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Molecular Characterization and DNA Fingerprinting of Superior Jackfruit Genotypes from Kerala using SSR Markers

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A B S T R A C T

The *Artocarpus heterophyllus* Lam commonly known as jackfruit belongs to the family Moraceae. The Western Ghats of India is believed to be the centre of origin of jackfruit. A study on Molecular characterization and DNA fingerprinting of promising selections of jackfruit (*Artocarpus heterophyllus* Lam.) using SSR markers was carried out at Regional Agricultural Research Station (R.A.R.S.), Kumarakom and Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara, Kerala during the period 2015-2017. The objectives of the study were to characterize jackfruit varieties using SSR markers and to develop DNA fingerprint with which the variety could be identified and its fidelity is detected. A total of eight jack genotypes including one KAU released jack variety (Sindhur), one Cultivar (Muttom varikka) and six superior jackfruit selections (Veloor varikka-1, Veloor varikka-2, Kavanar varikka-1, Pathamuttom varikka-1, Pathamuttom Varikka-2 and Chengalam varikka) identified at RARS, Kumarakom were utilized for the study. DNA extraction was done with CTAB method with slight modification. A set of 50 SSR primers reported from the related genera were screened for polymorphism. The PCR products obtained from SSR analysis were separated on 3 percent high resolution agarose gel and the amplification patterns were observed. Eleven SSR primers which showed maximum polymorphism were selected for fingerprinting. The amplification pattern obtained with these primers were scored and depicted to develop fingerprint for each variety. Most of the amplicons were found to be shared among the genotypes. However, the pattern of sharing was different and good enough to separate out most of the varieties. Unique amplicons were observed for Sindhur, Pathamuttom varikka-1, Pathamuttom varikka-2 and Chengalam varikka, which can act as specific fingerprint of these genotypes. Among the SSR primers, MAA145 showed 100% polymorphism. The PIC value for SSR primers ranged from 0.22 to 0.98 with an average of 0.79 and MI value varied from 0.66 to 2.7 with an average of 1.18.

Introduction

The jackfruit (*Artocarpus heterophyllus* Lam.) is a tetraploid (4n=56) which belongs to the family Moraceae. It is native to parts of South and Southeast Asia, and is believed to have originated in the south western rain forests of India (Rowe-Dutton, 1985). In Western Ghats it is found up to 1500m and has tremendous diversity (Muralidharan *et al.*, 1997). India is

one of the major jackfruit producing country. Malaysia, Bangladesh, Vietnam, Thailand, China, Myanmar, Indonesia, Sri Lanka are the other major jackfruit producers in world. Jackfruit is the National fruit of Bangladesh and is also known as "Poor man's food" (Rahman *et al.*, 1995). It is the World's largest edible fruit reaching up to 50 kg in weight and produces higher yield than any other fruit crops (Naik, 1949).

In India, Kerala is the largest producer of jackfruit and it is about 28 lakh tonnes from an area of 89702 ha. Tamil Nadu, Karnataka, Maharashtra, Andhra Pradesh, West Bengal, Assam, Tripura, Bihar and Uttar Pradesh are other jack growing states (APAARI, 2012).

The knowledge about genetic diversity of jack is important to identify superior genotypes for cultivation. In Kerala, such studies have resulted in the release of a new jack variety 'Sindhur' and selection of some superior genotypes. Regional Agricultural Research Station (RARS), Kumarakom had conducted studies on the variability of jack fruit in Kuttanad region and identified few promising genotypes (Krishnan *et al.*, 2015). The Central Seed Committee established under the Seed Act 1966 insists for DNA fingerprint data for the varieties released or proposed to be released.

The specific fingerprint data will serve as a mark for identifying the varieties and could be utilized for registration and documentation of varieties, settling IPR issues and to avoid bio piracy. There are no reports on the DNA fingerprinting of different jackfruit cultivars in Kerala.

The objectives of present study were to characterize the major jackfruit genotypes using SSR markers and to develop DNA fingerprint with which the variety could be identified and its fidelity can be detected.

Materials and Methods

Plant materials

Eight selected jackfruit genotypes viz., variety Sindhur, cultivar Muttom varikka and six superior jack selections (Veloor varikka-1, Veloor varikka-2, Pathamuttom varikka-1, Pathamuttom Varikka-2, Chengalam varikka and Kavanar Varikka) collected from Kuttanad tract and maintained at RARS, Kumarakom were used for the study. Tender emerging leaves were collected from each genotype early in the morning. After covering with aluminium foil, the leaves were brought to the laboratory in ice box. The leaf surface was washed with sterile water and wiped with 70 percent ethanol and was stored at -80 °C until use.

DNA extraction

CTAB method developed by Doyle and Doyle (1987) was used for the extraction of genomic DNA. CTAB isolation buffer (2X) was preheated in a 50 ml Oakridge centrifuge tube to 60°C in a water bath. Fresh leaf tissue (0.2 – 0.5g) is ground with a pinch of polyvinyl pyrrolidin (PVP), 50 µl of 2-βmercaptoethanol and liquid nitrogen. The powdered sample was transferred to 2 ml eppendorf tube containing 1 ml of preheated CTAB solution. The sample is incubated for 30 minutes at 60°C with occasional gentle swirling. Equal volume of chloroform – isoamyl alcohol (24:1) mixture was added to the tube. It was mixed gently by inversion and was centrifuged (Eppendorf) at 12000 rpm for 20 min at room temperature. The content got separated into three distinct phases. The top aqueous layer was transferred to a sterile eppendorf tube. After transferring the aqueous phase into a clean eppendorf tube, 0.6 ml of chilled isopropanol was added. This was mixed by gentle inversion till the DNA was precipitated. These tubes were kept at -20°C for half an hour for complete

precipitation. After this, it was centrifuged for 15 minutes at 12,000 rpm at 4°C. The supernatant was gently poured off. The DNA pellet was washed with 10-20 µl of wash buffer with centrifugation at 1000 rpm for 5 min. The supernatant was carefully removed. It was again washed with 70% ethanol. Again the tubes were spun for 5 minutes at 1000 rpm and ethanol was decanted. The remaining pellet was air dried and RNA contamination was removed by adding 1µl of 10 mg/l RNase A to 50 µl of sample DNA. The purified DNA pellets were dissolved in 50 µl of TE buffer and stored at -20 °C until use.

The quality and quantity of DNA were examined by using Nanodrop^{R} spectrophotometer (ND-1000). The quality and integrity of DNA were assessed through Agarose gel electrophoresis (Sambrook *et al.*, 1989).

SSR primer screening

A set of 50 SSR primers reported from the related genera, mulberry (Mathithumilan *et al.*, 2013) and breadfruit (Witherup *et al.*, 2013; De Bellis *et al.*, 2016) were selected for the study. Screening was done using bulked DNA from eight jackfruit genotypes. Out of the fifty SSR primers eleven primers were selected for further analysis based on polymorphism. PCR amplified product of these primers were resolved on 3 % high resolution agarose gel along with 100 bp DNA ladder (Thermo scientific) and scored for developing fingerprints and molecular characterization.

PCR amplification

The PCR amplifications of DNA with SSR primers were carried out in 25 µl reaction mix consisted of 1X Taq buffer with 1.5 mM $MgCl_2$, 125 µM dNTPs, 0.5 picomoles of each primers, 0.5 unit Taq DNA polymerase (TaKaRa) and 40 ng template DNA. The amplification was performed in Agilent PCR machine (Super Cycler 8800) using the programme; 94°C for 2 min, 35 cycles of 94°C for 30 sec, annealing 50-55°C (varied) for 30 sec, 72°C for 1 min 50 sec and final extension of 8 min. After amplification the PCR products were resolved on 3% high resolution agarose gel (stained with ethidium bromide) along with 100 bp DNA ladder. The gels were documented with Biorad Geldoc EZ imager.

Data analysis

The bands were scored by visual observation for their presence (1) or absence (0) on the gel. The sizes of bands were detected based on the ladder size. Genetic variability was estimated based on this manually scored bands using NTSYS version 2.1 (Rohlf, 1992) and cluster analysis was done using Unweighted Pair Group Method (UPGMA) (Sneath and Sokal, 1973). Fingerprints were developed using the scored bands of selected primers using Microsoft office excel. Different colour codes were provided to the bands of different genotypes based on their molecular weight, same colours were provided to the amplicones of same size. The Polymorphic information content (PIC) of the markers were calculated using the formula PICi =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).

Results and Discussion

Fifty SSR primer sets were screened for polymorphism in eight jack genotypes studied after bulking the DNA. Eleven SSR primer sets were selected based on their amplification pattern for developing fingerprints of eight jack genotypes. Witherup *et al.*, (2013)

reported 13 SSR markers and De Bellis *et al.,* (2016) reported 21 SSR makers in jackfruit. Six of the selected primers belonged to mAaCIR series and five belonged to MAA (Table 1). These primers yielded a total number of 29 alleles with an average of 2.63 alleles per primer (Table 2). An average of 1.81 polymorphic alleles was produced by each primer. The percentage of polymorphism varied from 50 % (mAaCIR 0049, mAaCIR 0078, mAaCIR 0127, mAaCIR 0134, mAaCIR 0141 and MAA 122) to 100 % (mAaCIR 0115 and MAA 145). Calculated SSR PIC value for selected SSR primers were ranged between 0.22 (MAA 54a) to 0.98 (mAaCIR 0078, mAaCIR 0134, mAaCIR 0141 and MAA 122) with an average of 0.75. The Marker index for SSR primers varied between 0.60 (mAaCIR 0115) to 2.7 (MAA 196a) with an average 1.18 (Table 2). This showed the efficiency of selected primers to determine the genetic diversity. Mandal *et al.,* (2016) conducted discrimination analysis and obtained that MI was the most efficient parameter for the identification of most capable primer. In jackfruit, only a few SSRs markers were reported.

DNA fingerprinting

Distinct polymorphic bands generated for eight jackfruit genotypes using eleven SSR primer sets were consolidated and was depicted in the colour chart. The fingerprint was generated using polymorphic bands which were shared among maximum three genotypes. All the selected SSR primers yielded at least one useful polymorphic band.

Amplification with 11 SSR primers yielded 28 useful distinct polymorphic bands over eight jackfruit genotypes (Fig. 1). Molecular size of these selected bands ranged from 100 bp to 340 bp. Among these, 9 bands were unique, two were shared by two genotypes and five were shared by three genotypes. Maximum number of 6 amplicons were observed in Pathamuttom varikka-2 and Sindhur, whereas, the genotypes Veloor varikka-1 and Veloor varikka-2 had only one useful amplicon. The primer MAA 105 generated maximum number of amplicons (7).

Among these selected primers, seven of them produced specific bands which can acts as variety specific DNA fingerprints. Primers mAaCIR 0078 (at 150 bp) and MAA 122 (at 270 bp) produced unique band in Pathamuttom varikka-1. Amplification with mAaCIR 0134 (at 240 bp) and MAA 105 (at 265 bp) yielded unique bands in Pathamuttom varikka-2. At a length of 230 bp, primer mAaCIR 0141 poduced a specific band in Sindhur. In Pathamuttom varikka-2 and Sindhur unique bands were developed by the primer MAA 196a at 340 bp and 320 bp respectively. The primer MAA 145 generated unique band in Pathamuttom varikka-2 at 287 bp and Chengalam varikka at 280 bp (Fig. 3).

Apart from these unique bands, amplicons which were shared among maximum of three varieties were also selected for developing final SSR fingerprints for each genotypes. Amplification with mAaCIR 0049 yielded polymorphic bands in Kavanar varikka-1 and Muttom varikka at 100 bp. A polymorphic band (at 175 bp) shared by Pathamuttom varikka-1, Pathamuttom varikka-2 and Sindhur, when amplified with the primer mAaCIR 0115. Polymorphic amplicones at 200 bp generated by the primer mAaCIR 0127 was shared by Kavanar varikka-1, Chengalam varikka and Muttom varikka. Polymorphic band at 220 bp obtained by amplification with the primer MAA 54a was observed in Kavanar Varikka-1, Muttom varikka and Sindhur. On amplification with the primer MAA 105, Kavanar Varikka-1, Muttom varikka and Sindhur shared a polymorphic band at 290 bp and Veloor varikka-1, Veloor varikka-2 and Chengalam varikka shared a band at 270 bp.

Amplification with the primer MAA 196a developed a polymorphic band at 290 bp in Pathamuttom varikka-2 and Sindhur. In the present investigation the genotypes Veloor varikka -1, Veloor varikka -2 and Kavanar varikka -1, Muttom varikka could not be distinguished with unique band in SSR marker systems. This may be because only 11 SSR primers were used in this study and if more primers were screened, unique bands could be brought out for these varieties also. However the DNA fingerprints developed were unique for all the other genotypes.

Literature on DNA fingerprinting using SSR markers of jackfruit cultivar was not available. Baraket *et al.*, (2011) opined that SSR markers are suitable for diversity analysis and cultivar fingerprinting in *Ficus carica.* De Bellis *et al.,* (2016) developed and validated 50 SSR markers in breadfruit (*Artocarpus altilis*) by next generation sequencing, which are polymorphic in 39 bread fruit accessions. Similar DNA fingerprint utilizing SSR markers were developed in eight cocoa varieties released from Kerala Agricultural University (Sujith, 2016). Gopalsamy *et al.,* (2012) reported molecular marker (RAPD) based fingerprinting to estimate genetic diversity among five jackfruit accessions at GKVK, Bangalore. Kanupriya *et al.,* (2011) characterized 9 guava cultivars using 23 microsatellite markers and developed molecular barcodes.

Table.1 List of polymorphic SSR markers amplified in jackfruit genotypes

Sl. No.	Primer	Annealing temperature (^{o}C)	Nucleotide sequence
1	**mAaCIR 0049**	**53**	**F:5'-TACATACAAGCCAACTTCCA-3'** **R:5'-CCTTTGTGAGGAAGACCA-3'**
2	**mAaCIR 0078**	**53**	**F:5'-CTTCAACTATTACTACTGCTGCT-3'** **R:5'-CTGTTCAGGTTGGTGCT-3'**
3	**mAaCIR 0127**	**52**	**F:5'-TGATTCTCTCTTTACAGGCAC-3'** **R:5'-GCTCAGGTGCTTACTTGTTC-3'**
4	**mAaCIR 0134**	**55**	**F:5'-AGCTGCCAATGATCCC-3'** **R:5'-ATGTGAAAAGGTTGGATTTG-3'**
5	**mAaCIR 0141**	**55**	**F:5'-TCAAGCCCCTCACTCAA-3'** **R:5'-ATGGCATAGCACAACACAA-3'**
6	**MAA 54a**	**55**	**F:5'-AACCTCCAAACACTAGGACAAC-3'** **R:5'-AGCTACTTCCAAAACGTGACA-3'**
7	**MAA 105**	**55**	**F:5'-GTTGGGACACTGTGAACTATTC-3'** **R:5'-AAAAGCTAGTGGATTAGATGCA-3'**
8	**MAA 122**	**55**	**F:5'-CTGGCCTTCAGTTTTGTCAAC-3'** **R:5'-CACCAGGCTTCAAGATGAAA-3'**
9	**MAA 145**	**55**	**F:5'-CCAACGCATAGCCAAATC-3'** **R:5'-AAATCCCAAACCCAACGT-3'**
10	**MAA 196a**	**55**	**F:5'-GGAATGTGGTAGATGAAACTCC-3'** **R:5'-CGACAAAAAAACAAAGGAAGAC-3'**

Table.2 Details of DNA amplification with Selected 11 SSR primers

Sl. No	SSR Primer	Total no of alleles	No of polymorphic alleles	Polymorphism (%)	Polymorphic information content (PIC)	Maker Index (MI)
1	mAaCIR 0049	2	1	50	0.94	0.94
2	mAaCIR 0078	2	1	50	0.98	0.98
3	mAaCIR 0115	2	2	100	0.30	0.60
4	mAaCIR 0127	2	1	50	0.86	0.86
5	mAaCIR 0134	2	1	50	0.98	0.98
6	mAaCIR 0141	2	1	50	0.98	0.98
7	MAA 54a	4	3	75	0.22	0.66
8	MAA 105	4	3	75	0.70	2.1
9	MAA 122	2	1	50	0.98	0.98
10	MAA 145	3	3	100	0.40	1.2
11	MAA 196a	4	3	75	0.90	2.7
Total		**29**	**20**	**725**	**8.24**	**12.98**
Average		**2.63**	**1.81**	**65.90**	**0.75**	**1.18**

Fig.1 DNA fingerprint of six genotypes of jackfruit using SSR markers

Mol. Size(bp)/ Primer	Veloor varikka-1	Veloor varikka-2	Pathamuttom vaikka-1	Pathamuttom varikka-2	Kavanar varikka-1	Chengalam varikka	Muttom varikka	Sindhur
340				MAA 196a				
320								MAA 196a
290				MAA 196a	MAA 105		MAA 105	MAA 196a MAA 105
287				MAA 145				
280						MAA 145		
270	MAA 105	MAA 105	MAA 122			MAA 105		
265				MAA 105				
240				mAa 0134				
230								mAa 0141
220					MAA 54a		MAA 54a	MAA 54a
200					mAa 0127	mAa 0127	mAa 0127	
175			mAa 0115	mAa 0115				mAa 0115
150			mAa 0078					
100					mAa 0049		mAa 0049	

Colour codes for bands

1	2	3

1. Unique band
2. Polymorphic band shared between two genotypes
3. Polymorphic band shared between three genotypes

Fig.2 Dendrogram based on similarity coefficient for SSR analysis of jackfruit genotypes

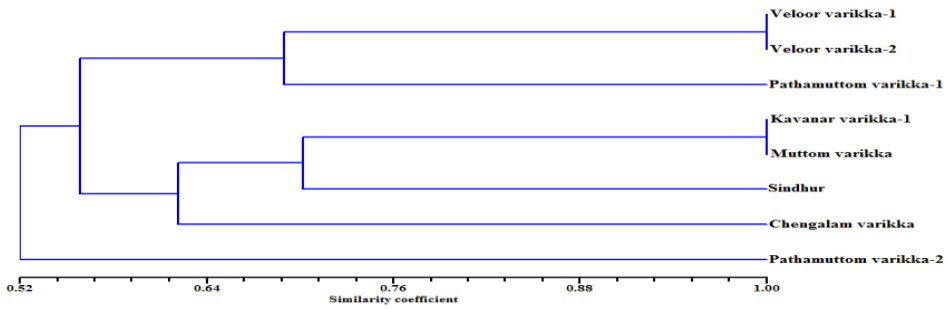
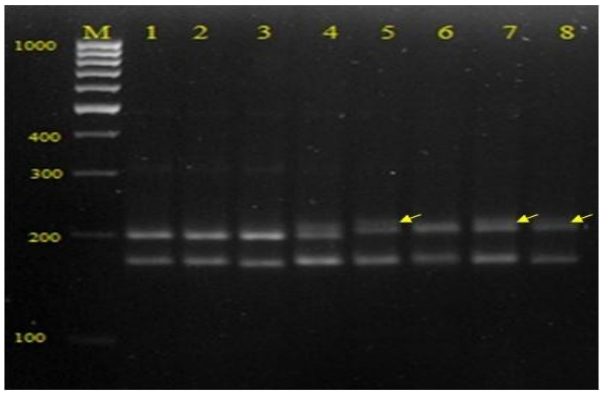


Fig.3 DNA amplification pattern generated with primer MAA 54a



M-Molecular weight marker (100bp), 1- Veloor varikka-1, 2- Veloor varikka-2, 3- Pathamuttom varikka-1, 4- Pathamuttom varikka-2, 5- Kavanar varikka-1, 6- Chengalam varikka, 7- Muttom varikka, 8- Sindhur

Cluster analysis

Polymorphism generated with SSR primers for the eight jack genotypes were also used for constructing dendrogram (Fig. 2). At 0.76 similarity coefficient, the jackfruit genotypes were clustered into six groups. Cluster I and III were formed with two members each *i.e*., cluster I contained Veloor varikka-1 and Veloor varikka-2 and Cluster III included Kavanar varikka-1 and Muttom varikka. Other four clusters were formed with only one member *i.e*., Cluster II, IV, V and VI with Pathamuttom varikka-1, Sindhur, Chengalam varikka and Pathamuttom varikka-2 respectively. Maximum variability observed was 48 percent for the variety Pathamuttom varikka-2. Studies on genetic diversity analysis in jack genotypes using SSR markers were meager. Shyamalamma *et al.,* (2008) evaluated genetic diversity among 50 jackfruit accessions was using 16 AFLP markers. Cluster analysis of these 50 accessions formed three major clusters. This information will be useful for further tree breeding programmes in jackfruit.

The present study has revealed the genetic relationship of eight Jackfruit genotypes using SSR markers. The specific DNA fingerprints developed for the jack variety Sindhur, promising selections Pathamuttom varikka-1, Pathamuttom varikka-2 and Chengalam varikka could be utilized for varietal identification and settling IPR issues.

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