Journal of Applied Horticulture, 27(3): 457-460, 2025
https://doi.org/10.37855/jah.2025.v27i03.83
Journal of Applied Horticulture
ISSN: 0972-1045

Genetic diversity analysis of rambutan (*Nephelium lappaceum* L.) collections from Kerala using microsatellite molecular markers

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Abstract

Rambutan (*Nephelium lappaceum* L.) cultivation shows promising potential in Kerala, a tropical region in India known for its diverse agricultural practices; however, despite existing morphological characterization and diversity studies of local collections, comprehensive genetic diversity analysis using molecular markers remains lacking in the country. This research, conducted between 2021 and 2023, aimed to fill this gap by assessing the genetic diversity of twenty rambutan collections from the major rambutan growing regions in Kerala using SSR molecular markers. DNA isolation was performed using CTAB with minor modifications to obtain high-quality DNA samples. A sorbitol buffer pre-wash was done to improve the DNA quality. From 16 SSR primers screened, five were selected based on reproducibility and distinct banding patterns. These primers exhibited 100 % polymorphism, with polymorphic information content values ranging from 0.60 to 0.72, indicating their informativeness. The marker index, which measures primer utility, ranged from 1.80 to 2.88. Cluster analysis revealed two major clusters at a similarity coefficient of 0.70, with Cluster I comprising eight accessions and Cluster II comprising 12 genotypes. Principal coordinate analysis confirmed this clustering pattern. Genetic diversity ranged from 61 % to 70 % among the studied accessions, with collections from the same area exhibiting closer genetic distances, indicating possible dispersal from related parents. This study provides valuable foundational information for future crop improvement programs and marker-assisted breeding initiatives for rambutan in Kerala.

Key words: Rambutan, genetic diversity, molecular characterization, SSR Markers, dendrogram, PCoA Analysis

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 Southeast 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, the edible part of the fruit, constitute 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. In India the commercial cultivation of rambutan is confined to a few regions in Southern parts. With a total area of 1500 acres under cultivation in India, rambutan is primarily grown in Kerala and Karnataka. Rambutan has great potential for commercial cultivation in Kerala owing to the warm humid climate that prevails here.

As many of the tropical fruit species rambutan is also heterozygous in nature due to their high degree of outcrossing, systematic morphological characterization, complemented by molecular characterization, is necessary to assess the extent of variability and to utilize the existing germplasm effectively. Morphological and biochemical markers are used commonly to detect genetic diversity in fruit crops. However, their environmental plasticity makes them ineffective in detecting inter-varietal and intra-varietal polymorphisms. 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). 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 from the available seedling population (Sijimon, 2009). Muhamed (2016) examined the existing variability and genetic resources present in the major rambutan growing regions of Kerala. But studies on the characterization of rambutan genotypes using molecular markers are meager. In this scenario, the current study was undertaken with the primary objective of characterizing rambutan genotypes using molecular markers to identify potential genotypes from locally available seedling population of rambutan for future development.

Materials and Methods

Plant materials: In this study, plant samples were collected from three districts in Kerala, namely, Kottayam, Pathanamthitta, and

Thrissur, which are renowned as major rambutan cultivation regions in Kerala (Table 1). The plant material of interest comprised tender emerging leaves, and the collection process was performed during the early morning hours. To ensure optimal preservation, each collection's leaves were carefully wrapped in aluminum foil immediately upon collection. Subsequently, the leaves were transported to the laboratory within an icebox to maintain their freshness and integrity.

Upon arrival at the laboratory, a thorough cleansing of the leaf surface was performed using sterile water. To further eliminate potential contaminants, the leaves were carefully wiped with a solution of 70 percent ethanol. Following these preparatory steps, the cleaned plant samples were stored at a temperature of -80 Â°C until they were ready for use in the research.

Table 1. Details of rambutan collections selected for the study

Sl. 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

DNA Extraction: The genomic DNA was extracted from leaf samples using the Doyle and Doyle (1987) method with minor modifications. 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 5 mL of sorbitol buffer was added. The mixture was then spun at 10,000 rpm for 5 minutes, and the aqueous phase was discarded. This sorbitol buffer pre-wash step was repeated 4 to 5 times. Subsequently the samples were 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 10,000 rpm 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 again. 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.

The assessment of DNA quality and quantity was assessed using a UV Visible spectrophotometer (Shimadzu UV 1800). To ascertain the quality and integrity of the DNA samples, Agarose gel electrophoresis was employed, following the protocol outlined by Sambrook *et al.* (1989).

SSR Primer screening: A total of 16 SSR primers, previously documented by Razak *et al.* (2020) and Arias *et al.* (2020) for rambutan were utilized in this study. Based on preliminary screening of these, five primers were subsequently selected for more detailed analysis due to their ability to exhibit polymorphism. The PCR-amplified products generated by these selected primers were separated and visualized using a 3 % high-resolution agarose gel, alongside a 100 bp DNA ladder (Thermo Scientific).

PCR amplification: 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. For each sample, a reaction mixture of 20µL was prepared. This mixture included of 1.5 mM MgCl2, 200 µM dNTP mix (Promega), 1x concentration of 10X PCR Buffer, 0.5 units of Taq DNA polymerase (TakaRa), and 10 picomoles of each primer. The amplification was conducted in an Agilent PCR machine (Super Cycler 8800) following a specific program. Initially, denaturation was carried out at 94°C for 4 minutes, followed by denaturation at 94°C for 30 seconds, annealing at a temperature ranging from 50°C to 55°C for 1 minute, extension at 72°C for 1 minute and 50 seconds, and a final extension at 72°C for 10 minutes. The reaction underwent 35 cycles, and afterward, it was stored at 4°C. Following the amplification, the PCR products were separated on a 3 % high-resolution agarose gel, which had been dyed with ethidium bromide along with 100 bp DNA ladder (Fig.1). The gel documentation was performed using the Biorad Geldoc EZ imager.

Data analysis: The bands were assessed through visual observation, and their existence was denoted as "1, " while absence was noted as "0" on the gel. The band sizes were determined by referencing the ladder sizes. Genetic diversity was estimated by analyzing these manually scored bands using NTSYS version 2.1 (Rohlf, 1992), and cluster analysis was conducted using the Unweighted Pair Group Method (UPGMA) (Sneath and Sokal, 1973). The Polymorphic information content (PIC) of the markers were calculated using the formula PIC =1-Σ $(pi)^2$, Where, pi is the frequency of i th allele (Milbourne *et al.,* 1997). Marker index (MI) of the markers were calculated using the formula MI = PIC × No. of polymorphic bands (Powell *et al.,* 1996).

Result and discussion

In the current SSR analysis, 16 SSR primers which were reported in earlier studies were screened. This included twelve Nla SSR

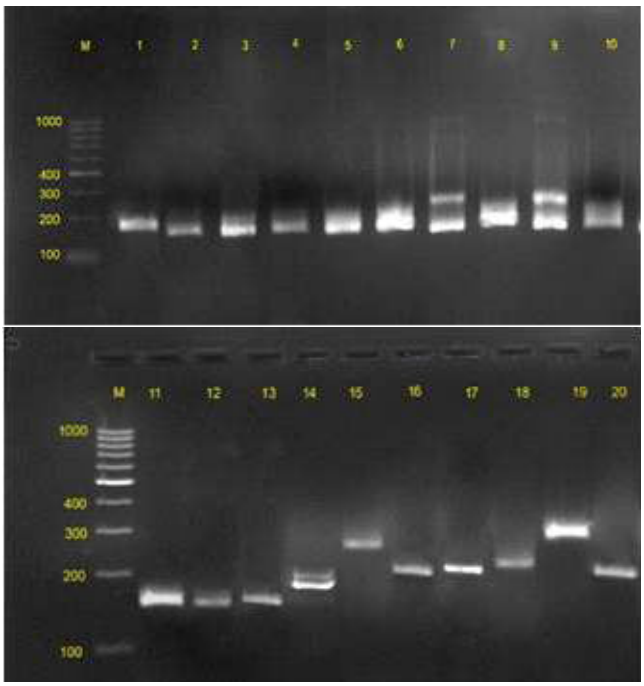

Fig. 1. DNA amplification pattern generated with the primer Nla SSR 3. M-DNA marker (100bp), 1-Col.19, 2-Col.20, 3-Col.01, 4-Col.02, 5-Col.03, 6-Col.04, 7-Col.05, 8-Col.52, 9-Col.53, 10-Col.06. M-DNA marker (100bp), 11-Col.48, 12-Col.42, 13-Col.15, 14-Col.61, 15-Col.62, 16-Col.81, 17-Col.86, 18-Col.87,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

and four Stv-nel series. Out of the sixteen primers, five primers were chosen for additional examination based on polymorphism pattern (Table 2). The research on SSR markers for rambutan is limited. Xing *et al.*(2019), Razak *et al.*(2020) and Arias *et al.*(2020) reported few SSR primers for rambutan.

The selected five SSR primers yielded a total of 18 amplicons with an average of 3.6 per primer (Table 3). The percentage of Polymorphism recorded was 100 %. The PIC Value for the selected SSR primers varied from 0.60 (NlaSSR 7) to 0.72(NlaSSR 23) with an average of 0.66. Xing *et al.* (2019) stated that the polymorphic information content (PIC) of SSR primers ranged between 0.36 and 0.49, with an average of 0.39 among 68 rambutan accessions. While Razak *et al.*(2020) reported 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 closely align with the findings of present study. These findings underscore the reliability and relevance of our findings in the context of genetic diversity assessment in rambutan populations. The marker index for SSR primers varied from 1.80 to 2.88, with an average of 2.42. The marker index provides insight into a primer's ability to identify polymorphic loci within different genotypes (Varshney *et al.,* 2007).These MI values provide an indication of the efficiency of the selected primers in capturing genetic variations within the rambutan population.

Cluster analysis: The polymorphism produced by the SSR primers for the twenty rambutan collections were employed to create the dendrogram as depicited in Fig. 2. At a coefficient of 0.70, the rambutan genotypes were divided into two main clusters. Cluster I comprised eight members (Col.19, 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.05, Col.53, Col.48, Col.15, and Col.42). Cluster I was further divided

Table 2. List of SSR primers selected for amplification in rambutan

Primer	Annealing temperature (°C)	Nucleotide sequence (5'-3')
NlaSSR 23	52	F: GTTGGTGCAGTCAAGGCTC R: GCTGACGACACCGCAGG
NlaSSR 3	55	F: AGAAGTTGTGAGTCAACTGAGAC R: AGATTTGGTACATACAGAGCAGG
NlaSSR 5	52	F: AGTCTACAATTTCGCCACAAAG R: GTAACTCCAAGCAACCGCC
NlaSSR 7	53	F: ATTCAACAGGGCTCCTGCC R: CAGTGCTCGTCTTCACC
NlaSSR 12	55	F: TCCAGTCTAAGAGCAGCAAATC R: TGGTGGTGGCTGCAATCTG

Table 3. Details of DNA amplification with selected SSR primers

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

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 and Col 87 and Col.97 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.05, and Col.53) and subcluster IIB (Col.48, 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 %. Research on genetic diversity analysis in rambutan using SSR markers is limited. In a study conducted by Razak *et al.* (2020), twenty rambutan varieties were grouped into two distinct clusters in neighbor joining dendrogram. The dendrogram analysis demonstrated that no clear morphological or phenotypical similarities or differences were evident among the clones within the same cluster.

The current study observed a genetic diversity of 70 % among the 20 genotypes studied. Collections from the same location showed lower genetic distance, indicating potential dispersal from related parents. These findings are supported by Chhetri *et al.* (2019). The Cluster I included all thirteen collections collected from the Pathanamthitta district and also the three collections from the Kurichy area of Kottayam. Cluster II consisted of seven collections within Kottayam district. A notable division was observed among the rambutan collections gathered from various districts in Kerala.

Principal coordinate analysis (PCoA analysis): 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 Fig. 3. Cluster I consists of Col.19, 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

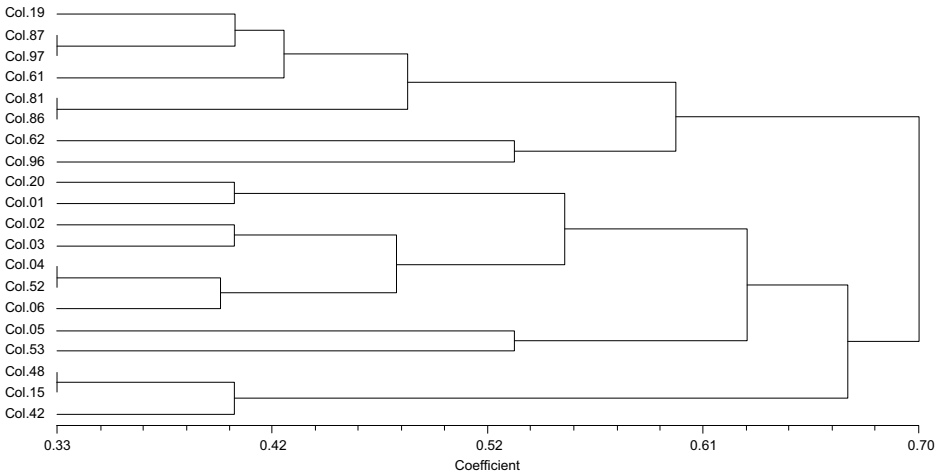

Fig. 2. UPGMA Dendrogram based on similarity coefficient for SSR analysis of rambutan collections

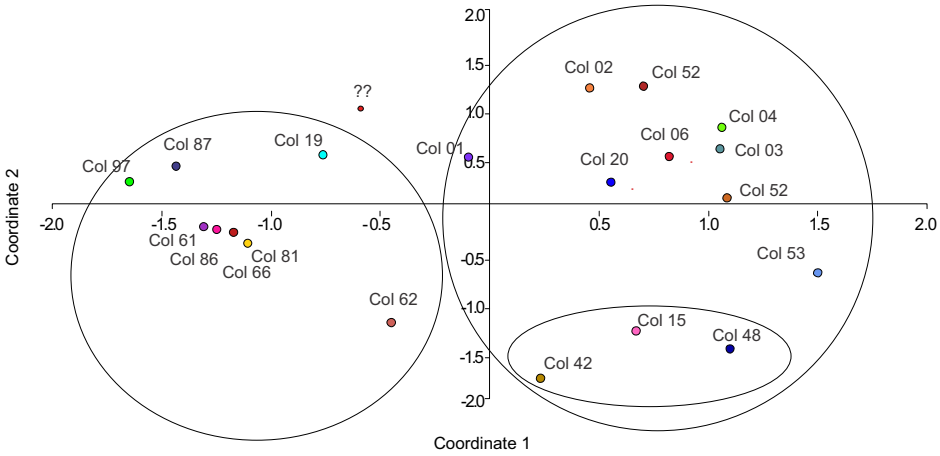

Fig. 3. Principal Coordinate Analysis based on SSR data

consistent with the results obtained from the UPGMA cluster analysis of the SSR data. There was a large degree of grouping based on their geographical distribution. Gajera *et al.* (2014) also found a similar clustering division in their dendrogram and PCoA plot in their study on the genetic variability in mango. Furthermore, in another study by Guo *et al.* (2011), the distribution of grape varieties in the PCoA plot and UPGMA dendrogram exhibited a striking resemblance.

The results of the current study revealed a genetic diversity of 70 % for the rambutan collections from major rambutan growing regions of Kerala. Lower genetic distance was observed between collections 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. 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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Received: February, 2025 ; Revised: March, 2025 ; Accepted: April, 2025