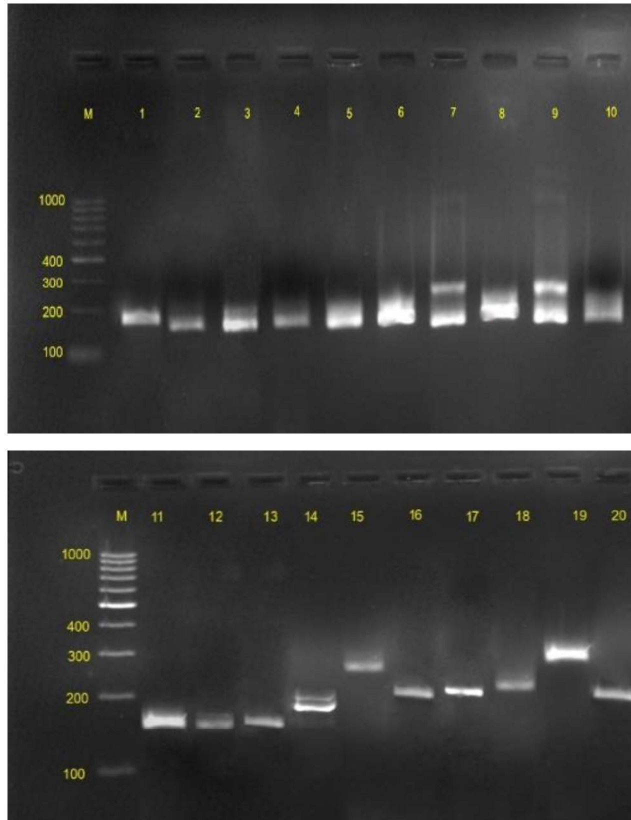
The primer NlaSSR 12 generated 3 polymorphic bands and molecular sizes of these ranged between 220 to 300bp. The amplicon at 300bp was shared by Col.04, Col.06, Col.48, Col.52, and Col.53.





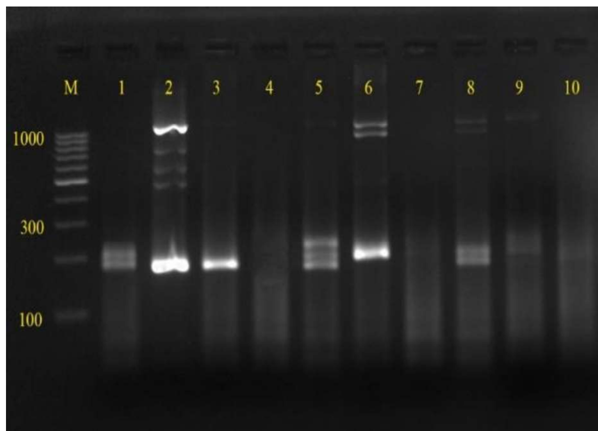
M-DNA marker (100bp), 1-Col.19, 2-Col.20, 3-Col.01, 4-Col.02, 5-Col.03, 6-Col.04, 7-Col.05, 8-Col.52, 9-Col.53,10-Col.06. M- DNA marker (100bp), 11-Col.48, 12-Col.42, 13-Col.15, 14-Col.61, 15-Col.62, 16-Col.81, 17-Col.86, 18-Col.87, 19-Col.96, 20-Col.97.

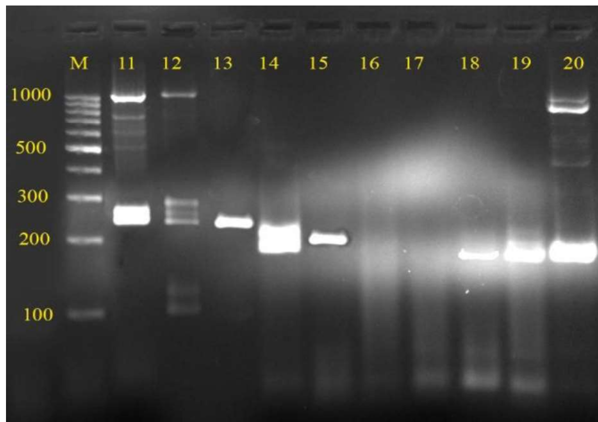
Plate 13. DNA amplification pattern generated with Nla SSR 23



M-DNA marker (100bp), 1-Col.19, 2-Col.20, 3-Col.01,4-Col.02, 5-Col.03, 6-Col.04, 7-Col.05, 8-Col.52, 9-Col.53,10-Col.06. M- DNA marker (100bp), 11-Col.48, 12-Col.42, 13-Col.15, 14-Col.61, 15-Col.62, 16-Col.81, 17-Col.86, 18-Col.87, 19-Col.96, 20-Col.97.

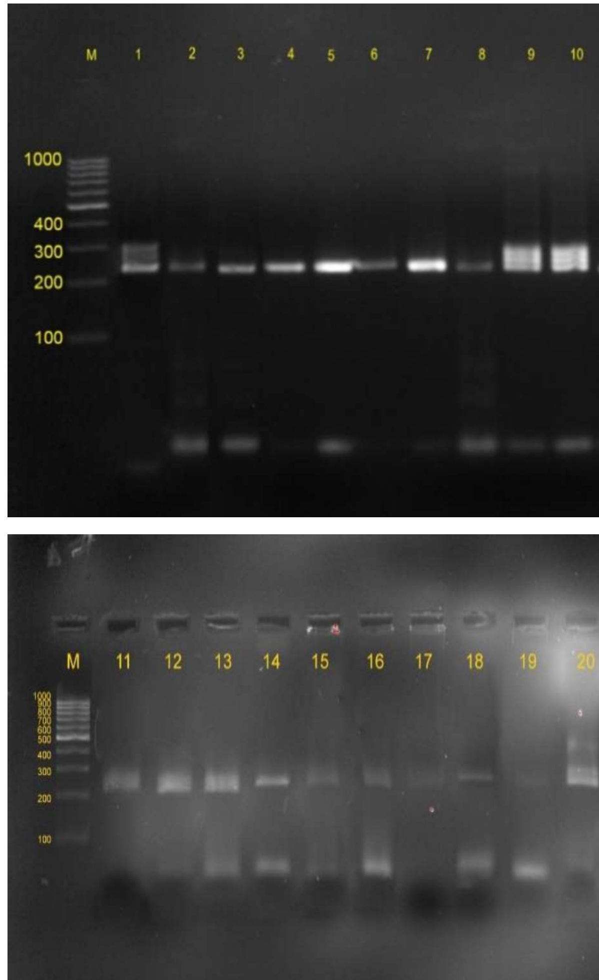
Plate 14. DNA amplification pattern generated with Nla SSR 3





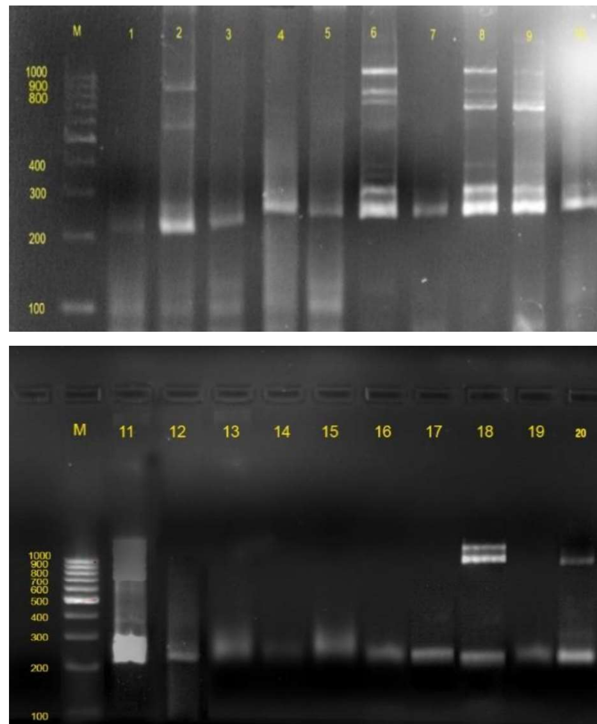
M-DNA marker (100bp), 1-Col.19, 2-Col.20, 3-Col.01, 4-Col.02, 5-Col.03, 6-Col.04, 7-Col.05, 8-Col.52, 9-Col.53, 10-Col.06. M- DNA marker (100bp), 11-Col.48, 12-Col.42, 13-Col.15, 14-Col.61, 15-Col.62, 16-Col.81, 17-Col.86,18-Col.87,19-Col.96, 20-Col.97.

Plate 15. DNA amplification pattern generated with Nla SSR 5



M-DNA marker (100bp), 1-Col.19, 2-Col.20, 3-Col.01, 4-Col.02, 5-Col.03, 6-Col.04, 7-Col.05, 8-Col.52, 9-Col.53,10-Col.06. M- DNA marker (100bp), 11-Col.48, 12-Col.42, 13-Col.15, 14-Col.61, 15-Col.62, 16-Col.81, 17-Col.86, 18-Col.87, 19-Col.96, 20-Col.97.

Plate 16. DNA amplification pattern generated with Nla SSR 7



M-DNA marker (100bp), 1-Col.19, 2-Col.20, 3-Col.01, 4-Col.02, 5-Col.03, 6-Col.04, 7-Col.05, 8-Col.52, 9-Col.53, 10-Col.06. M- DNA marker (100bp), 11-Col.48, 12-Col.42, 13-Col.15, 14-Col.61, 15-Col.62, 16-Col.81, 17-Col.86, 18-Col.87, 19-Col.96, 20-Col.97.

Plate 17. DNA amplification pattern generated with Nla SSR 12

4.3 Diversity analysis based on UPGMA cluster analysis of different rambutan genotypes

4.3.1 Cluster analysis based on ISSR data

Based on the data analysis of selected ISSR markers, a dendrogram (Figure 3) was constructed to analyze the genetic relationship among the genotypes studied.

UPGMA Cluster analysis separated the twenty rambutan accessions equally into two main clusters at a coefficient of 0.61 representing 39% overall similarity between the accessions. Cluster I consisted of Col.19, Col.52, Col.20, Col.01, Col.02, Col.03, Col.53, Col.06, Col.05, and Col.04 genotypes, while Cluster II included Col.48, Col.42, Col.62, Col.81, Col.86, Col.15, Col.97, Col.87, Col.96, and Col.61 genotypes (Table 13)

Cluster I was again divided into two subclusters at a coefficient of 0.51 with the genotype Col.04 alone forming a separate OTU (Subcluster I B). All the other genotypes fall into Subcluster I A. Within Subcluster IA, genotypes Col.03 and Col.53 showed closer genetic distance at a coefficient of 0.29 with 71% similarity. Subcluster I A was again separated into 5 groups at a coefficient of 0.37.

Cluster II was also divided into subclusters where IIA consisted of all 9 genotypes (Col.48, Col.42, Col.87, Col.15, Col.81, Col.86, Col.62, Col.97, and Col.96) except Col.61 which exclusively formed subcluster II B at 44% similarity. At 54% similarity (Coefficient 0.47) subcluster II A formed six groups with Col.96, Col.87, Col.97 and Col.15 individually forming separate groups.

4.3.2 Cluster analysis based on SSR data

Using SSR data a dendrogram (Figure 4) was constructed. At a coefficient of 0.70, the rambutan genotypes were divided into two main clusters. Cluster I comprised eight members (Col.09, Col.87, Col.97, Col.61, Col.81 Col.86, Col.62, and Col.96) while Cluster II consisted of twelve members (Col.20, Col.01, Col.02, Col.03, Col.04, Col.52, Col.06, Col.53, Col.05, Col.48, Col.15, and Col.42).

Cluster I was further divided into two subclusters at 59% of variability: subcluster IA and subcluster IB. Subcluster IA consisted of six members: Col.19, Col.87, Col.97,

Col.61, Col.81, and Col.86. In this group Col.81 and Col. 86 showed a closer genetic relationship with 67 % similarity. In Subcluster IB, only two genotypes, Col.62 and Col.96, were clustered together.

Cluster II further splits into subcluster IIA (Col.20, Col.01, Col.02, Col.03, Col.04, Col.52, Col.06, Col.53, Col.05, and Col.48) and subcluster IIB (Col.15 and Col.42) at a coefficient of 0.67.

In Subcluster II A, various genotypes exhibited different levels of similarity. This group was again divided into 4 groups at 48% of similarity. The maximum similarity was found between Col.04 and Col.52 in subcluster II A and Col.15 and Col.48 in subcluster II B with a similarity of 67%.

4.3.3 Cluster analysis based on ISSR and SSR combined data

UPGMA cluster analysis of twenty rambutan genotypes with combined data of ISSR and SSR analysis divided the genotypes into two major clusters with ten genotypes each at 62% of variability (figure 5).

Cluster I was divided into two subclusters at 50% variability with the genotype Col.04 solely falling into subcluster I B. The subcluster I A formed three groups at 57% similarity. The genotypes Col. 02 and Col. 03 showed closer similarity at a coefficient of 0.34.

Cluster II also further divided into two subclusters: subcluster IIA and Subcluster II B, at a similarity coefficient of 0.56. Subcluster II A comprised six genotypes: Col.48, Col.42, Col.15, Col.62, Col.81, and Col.86. Meanwhile, Subcluster II B contained the genotypes Col. 61, Col.87, Col.96, and Col.97.

Table 13. Clustering based on ISSR data

Cluster no.	No.of Cluster members	Cluster members
Cluster I	10	Col.19, Col.52, Col.20, Col.01, Col.02, Col.03, Col.53, Col.06, Col.05, and Col.04.
Cluster II	10	Col.48, Col.042, Col.62, Col.81, Col.86, Col.15, Col.97, Col.87, Col.96, and Col.61.

Sub cluster no.	No.of Cluster members	Cluster members
Subcluster I A	9	Col.19, Col.20, Col.01, Col.02, Col.03, Col.52, Col.53, Col.06 and Col.05.
Subcluster I B	1	Col.04
Subcluster II A	9	Col.48, Col.42, Col.87, Col.15, Col.81, Col.86, Col.62, Col.97, and Col.96.
Subcluster II B	1	Col.61

Table 14. Clustering based on SSR data

Cluster no.	No. of Cluster members	Cluster members
Cluster I	8	Col.09, Col.87, Col.97, Col.61, Col.81 Col.86, Col.62, and Col.96.
Cluster II	12	Col.20, Col.01, Col.02, Col.03, Col.04, Col.52, Col.06, Col.53, Col.05, Col.48, Col.15, and Col.42.

Sub cluster no.	No. of Cluster members	Cluster members
Subcluster I A	6	Col.19, Col.87, Col.97, Col.61, Col.81 Col.86.
Subcluster I B	2	Col.62 and Col.96.
Subcluster II A	10	Col.20, Col.01, Col.02, Col.03, Col.04, Col.52, Col.06, Col.53, Col.05, and Col.48.
Subcluster II B	2	Col.015 and Col.42.

Table 15. Clustering based on both ISSR and SSR combined data

Cluster no.	No.of Cluster members	Cluster members
Cluster I	10	Col.19, Col.52, Col.20, Col.01, Col.02, Col.03, Col.53, Col.06, Col.05, and Col.04.
Cluster II	10	Col.48, Col.42, Col.15, Col.62, Col.81, Col.86, Col.61.Col.87, Col.97, and Col.96.

Sub cluster no.	No.of Cluster members	Cluster members
Subcluster I A	9	Col.19, Col.20, Col.01, Col.02, Col.03, Col.52, Col.53, Col.06 and Col.05
Subcluster I B	1	Col.04
Subcluster II A	9	Col.48, Col.42, Col.15, Col.62, Col.81, Col.86,
Subcluster II B	1	Col.61, Col.87, Col.96, and Col.97

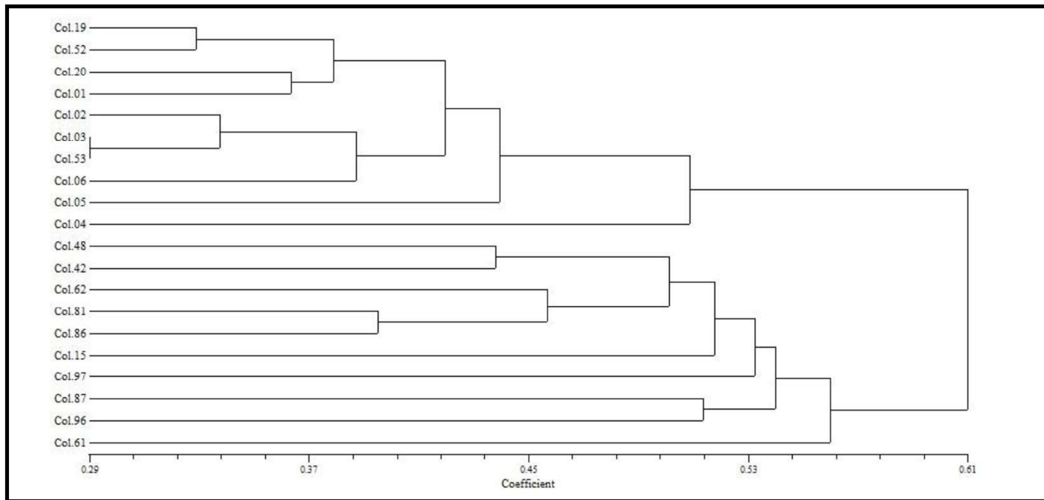


Figure 3. Dendrogram based on ISSR data

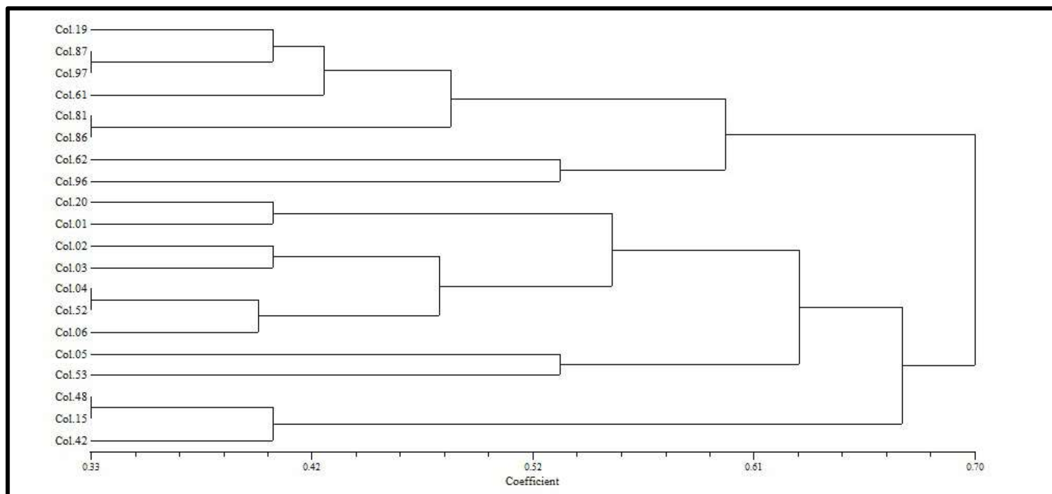


Figure 4. Dendrogram based on SSR Data

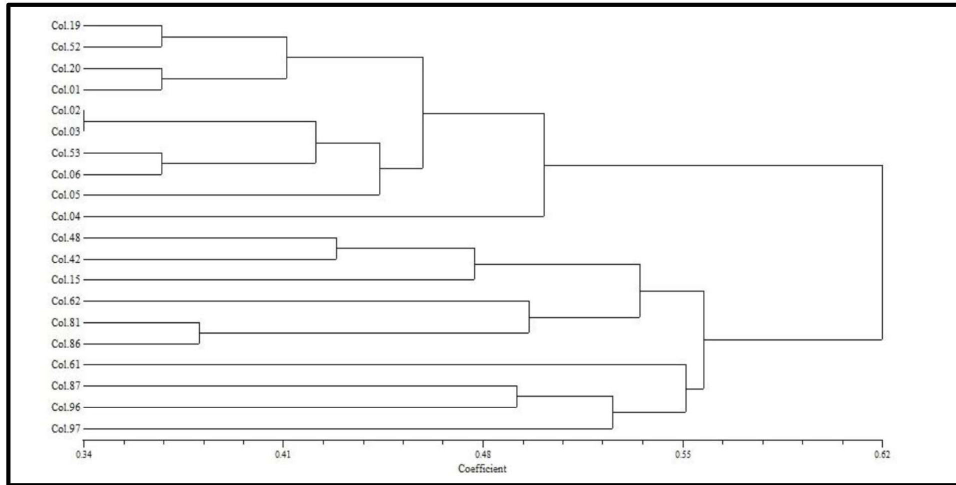


Figure 5. Dendrogram based on SSR + ISSR combined data

4.4 Diversity analysis based on Principal Coordinate Analysis (PCoA) of different rambutan genotypes.

4.4.1 PCoA analysis based on ISSR data

The spatial representation obtained from the PCoA analysis of ISSR primer data provided insights into the relative genetic distance between individuals. The analysis revealed the existence of two distinct clusters, as illustrated in (Figure 6). Cluster I comprised the genotypes Col.48, Col.42, Col.62, Col.81, Col.86, Col.15, Col.97, Col.87, Col.96, and Col.061, while Cluster II consisted of the genotypes Col.19, Col.52, Col.20, Col.01, Col.02, Col.03, Col.53, Col.06, Col.05, and Col.04. Notably, Col.61 in Cluster I and Col.04 in Cluster II formed separate individual clusters, which is consistent with the results obtained from the UPGMA cluster analysis based on ISSR data (as presented in Figure 3). The clustering patterns and members were found to be similar, with the only variation being the coordinate axis of the PCoA analysis.

4.4.2 PCoA analysis based on SSR data

The PCoA analysis conducted on SSR primer data provided a spatial representation that depicted the relative genetic distance between individuals. This representation revealed the presence of two distinct clusters, as shown in (Figure 7). Cluster I consists of Col.09, Col.87, Col.97, Col.61, Col.81, Col.86, Col.62, and Col.96, while Cluster II consists of Col.20, Col.01, Col.02, Col.03, Col.04, Col.52, Col.06, Col.53, Col.05, Col.48, Col.15, and Col.42. This clustering pattern observed in the PCoA analysis is consistent with the results obtained from the UPGMA cluster analysis of the SSR data (Figure 3).

4.4.3 PCoA analysis based on ISSR + SSR combined data

The PCoA plot generated using the combined data from ISSR and SSR markers (Figure 8) showed a consistent pattern with the results of the cluster analysis performed on the combined ISSR and SSR data (Figure 5). Cluster I consisted of the genotypes Col.48, Col.42, Col.62, Col.81, Col.86, Col.15, Col.97, Col.87, Col.96, and Col.061, while Cluster II comprised the genotypes Col.19, Col.52, Col.20, Col.01, Col.02, Col.03, Col.53, Col.06, Col.05, and Col.04.

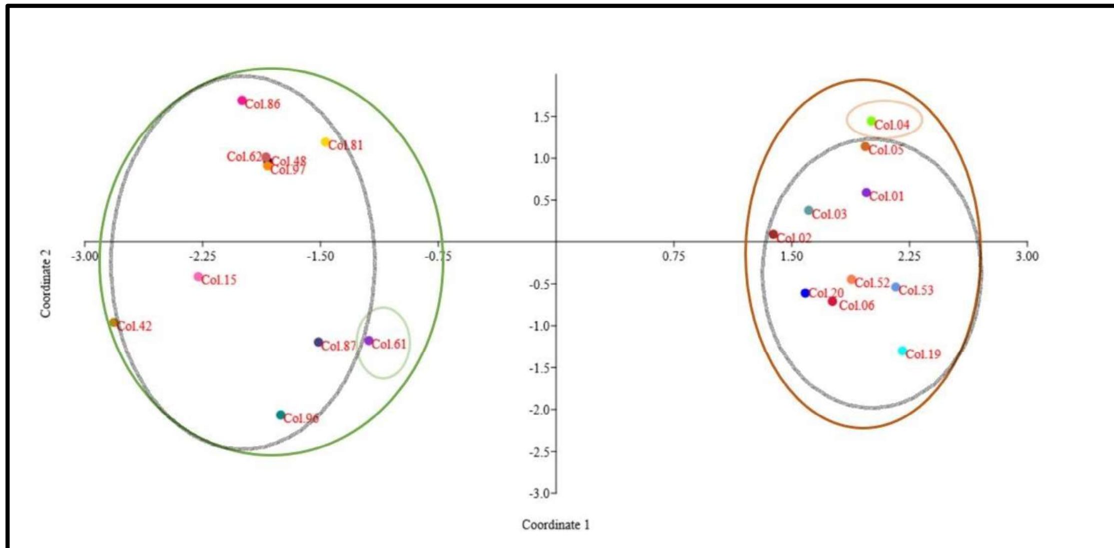


Fig 6: Principal Coordinate Analysis based on ISSR data

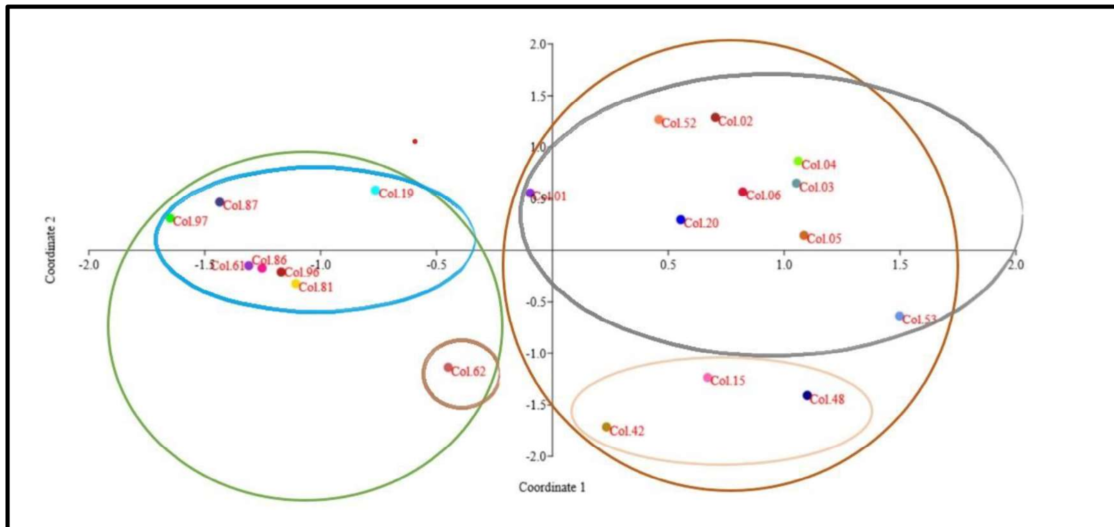


Fig 7: Principal Coordinate Analysis based on SSR data

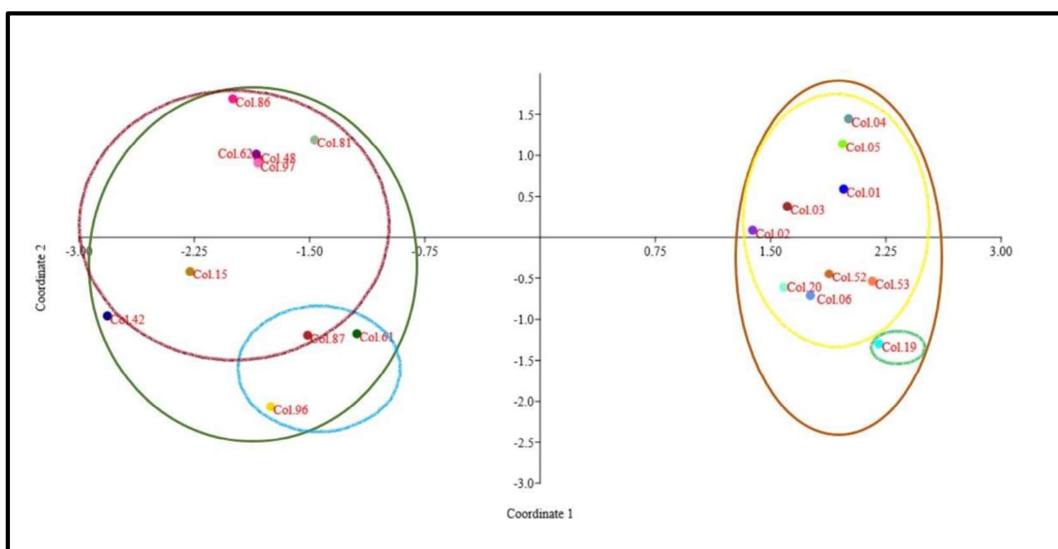


Fig 8: Principal Coordinate Analysis based on SSR + ISSR combined data

Discussion

5. DISCUSSION

Rambutan (*Nephelium lappaceum* L.), a member of the Sapindaceae family, has immense potential in Kerala, being a tropical region. This family comprises numerous trees and shrubs, with over 125 genera and more than 1000 species found across warm regions and the tropics. Rambutan plants are adaptable to various soil types, ranging from heavy soils in low-lying areas to hilly soils in upland regions. Rambutan cultivation is rapidly expanding along the western coast of India, particularly in Pathanamthitta, Kottayam, and Thrissur districts of Kerala. Even though morphological characterization and diversity studies have been conducted on collections from different localities in Kerala, no genetic diversity analysis using molecular markers has been carried out so far. Hence, the current research was carried out to investigate the genetic diversity of rambutan collections from these significant growing regions of Kerala using molecular markers.

5.1 Isolation and quantification of DNA

For the extraction of genomic DNA fresh tender leaves were collected from each rambutan genotype in the early morning. According to Mir *et al.,* (2018), fresh tender leaves are better than mature leaves in reducing the interference of secondary metabolites during DNA extraction in litchi.

The isolation of DNA was performed by the modified CTAB method described by Doyle and Doyle (1987). Few more modifications were made in the current study as per the suggestions reported by Manggabarani *et al.,* (2018) in a previous study in rambutan. They opined that the sorbitol buffer pre-wash reduced the interference of high mucus content in rambutan leaves and repeated purification procedures separated secondary metabolites and polysaccharides from DNA samples. Napitu *et al.,* (2016) also employed the DNA isolation technique proposed by Doyle & Doyle (1990) with modifications for wild relatives of rambutan by repeating purification steps. Similarly, Feng *et al.,* (2017) also employed a modified CTAB method for extracting genomic DNA from litchi, a close relative of rambutan. This modified method involved repeating certain steps in the DNA purification process.

Several researchers have made modifications to the CTAB protocol for DNA extraction. Lade *et al.,* (2014) used 5M NaCl and PVP to remove polyphenols, while Afshar *et al.,* (2018) added LiCl, sodium acetate, β-mercaptoethanol, and PVP. Inglis *et al.,* (2018) included a sorbitol pre-wash step, and Rani *et al.,* (2019) increased PVP and CTAB concentrations. Choudhary *et al.,* (2020) used higher concentrations of mercaptoethanol, and Mawarni *et al.,* (2021) eliminated liquid nitrogen and adjusted several chemical concentrations. Ramachandran *et al.,* (2022) avoided toxic chemicals, and Nath *et al.,* (2022) optimized the usage of various DNA extraction chemicals and reduced the amount of leaf tissue used.

In the current study, DNA samples were run in 0.8 percent agarose gel and stained with ethidium bromide to determine the DNA's purity. In order to prevent RNA contamination, RNase treatment was performed. The utilization of RNAase in the purification process has been reported by Gallego and Martinez (1996) and Ravel *et al.,* (1998).

The DNA sample isolated in this study exhibited a high molecular weight band on the electrophoresis gel after undergoing RNase treatment, indicating its good quality. According to the findings of Wettasinghe and Peffley (1998), a DNA sample is of high quality when it displays a distinct band of high molecular weight with minimal smearing and low presence of RNA.

To evaluate the purity of the isolated DNA, from the UV spectrophotometric readings, the OD 260/OD 280 ratio was calculated. The absorbance ratio of the DNA samples from various accessions ranged between 1.81 and 1.95, which indicated the presence of highly pure DNA. According to Sambrook *et al.,* (1989), the average OD 260/OD 280 value for pure DNA preparations is between 1.8 and 2.0. The DNA concentration in the current study ranged between 220-386 ng/µl.

5.2 Molecular marker analysis

Molecular primers that target DNA sequences can detect a greater level of polymorphism compared to protein-based markers and morphological markers. This advancement in genetic markers, as described by Botstein *et al.,* (1980) and Tanksley

et al., (1989), has the potential to overcome limitations associated with morphological and biochemical data in varietal characterization methods. PCR-based markers are mostly employed for molecular characterization. Depending on the primer employed, they amplify a certain area of the genomic DNA. For the current investigation, ISSR and SSR, two PCR-based marker methods, were employed.
To ensure accurate molecular analysis, it is crucial to optimize the concentration of reagents and temperature conditions in PCR reactions. This was achieved in the current study by repeating the reactions. The DNA samples were diluted to a concentration of 40ng/µl. Previous studies indicated that a genomic DNA concentration of more than 30 ng/µl provides favorable amplification results in PCR (Henegariu *et al.,* 1997).

5.2.1 ISSR (Inter Simple Sequence Repeat) Analysis

In the current study thirty ISSR primers were used for screening, among these 16 primers belong to the UBC (University of Columbia) series and two primers belong to the PIET series and the rest come under ISSR and N/ISSR series. Out of the thirty primers, eleven primers were selected based on polymorphism in initial screening (Table 10). Zietkiewicz *et al.,* (1994) introduced ISSR as a novel molecular primer that utilizes information from SSR (Simple Sequence Repeat) sequences in plant genomes. ISSR primers have the advantage of generating highly reproducible DNA results, making them useful for identifying genetic variations within lower taxonomic groups. In ISSR primer analysis, the amplification occurs between two binding sites that are positioned in opposing orientations and within suitable proximity. Polymorphism in ISSR analysis is determined by observing variations in the presence or absence of bands, which are indicative of binding site additions or reductions (Yang *et al.,* 1996). ISSR markers are widely regarded as highly effective due to their capacity to reveal more pertinent DNA bands through a single amplification process (Farajpour *et al.,* 2011).

In rambutan, only a few studies were conducted using ISSR primers. Manggabarani *et al.,* (2018) utilized 31 ISSR markers to analyze the genetic diversity among 30 rambutan cultivars from Indonesia. After a screening process, the

researchers identified six specific ISSR primers that showed promising results and were selected for further analysis. Napitu *et al.* (2016) conducted a study on the wild relatives of rambutan in Kalimantan, employing ISSR markers to examine their genetic diversity.

Long *et al.,* (2015) employed 12 ISSR primers for understanding the genetic diversity among seven genotypes of Litchi, a close relative of rambutan in the Sapindaceae family.

5.2.2 SSR (Simple Sequence Repeat) analysis

In the current study, twenty rambutan collections were screened using sixteen SSR primers. This included twelve Nla SSR and four Stv-nel series. Out of the sixteen primers, five primers were selected for further analysis based on polymorphism (Table 12).

Only small quantities of DNA are needed for the SSR analysis (Rafalski *et al.,* 1996), and the presence of polymorphism in SSR depends on the number of repeated units (Weber, 1990; Rafalski *et al.,* 1996; Staub and Serquen, 1996). Due to its high level of polymorphism (Jones *et al.,* 1997; Mohan *et al.,* 1997), SSR serves as a valuable marker for applications such as mapping, genetic diversity studies, fingerprinting, and population genetics.

According to Ziya *et al.,* (2016), one of the notable advantages of SSR primers is their remarkable transferability among closely related species. This quality is highly valuable in genetic studies. Despite the availability of alternative genotyping technologies like genotyping by sequencing (GBS), SSR primers continue to be widely preferred due to their efficiency, reliability, reproducibility, and user-friendly nature, as highlighted by Kanashiro *et al.,* (2017). These qualities make SSR primers the preferred choice for genotyping, even in the current era.

The research on SSR markers for rambutan is limited. Xing *et al.,*(2019) reported 10 SSR primers, while Razak *et al.,*(2020) identified 12 SSR primers. In contrast, Arias *et al.,*(2020) developed a large set of (35 numbers) SSR primers for rambutan.

5.2.3 Polymorphic information content of selected ISSR and SSR primers

In the current study, The PIC values of the selected ISSR markers ranged from 0.12 to 0.35 with an average of 0.29 (Table 13; Fig 1a) while the PIC values of the SSR markers ranged between 0.60 to 0.72 with an average of 0.66 (Table 14; Fig 1b).

The PIC value is an indicator of allele diversity and frequency within genotypes (Sheeja *et al.,*2013) and aids in understanding the utility of a primer for characterizing a variety (Hollman *et al.,* 2005). The polymorphic information content (PIC) of ISSR primers among Garcinia accessions varied between 0.08 to 0.46 with an average of 0.33 (Krishnan, *et al.*,2020).

In a study, Xing *et al.,* (2019) reported that the polymorphic information content (PIC) of SSR primers ranged between 0.36 to 0.49 with an average of 0.39 among 68 rambutan accessions. Razak *et al.,*(2020) stated that the values of SSR Polymorphic information content(PIC) varied from 0.32 to 0.83 with an average of 0.62 in rambutan collections which is in close agreement with the present study.

In a study conducted by Sulu *et al.,*(2020), the PIC (Polymorphic Information Content) values for ISSR primers ranged from 0.31 to 0.96, while for SSR primers, they varied between 0.33 and 0.96 in citrus genotypes. In cocoa, Sujith (2016) observed that ISSR primers had a PIC value ranging from 0.17 to 0.68, while SSR primers had a PIC value ranging between 0.42 to 0.80.

5.2.4 Marker Index of Selected ISSR and SSR Primers

In the current study, ISSR markers recorded a Marker index ranging from 0.36 to 2.88, with an average of 1.70. On the other hand, Marker index for SSR markers varied from 1.80 to 2.88, with an average of 2.42.

The marker index is a statistical tool used to determine the effectiveness of a marker system in detecting genetic variations among different genotypes. It quantifies the capacity of each primer to identify polymorphic loci. The marker index provides insight into a primer's ability to identify polymorphic loci within

different genotypes (Varshney *et al.,* 2007).Discrimination analysis was carried out by Mandal *et al.,*(2016), who discovered that MI was the most effective metric for identifying the most suitable primer.

Ariffin *et al.,* (2015) observed that the marker index (MI) for ISSR primer ranged from 0.56 to 2.34, with an average of 1.25 in Mango genotypes. In a study on the diversity of garcinia accessions, the marker index (MI) values varied from 0.24 to 3.06, with an average of 1.30 for ISSR primers (Krishnan *et al.,* 2020). Sandeep Kumar *et al.,* (2023) reported a marker index (MI) ranging between 0.012 to 0.015 in guava genotypes for SSR markers.

5.3 Diversity Analysis

The current study observed a genetic diversity of 61% to 70% among the examined accessions using SSR, ISSR, and combined marker analysis. Accessions from the same location showed lower genetic distance, indicating potential dispersal from related parents. These findings are supported by Chhetri *et al.,* (2019).

In this study, Col.19, Col.52, Col.02, Col.03, and Col.53 showed 67% similarity in ISSR analysis and Col.87, Col.97, Col.81, Col.86, Col.04, Col.52, Col.48 and Col.15 showed closer similarity at a coefficient of 0.33 (Fig .3 & 4). The differences observed between the ISSR clustering pattern and the SSR clustering pattern could be attributed to the characteristics of ISSR primers, which focus on variable microsatellite regions and generate numerous polymorphic fragments (Gupta *et al.,* 1994). However, previous studies on genetic diversity using ISSR markers have shown their effectiveness in characterizing genetic diversity in woody species (Mohammad *et al.,* 2018 & Chhajer *et.al.,* 2018). They have also been successfully utilized for fruit-bearing species like apples (Goulao *et. al.,*2001), strawberries (Arnau *et al.,* 2003), Citrus (Biswas *et al.,* 2010), and apricot (Li *et al.,* 2013).

While analyzing the clustering patterns of current research it is observed that the dendrograms generated based on ISSR, SSR, and the combined ISSR-SSR data were found to be in conformity with the geographic locations of their collection. The ISSR-based dendrogram and ISSR – SSR combined dendrogram developed two major clusters. Cluster I consisted of genotypes collected from the Kottayam district,

while Cluster II consisted of the genotypes collected from the Pathanamthitta and Thrissur districts. Tran *et al.,* (2019) observed that the ISSR-based UPGMA dendrogram of *Litchi chinensis*, a close relative of rambutan, showed a clustering pattern where the subclusters generally corresponded to their geographic origins.

The dendrogram acquired with SSR primers formed two main clusters at a coefficient of 0.70. Cluster I included all thirteen accessions collected from the Pathanamthitta district and also the three accessions from the Kurichy area of Kottayam. Cluster II consisted of seven genotypes from different regions within Kottayam. The clustering pattern is almost comparable with that generated with ISSR data and collective data of ISSR and SSR. Sabreena *et al.,* (2021) reported that both ISSR and SSR marker approaches were equally effective in classifying buckwheat genotypes based on their geographical locations. They also opined that both markers are beneficial for characterization works.

5.4 Principal Coordinate Analysis (PCoA)

Principal Coordinate Analysis (PCoA) is a useful tool for summarizing and visualizing genetic distances between genotypes or populations. It operates based on Euclidean distances, allowing for the representation of genetic variability without explicitly showcasing the specific alleles involved. This approach offers certain advantages as it relies on measures of genetic variability that are directly linked to population genetics models. For instance, PCoA has been effectively applied to summarize matrices of pairwise FST, as demonstrated in the research conducted by Zhivotovsky *et al.*(2003). In this context, PCoA enables the exploration of population structure and differentiation by capturing the underlying genetic distances between populations. By employing PCoA on pairwise FST data, researchers can gain insights into the genetic relationships and variations among different populations without explicitly analyzing the alleles themselves. By constructing a geometrical arrangement of points in reduced space, PCoA reflects the relationships between items in the original data matrix. Points that are close together in the visualization represent similar items, while points that are far apart indicate dissimilar items (Gordon, 1980).

In the present study, Principal Coordinate Analysis (PCoA) plot showed a similar partition of clusters as that in the UPGMA dendrogram. There was a large degree of grouping based on their geographical distribution. Gajera *et al.,* (2013) obtained a similar pattern for the division of clusters in dendrogram and PCoA plot in their study on genetic variability in mango. In a study on the genetic relationship of bamboo species, PCoA revealed that the grouping of different bamboo species in the plot was in agreement with the UPGMA dendrogram (Amom *et al*., 2018). In another study, the distribution of grape varieties in the PCoA plot and UPGMA dendrogram was found similar (Guo *et al.,* 2012).

This study could find out the extent of genetic variability existing among the twenty rambutan genotypes collected from major rambutan growing regions of Kerala. Future crop improvement initiatives for this exotic fruit crop could make use of existing germplasm from different regions of the state and molecular analysis using more markers. Identification of markers associated with economically important trails will be useful for marker assisted breeding programmes. The current study on genetic diversity using molecular markers is the first of its type in Kerala and hence can be considered as basic information for future related works.

Summary

6. SUMMARY

The study titled "Genetic diversity analysis of rambutan (*Nephelium lappaceum. L*) accessions using molecular markers" was conducted at the Regional Agricultural Research Station, Kumarakom, and the Department of Fruit Science, College of Agriculture, Vellayani between 2020 and 2022. The main objective of the research was to assess and describe the genetic variability of twenty rambutan accessions collected from the Kottayam, Pathanamthitta, and Thrissur districts using SSR and ISSR molecular markers.

The findings of the study were as follows.

1. The molecular characterization of twenty rambutan accessions previously identified as promising by Muhamed (2016) was conducted in this study.
2. Modified CTAB protocol (Doyle and Doyle 1987) was used for DNA extraction. A pre-washing with sorbitol buffer was done to improve the DNA quality.
3. The DNA quantification was carried out utilizing a UV spectrophotometer. The absorbance ratio at 260nm to 280nm (A260/A280) was determined and used as an indicator of DNA purity. The obtained A260/A280 readings were within the range of 1.80 to 1.95, indicating DNA samples' suitability for subsequent analysis.
4. A total of thirty ISSR (Inter Simple Sequence Repeats) primers and sixteen SSR (Simple Sequence Repeats) primers were initially screened for polymorphism. Based on the ability to produce significant polymorphic patterns eleven ISSR primers and five SSR primers were selected for further studies.
5. The amplification patterns generated by the selected primers were recorded and scored. The percentage of polymorphism, Polymorphic Information Content (PIC), and Marker Index (MI) were calculated for assessing the efficiency of selected primers.

6. The ISSR primers used in the study exhibited a polymorphism percentage ranging from 55.56% (ISSR 10) to 100% (ISSR-1 &UBC 828), with an average value of 79.44%. On the other hand, the selected SSR primers displayed a 100% polymorphism percentage.
7. In order to evaluate the usefulness of the selected primers in detecting polymorphism within the population, the Polymorphic Information Content (PIC) was determined. The PIC values for the ISSR primers ranged from 0.12 (UBC-819) to 0.41 (ISSR-23), with an average PIC value of 0.29. Similarly, for the SSR primers, the PIC values ranged from 0.60 (NlaSSR 7) to 0.72 (NlaSSR 23), with an average PIC value of 0.66. These PIC values provide insights into the informativeness and discriminatory power of the selected primers in detecting genetic variations within the rambutan population studied.
8. To assess the effectiveness of the selected primers in detecting polymorphism, the Marker Index (MI) was calculated. In the case of ISSR primers, the MI ranged from 0.36 (UBC 819)to 2.88 (UBC 825), with an average MI of 1.70. Similarly, for the SSR primers, the MI varied between 1.80 (NlaSSR 7) to 2.88 (NlaSSR 23), with an average value of 2.42. These MI values provide an indication of the efficiency of the selected primers in capturing genetic variations within the rambutan population.
9. In ISSR analysis, primer UBC-828 amplified two specific bands at 350bp and700bp for the genotype Col.04 and hence it can be used for identifying this accession.
10. The cluster analysis was conducted using the NTSYS-Pc software. Separate cluster analyses were performed using the scored data of ISSR primers, SSR primers, and the combined data of both SSR and ISSR primers.
11. In the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) cluster analysis based on ISSR data, the rambutan accessions were divided into two distinct clusters at a coefficient of 0.61. Cluster I comprised the rambutan selections Col.19, Col.52, Col.20, Col.01, Col.02, Col.03, Col.53, Col.06, Col.05, and Col.04 which were collected from the Kottayam district

of Kerala. Cluster II consisted of Col.48, Col.42, Col.62, Col.81, Col.86, Col.15, Col.97, Col.87, Col.96, and Col.61 and most of them belonged to the Pathanamthitta district. Col.015, which was included in cluster II was from Thrissur district. The highest degree of similarity was observed between Col.03 and Col.53, with a similarity of 71%.

12. In the SSR data-based cluster analysis, the rambutan accessions were divided into two major clusters at a coefficient of 0.70. Cluster I comprised a total of eight rambutan accessions viz., Col.09, Col.87, Col.97, Col.61, Col.81, Col.86, Col.62, and Col.96. The Cluster II included the remaining twelve genotypes Col.20, Col.01, Col.02, Col.03, Col.04, Col.52, Col.06, Col.53, Col.05, Col.48, Col.15, and Col.42. The highest similarity of 67% was observed between the genotypes Col.87 and Col.97, Col. 81 and Col.86, Col.04 and Col.52, and Col.48 and Col.15.
13. In the combined SSR-ISSR cluster analysis, at a similarity coefficient of 60% the rambutan accessions were divided into two clusters, showing a similar dendrogram pattern as observed in the ISSR data-based dendrogram. Each cluster consisted of ten members. The highest similarity of 66% was observed between Col.02 and Col.03 collected from the same location, indicating a close genetic relationship between these two genotypes.
14. The Principal Coordinate Analysis (PCoA) also revealed a similar pattern of distribution of the accessions as recorded in cluster analysis
15. The results of the current study revealed a genetic diversity ranging from 61% to 70% for the accessions under study using SSR, ISSR, and combined marker analysis. Lower genetic distance was observed between accessions from the same location indicating a possible occurrence of dispersal from related parents. The use of an extensive germplasm from different locations of the state and molecular analysis using more number of markers will be useful for utilizing the genetic diversity in further crop improvement programmes of this exotic crop. Moreover, the possible identification of markers associated with economically important traits helps in marker assisted breeding programmes in rambutan. The present study is a

preliminary evaluation of genetic diversity using molecular markers, which is the first of its kind in the state and hence considered as a basic information for further research.

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ANNEXURE I

List of laboratory equipments used for study

Refrigerated centrifuge	: ppendorf 5430R
SureCycler 8800 PCR	: Agilent Technologies
Electrophoresis unit	: Bio-Rad, USA
Spectrophotometer	: Shimadzu UV-1800 Spectrophotometer.
Gel documentation system	: Bio – Rad
Micropipettes	: Eppendorf

ANNEXURE II

Reagents required for DNA isolation

I. CTAB buffer (2x):

- CTAB (w/v)	: 2 g
- 100 mM Tris base (PH8)	: 1.21 g
- 20 mM EDTA(PH8)	: 0.745 g
- 14 M NaC1	: 8.18 g
- PVP	: 1.0 g

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

II.1M Sorbitol wash buffer

-Pure Sorbitol	: 182.17gm
- Double distilled water	: 1L

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

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

IV. Chilled isopropanol

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

V. Wash buffer

- 76% ethyl alcohol
- 10 mM ammonium acetate

VIII. TE buffer:

- 10 mM Tris (PH8)

- 1 mM EDTA

ANNEXURE III

Composition of buffers and dyes used for gel electrophoresis

I. **50x TAE Buffer(pH8)**

- 2M Triss (pH8)	: 242g
-1M Glacial Acetic acid	: 57.1 ml
- 50mM EDTA (pH 8.0)	: 100 ml

II. Loading dye 6X

0.25 per cent bromophenol blue

0.25 per cent xylene cyanol

30 per cent glyceol in water

III. Ethidium bromide(0.5µg/l)

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

GENETIC DIVERSITY ANALYSIS OF RAMBUTAN (*Nephelium lappaceum* L.) ACCESSIONS USING MOLECULAR MARKERS

by

GAZEL M GADDAFI

(2019-12-051)

Abstract of the thesis

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ABSTRACT

Rambutan (*Nephelium lappaceum* L.) holds great potential in Kerala being a tropical region. It belongs to the Sapindaceae family, which consists of numerous trees and shrubs, comprising over 125 genera and more than 1000 species that are found across the tropics and warm regions. These plants are adaptable to various soil types, ranging from heavy soils in low-lying areas to hilly soils in upland regions. The origin of rambutan can be traced back to Southeast Asia, specifically Indonesia and Malaysia. Rambutan cultivation is rapidly expanding along India's western coast, particularly in the districts of Pathanamthitta, Kottayam, and Thrissur of Kerala. Even though morphological characterization and diversity studies of the collections from various localities in Kerala have been carried out, the genetic diversity analysis using molecular markers has not yet been conducted. Hence, the current research titled "Genetic diversity analysis of rambutan (*Nephelium lappaceum* L.) accessions using molecular markers" was conducted at the Regional Agricultural Research Station Kumarakom and at the Department of Fruit Science, College of Vellayani between 2020 and 2022. The main objective of this study was to assess the diversity of twenty rambutan accessions using SSR and ISSR molecular markers collected from the districts of Kottayam, Pathanamthitta, and Thrissur.

DNA isolation was performed using the CTAB method (Doyle and Doyle,1987) with minor modifications. A pre-washing with sorbitol buffer was done to improve the DNA quality. The DNA samples showed UV absorbance ratios (A260/A280) between 1.80 and 1.95 indicating their purity. The initial primer screening was conducted with thirty ISSR and sixteen SSR primers. Based on their ability to yield reproducible and distinct banding patterns, eleven ISSR and five SSR primers were selected for subsequent analysis.

The ISSR primers used in the study exhibited a polymorphism percentage ranging from 55.56% (ISSR 10) to 100% (ISSR-1 and UBC 828), with an average value of 79.44%. On the other hand, the selected SSR primers displayed a 100% polymorphism percentage. The Polymorphic Information Content (PIC) values ranged from 0.12 (UBC-819) to 0.41 (ISSR-23) for ISSR markers and from

0.60 (NlaSSR 7) to 0.72 (NlaSSR 23) for SSR markers. PIC is an indication of the informativeness of the primers. The Marker index (MI) which measures the utility of the primers ranged between 0.36 (UBC 819) to 2.88 (UBC 825) for ISSR markers and it varied between 1.80(NlaSSR 7) to 2.88 (NlaSSR 23) for SSR markers.

The diversity analysis of rambutan accessions was performed using the NTSYS-Pc software. In the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) cluster analysis based on ISSR data, the rambutan accessions were divided into two distinct clusters at a similarity coefficient of 0.61 with 10 accessions in each cluster. The Col.03 and Col.53 were found to be closely related with a similarity of 71%.

In the SSR data-based cluster analysis, the rambutan accessions were divided into two major clusters at a similarity coefficient of 0.70. Cluster I comprised a total of eight rambutan accessions and Cluster II included the remaining twelve genotypes. The highest similarity of 67% was observed between Col.87 and Col.97, Col. 81 and Col.86, Col.04 and Col.52, and Col.48 and Col.15. In the combined SSR-ISSR cluster analysis, at a similarity coefficient of 0.62, the rambutan accessions were divided into two clusters, showing a similar dendrogram pattern as observed in the ISSR data-based dendrogram. The Principal Coordinate Analysis (PCoA) also revealed a similar pattern of distribution of the accessions as recorded in cluster analysis.The results of the present study revealed that the accessions studied had genetic diversity ranging from 61% to 70% under different marker systems. The accessions from the same area have shown a closer genetic distance, suggesting that dispersal from related parents may have occurred. In future crop improvement programmes for this exotic crop, existing germplasm from different locations of the state and molecular analysis employing more markers can be exploited. Identification of markers associated with economically important trails will be useful for marker assisted breeding programmes. The current study on genetic diversity using molecular markers is the first of its type in Kerala and hence can be considered as a basic information for future related works.

സംഗ്രഹം

ഉഷ്ണമേഖലാ പ്രദേശമായ കേരളത്തിൽ റംബുട്ടാൻ (*Nephelium lappaceum* L.) വലിയ കൃഷി സാധ്യതകളാണ് തുറന്നിടുന്നത്. ഉഷ്ണമേഖലാ പ്രദേശങ്ങളിൽ കാണപ്പെടുന്ന 125-ലധികം ജനുസ്സുകളും 1000-ലധികം സ്പീഷീസുകളും ഉൾക്കൊള്ളുന്ന നിരവധി മരങ്ങളും കുറ്റിച്ചെടികളും അടങ്ങുന്ന സാപിണ്ടെസിയ ഫാമിലിയിലാണ് റംബൂട്ടാനും ഉൾപ്പെടുന്നത്. താഴ്ന്ന പ്രദേശങ്ങളിലെ കനത്ത മണ്ണ് മുതൽ ഉയർന്ന പ്രദേശങ്ങളിലെ കുന്നിൻ മണ്ണ് വരെയുള്ള വിവിധ തരം മണ്ണിന് റംബുട്ടാൻ അനുയോജ്യമാണ്. തെക്കുകിഴക്കൻ ഏഷ്യ, പ്രത്യേകിച്ച് ഇന്തോനേഷ്യ, മലേഷ്യ എന്നിവിടങ്ങളിലാണ് റംബുട്ടാന്റെ ഉത്ഭവം. ഇന്ത്യയുടെ പടിഞ്ഞാറൻ തീരങ്ങളിൽ, പ്രത്യേകിച്ച് കേരളത്തിലെ പത്തനംതിട്ട, കോട്ടയം, തൃശൂർ ജില്ലകളിൽ റംബുട്ടാൻ കൃഷി അതിവേഗം വികസിച്ചുകൊണ്ടിരിക്കുന്നു. കേരളത്തിലെ വിവിധ പ്രദേശങ്ങളിൽ നിന്നുള്ള റംബുട്ടാൻ ശേഖരങ്ങളുടെ രൂപഘടനയും മോർഫോളജിയും ഉപയോഗിച്ച് മുൻപഠനങ്ങൾ നടത്തിയിട്ടുണ്ടെങ്കിലും, ജനറ്റിക് മാർക്കറുകൾ ഉപയോഗിച്ച് ജനിതക വൈവിധ്യ വിശകലനം ഇതുവരെ നടത്തിയിട്ടില്ല. അതിനാൽ, " ജനറ്റിക് ഡിവേർസിറ്റി അനാലിസിസ് ഓഫ് റംബുട്ടാൻ(*Nephelium lappaceum* L.) യൂസിങ് മോളിക്യൂലാർ മാർക്കേഴ്സ് " എന്ന തലക്കെട്ടിലുള്ള നിലവിലെ ഗവേഷണം കുമരകത്തെ പ്രാദേശിക കാർഷിക ഗവേഷണ കേന്ദ്രത്തിലും വെള്ളായണി കോളേജിലെ ഫ്രൂട്ട് സയൻസ് ഡിപ്പാർട്മെന്റിലും 2020 നും 2022 നും കാലയളവിൽ ഇടയിൽ നടത്തുകയുണ്ടായി. ISSR SSR മോളിക്യുലാർ മാർക്കറുകൾ ഉപയോഗിച്ച് കോട്ടയം, പത്തനംതിട്ട, തൃശൂർ ജില്ലകളിൽ നിന്ന് ശേഖരിച്ച ഇരുപത് റമ്പൂട്ടാൻ ശേഖരങ്ങളുടെ വൈവിധ്യം വിലയിരുത്തുക എന്നതായിരുന്നു ഈ പഠനത്തിന്റെ പ്രധാന ലക്ഷ്യം.

ചെറിയ പരിഷ്കാരങ്ങളോടെ CTAB രീതി (Doyle and Doyle,1987) ഉപയോഗിച്ചാണ് DNA ഐസൊലേഷൻ നടത്തിയത്. DNA ഗുണനിലവാരം മെച്ചപ്പെടുത്തുന്നതിനായി സോർബിറ്റോൾ ബഫർ ഉപയോഗിച്ച് പ്രീ-വാഷിംഗ് നടത്തി. DNA സാമ്പിളുകൾ 1.80 നും 1.95 നും ഇടയിൽ U.V അബ്സോർബൻസ് അനുപാതം (A260/A280) കാണിച്ചു, ഇത് അവയുടെ പരിശുദ്ധി സൂചിപ്പിക്കുന്നു. 30 ISSR, 16 SSR പ്രൈമറുകൾ ഉപയോഗിച്ചാണ് പ്രാഥമിക പ്രൈമർ സ്ക്രീനിംഗ്

നടത്തിയത്. പുനരുൽപ്പാദിപ്പിക്കാവുന്നതും വ്യത്യസ്തവുമായ ബാൻഡിംഗ് പാറ്റേണുകൾ നൽകാനുള്ള അവരുടെ കഴിവിനെ അടിസ്ഥാനമാക്കി, തുടർന്നുള്ള വിശകലനത്തിനായി 11 ISSR പ്രൈമറുകളും 5 SSR പ്രൈമറുകളും തിരഞ്ഞെടുത്തു.

ഈ പഠനത്തിൽ ഉപയോഗിച്ച ISSR പ്രൈമറുകൾ 55.56% (ISSR 10) മുതൽ 100% (ISSR-1, UBC 828) വരെയുള്ള പോളിമോർഫിസം ശതമാനം പ്രദർശിപ്പിച്ചു, ശരാശരി മൂല്യം 79.44% ആണ്. അതേ സമയം മറുവശത്ത്, തിരഞ്ഞെടുത്ത SSR പ്രൈമറുകൾ 100% പോളിമോർഫിസം ശതമാനം പ്രദർശിപ്പിച്ചു. ISSR മാർക്കറുകൾക്ക് 0.12 (UBC-819) മുതൽ 0.41 (ISSR-23) വരെയും SSR മാർക്കറുകൾക്ക് 0.60 (NlaSSR 7) മുതൽ 0.72 (NlaSSR 23) വരെയും പോളിമോർഫിക് ഇൻഫർമേഷൻ കോൺടെന്റ് (PIC) മൂല്യങ്ങൾ. പ്രൈമറുകളുടെ വിവരദായകതയുടെ ഒരു സൂചനയാണ് PIC. പ്രൈമറുകളുടെ പ്രയോജനം അളക്കുന്ന മാർക്കർ സൂചിക - മാർക്കർ ഇൻഡക്സ് (MI) ISSR മാർക്കറുകൾക്ക് 0.36 (UBC 819) മുതൽ 2.88 (UBC 825) വരെയാണ്, എസ്എസ്ആർ മാർക്കറുകൾക്ക് ഇത് 1.80 (NlaSSR 7) മുതൽ 2.88 (NlaSSR 23) വരെ വ്യത്യാസപ്പെടുന്നു.

NTSYS-Pc സോഫ്റ്റ്‌വെയർ ഉപയോഗിച്ചാണ് റംബുട്ടാൻ ശേഖരങ്ങളുടെ UPGMA(Unweighted Pair Group Method with Arithmetic Mean) വൈവിധ്യ വിശകലനം നടത്തിയത്. ISSR ഡാറ്റയെ അടിസ്ഥാനമാക്കിയുള്ള ക്ലസ്റ്റർ വിശകലനത്തിൽ, ഓരോ ക്ലസ്റ്ററിലും 10 ആക്സഷനുകൾ വീതം 0.61 എന്ന സാമ്യത ഗുണകത്തിൽ റംബുട്ടാൻ ശേഖരങ്ങളെ രണ്ട് വ്യത്യസ്ത ക്ലസ്റ്ററുകളായി തിരിച്ചിരിക്കുന്നു. Col.03 ഉം Col.53 ഉം 71% സമാനതയുമായി അടുത്ത ബന്ധമുള്ളതായി കണ്ടെത്തി.

SSR ഡാറ്റ അടിസ്ഥാനമാക്കിയുള്ള ക്ലസ്റ്റർ വിശകലനത്തിൽ, റംബുട്ടാൻ ശേഖരങ്ങളെ 0.70 എന്ന സാമ്യത ഗുണകത്തിൽ രണ്ട് പ്രധാന ക്ലസ്റ്ററുകളായി തിരിച്ചിരിക്കുന്നു. ക്ലസ്റ്റർ I-ൽ ആകെ എട്ട് റംബുട്ടാൻ പ്രവേശനങ്ങളും ക്ലസ്റ്റർ II-ൽ ശേഷിക്കുന്ന പന്ത്രണ്ട് ജനിതകരൂപങ്ങളും ഉൾപ്പെടുന്നു. ഇതിൽ Col.87 ഉം Col.97 ഉം Col.81 ഉം Col.86 ഉം Col.04 ഉം Col.52 ഉം Col.48 ഉം Col.15 ഉം തമ്മിൽ 67% ഉയർന്ന സാമ്യം നിരീക്ഷിക്കപ്പെട്ടു. സംയോജിത SSR-ISSR ക്ലസ്റ്റർ വിശകലനത്തിൽ, 0.62 എന്ന സാമ്യത ഗുണകത്തിൽ, റംബുട്ടാൻ ശേഖരങ്ങളെ രണ്ട് ക്ലസ്റ്ററുകളായി വിഭജിച്ചു, ISSR ഡാറ്റ അടിസ്ഥാനമാക്കിയുള്ള ഡെൻഡ്രോഗ്രാമിൽ നിരീക്ഷിച്ചതിന് സമാനമായ ഡെൻഡ്രോഗ്രാം പാറ്റേൺ ഇതിലും കാണാൻ കഴിഞ്ഞത്.

പ്രിൻസിപ്പൽ കോർഡിനേറ്റ് അനാലിസിസും (PCoA) ക്ലസ്റ്റർ വിശകലനത്തിൽ രേഖപ്പെടുത്തിയിരിക്കുന്ന ശേഖരങ്ങളുടെ വിതരണത്തിന്റെ സമാനമായ പാറ്റേൺ വെളിപ്പെടുത്തി. വിവിധ മാർക്കർ സിസ്റ്റങ്ങൾക്ക് കീഴിൽ പഠിച്ച ശേഖരങ്ങളിൽ 61% മുതൽ 70% വരെ ജനിതക വൈവിധ്യം ഉണ്ടെന്ന് നിലവിലെ പഠനത്തിന്റെ ഫലങ്ങൾ വെളിപ്പെടുത്തി. ഒരേ പ്രദേശത്തു നിന്നുള്ള റംബുട്ടാൻ ശേഖരങ്ങൾ അടുത്ത ജനിതക അകലം കാണിക്കുന്നു, ഇത് സന്തതി തൈകളുടെ വ്യാപനത്തെ അവയുടെ മാതൃസസ്യങ്ങളുടെ ജനിതകബന്ധം സ്വാധീനിക്കുന്നുവെന്ന് സൂചിപ്പിക്കുന്നു. ഈ വിദേശ വിളയുടെ ഭാവിയിലെ ക്രോപ് ഇമ്പ്രൂവ്മെന്റ് പരിപാടികളിൽ, സംസ്ഥാനത്തിന്റെ വിവിധ സ്ഥലങ്ങളിൽ നിന്ന് നിലവിലുള്ള വൈവിദ്ജ്യമാർന്ന ജെപ്ലാസങ്ങളിൽ കൂടുതൽ ജനറ്റിക് മാർക്കറുകൾ ഉപയോഗിക്കുന്ന തന്മാത്രാ വിശകലന പഠനങ്ങൾ നടത്താം. സാമ്പത്തികമായി പ്രാധാന്യമുള്ള പാതകളുമായി ബന്ധപ്പെട്ട മാർക്കറുകൾ തിരിച്ചറിയുന്നത് മാർക്കർ അസിസ്റ്റഡ് ബ്രീഡിംഗ് പ്രോഗ്രാമുകൾക്ക് ഉപയോഗപ്രദമാകും. മോളിക്യൂലർ മാർക്കറുകൾ ഉപയോഗിച്ചുള്ള റംബുട്ടാൻറെ ജനിതക വൈവിധ്യത്തെക്കുറിച്ചുള്ള നിലവിലെ പഠനം കേരളത്തി ലും ഇന്ത്യയിലും ആദ്യത്തേതാണ്, അതിനാൽ ഭാവിയിലെ അനുബന്ധ കൃതികളുടെ അടിസ്ഥാന വിവരമായി ഈ പഠനം കണക്കാക്കാം.