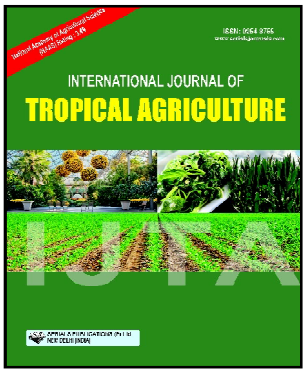

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Molecular Characterization of Jackfruit (*Artocarpus heterophyllus* L.) Accessions using ISSR Markers

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Abstract: Genetic variation among firm and soft flesh jackfruit accessions was assessed using inter-simple sequence repeat (ISSR) markers. Ten ISSR primers were used to evaluate ten accessions (five soft and five firm fleshes) and three varieties namely Sindoor, Muttom Varikka and Thamarachakka. In the present ISSR assay, total numbers of bands observed among the accessions and varieties with ten ISSR primers were 86 with average of 8.6 bands per primer. And out of them 48 were polymorphic which gave a percentage of polymorphism of 55.01 per cent with an average of 4.8 polymorphic bands per primer. Dendrogram for ISSR cluster analysis was generated by the unweighed pair group method with arithmetic mean (UPGMA) using the software package NTsys pc version 2.02i. Five main clusters were formed at 77 per cent similarity. Jaccard's similarity coefficient ranged from 70 to 100. Results indicate that ISSR can be useful for genetic diversity studies, to provide practical information for parental selection and to assist breeding and conservation strategies. Molecular markers could be successfully employed in determining the texture of the jackfruit flakes (firm/soft flesh types).

Keywords: Jackfruit, molecular markers, ISSR, genetic variability

INTRODUCTION

Jackfruit (*Artocarpus heterophyllus* L.) bearing world's largest fruit belongs to the family Moraceae. It is indigenous to the rainforests of Western Ghats of India. India is the largest producer of jackfruit in the World (Haq, 2006; APAARI, 2012). It is grown in states like Kerala, Karnataka, Tamil Nadu, Andhra Pradesh, Maharashtra, Assam *etc*. The estimated area of this fruit crop in Kerala is 89702 ha and the production is about 28 lakh tonnes. There are two

types of jackfruit *i.e.,* soft and firm flesh types according to the texture of the flakes. Due to its nutritional and economical value and food security, efforts have been made to promote its cultivation. Internationally, efforts have been made to promote jackfruit as an underutilized crop especially in countries plagued by malnutrition and economic depression. Several studies were conducted to explore the genetic variability in jackfruit in India and also in Kerala (Hossain, 1996; Anand, 1998; Muthulakshmi, 2003; Mathew *et al.,* 2003; Azad, 2007; Jagadeesh *et al.,* 2007; Shyamalamma *et al.*, 2008; Ullal and Haque, 2008 and Krishnan *et al.*, 2015). Variability studies were conducted and the selected accessions of jackfruit were maintained in college orchard of Kerala Agricultural University. Detailed evaluation of these accessions is very much essential for identifying trait specific types. Hence the present study is taken up with the objective to characterise the selected accessions of jackfruit based on the molecular analysis to facilitate future breeding programme.

MATERIALS AND METHODS

Plant material and molecular markers

The jackfruit germplasm maintained in the in college orchard and research stations under Kerala Agricultural University, Thrissur, Kerala, India, was selected for the study during 2014-2015. Tender emerging leaves were collected early in the morning from individual plants. The collected leaves were quickly covered in aluminum foils and transported to the laboratory in ice box. The surface was cleaned by washing with sterile water and wiping with 70 per cent ethanol and stored at -80 ^{o}C till being used. CTAB method developed by Doyle and Doyle (1987) was used for the extraction of genomic DNA. The RNase A from Sigma, USA was used for the present study. One per cent solution was prepared by dissolving RNase in TE buffer at 100 ^{0}C for 15 minutes. The solution was cooled to room temperature, dispensed into aliquots and stored at -20 ^{0}C.

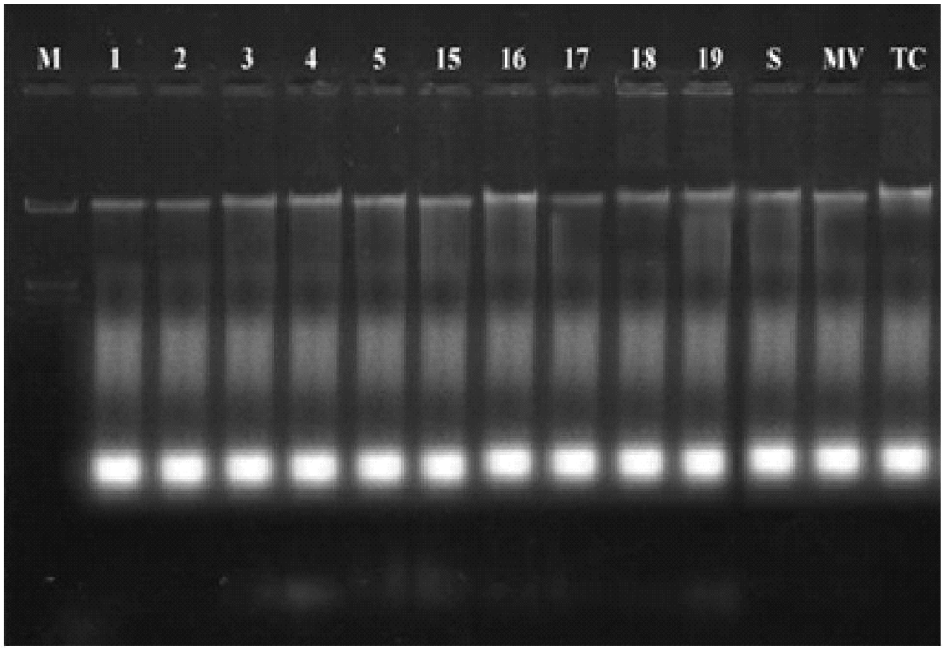
RNase solution (5 µl) was added to 100 µl DNA sample and incubated at 37 ^{0}C in dry bath (Genei, Thermocon) for one hour. Then added equal volume of chloroform: isoamyl alcohol (24: 1) and centrifuged at 10000 Xg for 20 min at 4 ^{0}C. The upper aqueous phase transferred to another tube. Repeated above step and finally precipitated the DNA from the aqueous phase with 0.6 ml volume of chilled isopropanol. The mixture was then incubated at -20 ^{0}C for 30 min and centrifuged at 10000 rpm for 15 min at 4 ^{0}C. The pellet of DNA was washed with 70 per cent ethanol. The pellet was air dried and dissolved in 50 to 100µl autoclaved distilled water. Electrophoresis was carried out 0.8 per cent agarose gel at constant voltage of 100V to test the quality and to find whether there was any shearing during RNase treatment.

RNase treatment and further precipitation gave sufficient quantity of good quality DNA from leaf sample. The agarose gel electrophoresis indicated clear discrete band without RNA contamination (Plate 1) and spectrophotometric analysis gave ratio of UV absorbance (A 260/280) between 1.8 and 2.0. Quality and quantity of DNA isolated through the Doyle and Doyle method for jackfruits are depicted in Plate 1 and Table 1.

Screening of ISSR primers and analysis

Fifty primers were screened for ISSR analysis (Table 2). And out of 50 screened primers, 10 primers were selected based on their good amplification power.

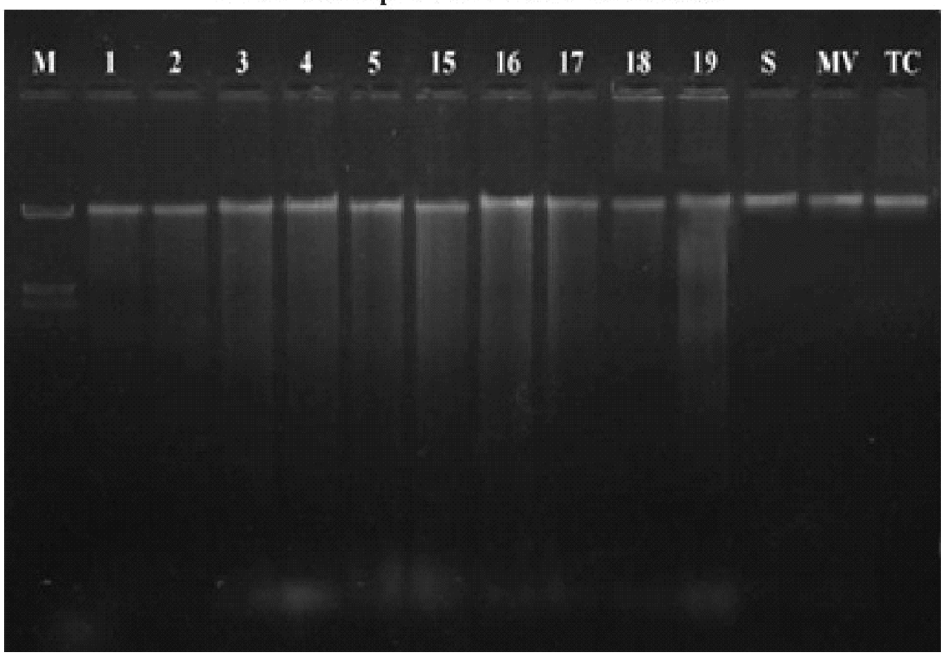
The amplified products were run on 2 per cent agarose gel using 1X TAE buffer stained with ethidium bromide along with marker (100-bp DNA ladder). The profile was visualized under UV (312 nm) transilluminator and documented by using gel documentation system (BIO-RAD Imaging system, USA) for further analysis. The documented ISSR profiles were carefully examined for amplification



M: Molecular weight marker λ DNA (Hind III digest) Lane 1 to 19,S,MV and TC jack fruit DNA samples

1-Acc 1, 2-Acc 2, 3-Acc 3, 4-Acc 4, 5-Acc 5, 15-Acc 15, 16-Acc 16, 17-Acc 17, 18-Acc 18, 19-Acc 19, S-Sindoor, MV-Muttom Varikka and TC-Thamarachakka

1a. DNA samples before RNase treatment



M: Molecular weight marker λ DNA (Hind III digest) Lane 1 to 19,S,MV and TC jack fruit DNA samples

1b. DNA samples after RNase treatment

Plate 1: Isolation of DNA and its purification

Table 1
Quality and quantity of DNA isolated from jackfruit genotypes by Nano Drop spectrophotometer

Accessions/ Varieties	*UV absorbance at 260 nm (A_{260})*	*UV absorbance at 280 nm (A_{280})*	*A_{260}/A_{280}*	*Quantity (ng/µl)*
Acc. 1	2.072	1.129	1.84	1981.80
Acc. 2	12.855	6.505	1.98	642.74
Acc. 3	3.897	1.959	1.99	194.83
Acc. 4	3.837	1.950	1.97	191.84
Acc. 5	9.375	4.703	1.99	468.73
Acc. 6	7.835	4.061	1.93	391.75
Acc. 7	9.006	4.617	1.95	450.30
Acc. 8	9.202	4.621	1.99	460.10
Acc. 9	10.833	5.685	1.91	541.66
Acc. 10	10.419	5.399	1.93	520.95
Acc. 11	9.865	5.070	1.95	493.24
Acc. 12	9.045	4.829	1.87	452.26
Acc. 13	2.586	1.348	1.92	132.02
Acc. 14	1.567	0.789	1.96	178.33
Acc. 15	4.343	2.308	1.88	345.76
Acc. 16	5.529	2.833	1.95	786.98
Acc. 17	17.498	8.604	2.03	874.89
Acc. 18	10.294	5.000	2.06	917.50
Acc. 19	16.607	8.231	2.02	118.89
Acc. 20	6.676	3.267	2.04	149.49
S	4.956	2.588	1.82	372.82
MV	0.640	0.354	1.81	256.81
TC	0.889	0.478	1.86	139.53

of DNA. The numbers of monomorphic and polymorphic bands were recorded for further analysis.

Analysis of molecular data

Scoring of bands in agarose gel was done with the Quantity one software (BIO-RAD) in the Gel Doc imagination system. 100 - bp ladder was used as molecular weight size marker for each gel along with DNA samples. Jaccard's coefficient of similarity was measured and a dendrogram based on similarity coefficient was generated by using Unweighed Pair Group Method with Arithmetic means (UPGMA). Only the distinct and well resolved fragments were scored. The resulting data were analysed using the software package NTsys (Rohlf, 2005).

Molecular Marker Analysis

The protocols for marker assays for ISSR are validated with bulked DNA of jackfruit varieties. Different primers were screened with the genomic DNA of 10 accessions and 3 varieties utilizing the validated protocols.

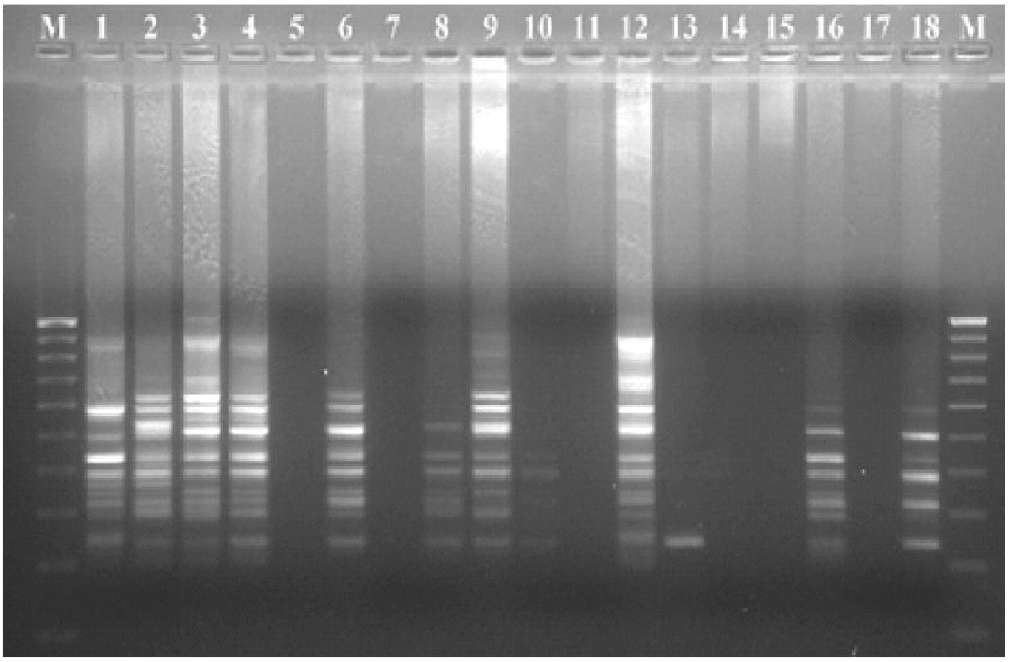
Table 2
Primers screened for ISSR analysis

Sl. No	*Primer*	*Nucleotide Sequence*
1	UBC 811	5'GAGAGAGAGAGAGAGAC3'
2	UBC 813	5'CTCTCTCTCTCTCTCTT3'
3	UBC 814	5'CTCTCTCTCTCTCTCTA3'
4	UBC 815	5'CTCTCTCTCTCTCTCTG3'
5	UBC 834	5'AGAGAGAGAGAGAGAGYT3'
6	UBC 835	5'AGAGAGAGAGAGAGAGYC3'
7	UBC 836	5'AGAGAGAGAGAGAGAGYA3'
8	UBC 840	5'GAGAGAGAGAGAGAGAYT3'
9	UBC 844	5'CTCTCTCTCTCTCTCTRC3'
10	UBC 890	5'VHVGTGTGTGTGTGTGT3'
11	UBC 866	5'CTCCTCCTCCTCCTCCTC3'
12	1UBC 807	5'AGAGAGAGAGAGAGAGT3'
13	UBC 843	5'CTCTCTCTCTCTCTCTRA3'
14	UBC 812	5'GAGAGAGAGAGAGAGAA3'
15	UBC 820	5'GTGTGTGTGTGTGTGTC3'
16	UBC 854	5'TCTCTCTCTCTCTCTCRG3'
17	UBC 845	5'CTCTCTCTCTCTCTCTRG3'
18	UBC 817	5'CACACACACACACACAA3'
19	UBC 826	5'ACACACACACACACACC3'
20	UBC 818	5'CACACACACACACACAG3'
21	ISSR 04	5'ACACACACACACACACC3'
22	ISSR 05	5'CTCTCTCTCTCTCTTG3'
23	ISSR 06	5'GAGAGAGAGAGAGAGAC3'
24	ISSR 07	5'CTCTCTCTCTCTCTTG3'
25	ISSR 08	5'GAGAGAGAGAGAGAGAT3'
26	ISSR 09	5'CTCTCTCTCTCTCTCTCG3'
27	ISSR 10	5'ACACACACACACACACG3'
28	ISSR 15	5'TCCTCCTCCTCCTCC3'
29	2UBC 808	5'AGAGAGAGAGAGAGAGC3'
30	3UBC 809	5'AGAGAGAGAGAGAGAGG3'
31	UBC 868	5'GAAGAAGAAGAAGAAGA3'
32	UBC 895	5'AGAGTTGGTAGCTCTTGATC3'
33	UBC 899	5'CATGGTGTTGGTCATTGTTCCA3'
34	UBC 880	5'GGAGAGGAGAGGAGA3'
35	UBC 892	5'TAGATCTGATATCTGAATTCCC3'
36	UBC 855	5'ACACACACACACACACYT3'
37	UBC 858	5'TGTGTGTGTGTGTGTGRT3'
38	UBC 864	5'ATGATGATGATGATGATG3'
39	(ACTG)4	5'ACTGACTGACTGACTG3'
40	UBC 873	5'GACAGACAGACAGACA3'
41	(GACAC)4	5'GACACGACACGACACGACAC3'
42	(TC)10G	5'TCTCTCTCTCTCTCTCTCTCG3'
43	(CT)10A	5'CTCTCTCTCTCTCTCTCTCTA3'
44	(CT)10G	5'CTCTCTCTCTCTCTCTCTCTG3'
45	UBC 841	5'GAGAGAGAGAGAGAGATYC3'
46	UBC 830	5'TGTGTGTGTGTGTGTGG3'
47	UBC 900	5' ACTTCCCCACAGGTTAACACA3'
48	UBC 825	5'ACACACACACACACACT3'
49	UBC S2	5'CTCTCTCTCGTGTGTGTG3'
50	4UBC 810	5'GAGAGAGAGAGAGAGAT3'

RESULTS AND DISCUSSION

The good quality genomic DNA isolated from jackfruit accessions were subjected to the ISSR assay. Fifty ISSR primers used for amplification of the genomic DNA with thermal settings mentioned earlier gave different amplification pattern (Table 2 and Plate 2) for the bulked DNA. From the amplified primers, 10 were selected for their reproducibility and polymorphism and used in the study and details are provided in Table 4. Characterization was done with 10 primers (Plate 3 and 4) so as to generate the ISSR data (Table 4).

Dendrogram for ISSR (Fig.1) cluster analysis was generated by the unweighed pair group method with arithmetic mean (UPGMA) using the software package NTsys version 2.02i . Jaccard's similarity coefficient ranged from 70 to 100. Five main clusters were formed at 77% similarity. The first cluster grouped 4 accessions (Acc 1, Acc 3, Acc 5, Acc 4) (Table 5). The second cluster consists of Sindoor. Third cluster consists of Sindoor. Third cluster consisits of Thamarachakka, fourth cluster consists of Acc 2 and Muttom Varikka and fifth cluster



M: Marker (100bp) ladder, 1-18: Amplification pattern with different ISSR primer

1-18: UBC 834, UBC 840, UBC 866, UBC 807, UBC 818, UBC 812, UBC 808 UBC 809, UBC 855, UBC 858, UBC 845, (TC) 10 G, UBC 817, UBC 818, UBC 810, UBC 825, UBC 880, UBC 826

Plate 2: Screening of ISSR primers for amplification of jackfruit genomic DNA

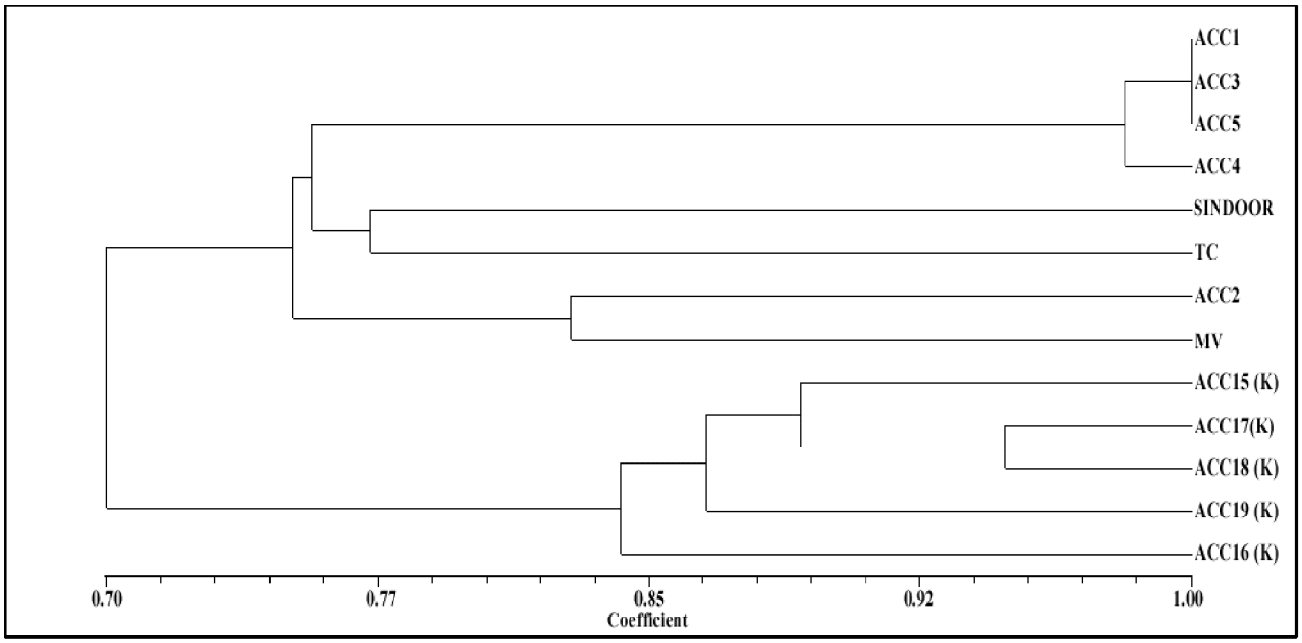


Figure 1: Dendrogram representing the molecular characterization

Table 3
Details of amplification with 50 primers screened for ISSR assay in jackfruit genotypes

Sl. No.	*Amplification pattern*				*Remarks*
	Primer	*No. of bands*	*Types of bands*		
			Distinct	*Faint*	
1	UBC 811	1	1	0	-
2	UBC 813	1	1	0	-
3	UBC 814	2	1	1	-
4	UBC 815	0	0	0	-
5	UBC 834	13	7	6	Selected
6	UBC 835	0	0	0	-
7	UBC 836	2	1	1	-
8	UBC 840	14	6	8	Selected
9	UBC 844	3	1	2	-
10	UBC 890	0	0	0	-
11	UBC 866	13	8	5	Selected
12	UBC 807	8	6	2	Selected
13	UBC 843	0	0	0	-
14	UBC 812	13	8	5	Selected
15	UBC 820	5	5	0	-
16	UBC 854	4	0	4	-
17	UBC 845	0	0	0	-
18	UBC 817	0	0	0	-
19	UBC 826	0	0	0	-
20	UBC 818	0	0	0	-
21	ISSR 04	0	0	0	-
22	ISSR 05	0	0	0	-
23	ISSR 06	0	0	0	-
24	ISSR 07	2	2	0	-
25	ISSR 08	2	0	2	-
26	ISSR 09	4	4	0	-
27	ISSR 10	2	1	1	-
28	ISSR 15	4	2	2	-
29	UBC 808	4	0	4	-
30	UBC 809	10	8	2	Selected
31	UBC 868	11	5	6	-
32	UBC 895	2	1	1	-
33	UBC 899	3	2	1	-
34	UBC 880	0	0	0	-
35	UBC 892	0	0	0	-
36	UBC 855	11	6	5	Selected
37	UBC 858	14	6	8	Selected
38	UBC 864	7	3	4	-
39	(ACTG)4	5	5	0	-
40	UBC 873	3	1	2	-
41	(GACAC)4	0	0	0	-
42	(TC)10G	14	10	4	Selected
43	(CT)10A	1	1	0	-
44	(CT)10G	2	1	1	-
45	UBC 841	14	5	9	Selected
46	UBC 830	0	0	0	-
47	UBC 900	0	0	0	-
48	UBC 825	0	0	0	-
49	UBC S2	0	0	0	-
50	UBC 810	0	0	0	-

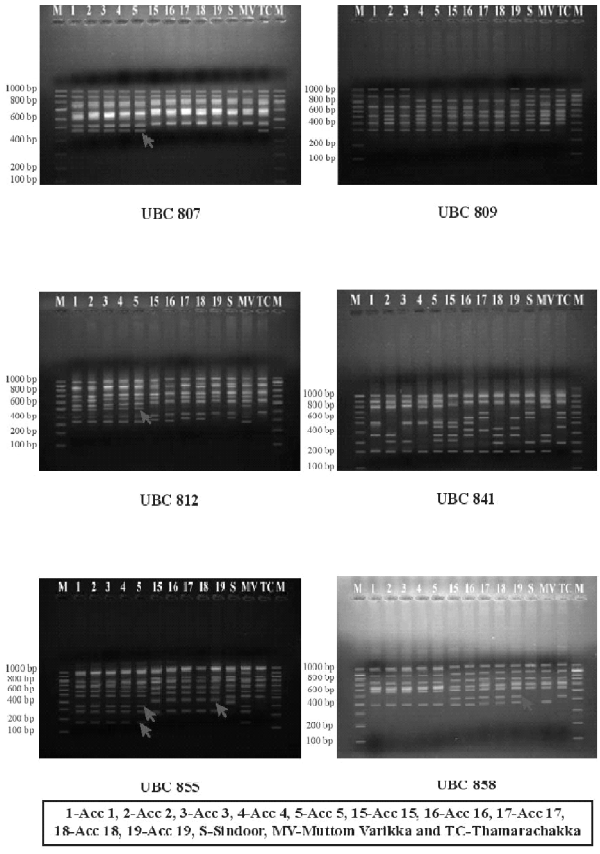


Plate 3: Amplification patterns of jackfruit genotypes with ISSR primers-UBC 807, UBC 809, UBC 812, UBC 841, UBC 855 and UBC 858

Table 4
Details of amplification with selected primers for ISSR assay in jackfruit

Sl.No	*Primer*	*Total number of amplicons*	*No. of monomorphic amplicons*	*No. of polymorphic amplicons*	*Polymorphism (%)*	*Size of the amplicons from range (bp)*
1	UBC 807	9	5	4	44.45	400-1000
2	UBC 809	10	2	8	80.00	300-1000
3	UBC 812	9	4	5	55.56	300- 1000
4	UBC 841	11	2	9	81.82	200-1000
5	UBC 855	9	5	4	44.45	200 -1000
6	UBC 858	7	5	2	28.57	400- 1000
7	UBC 866	8	5	3	37.50	400-1000
8	UBC 834	9	5	4	44.45	200-1000
9	UBC 840	8	4	4	50.00	200-1000
10	TC10G	6	1	5	83.34	200-1000
	Total	86	38	48	550.14	
	Average	8.6	3.8	4.8	55.01	

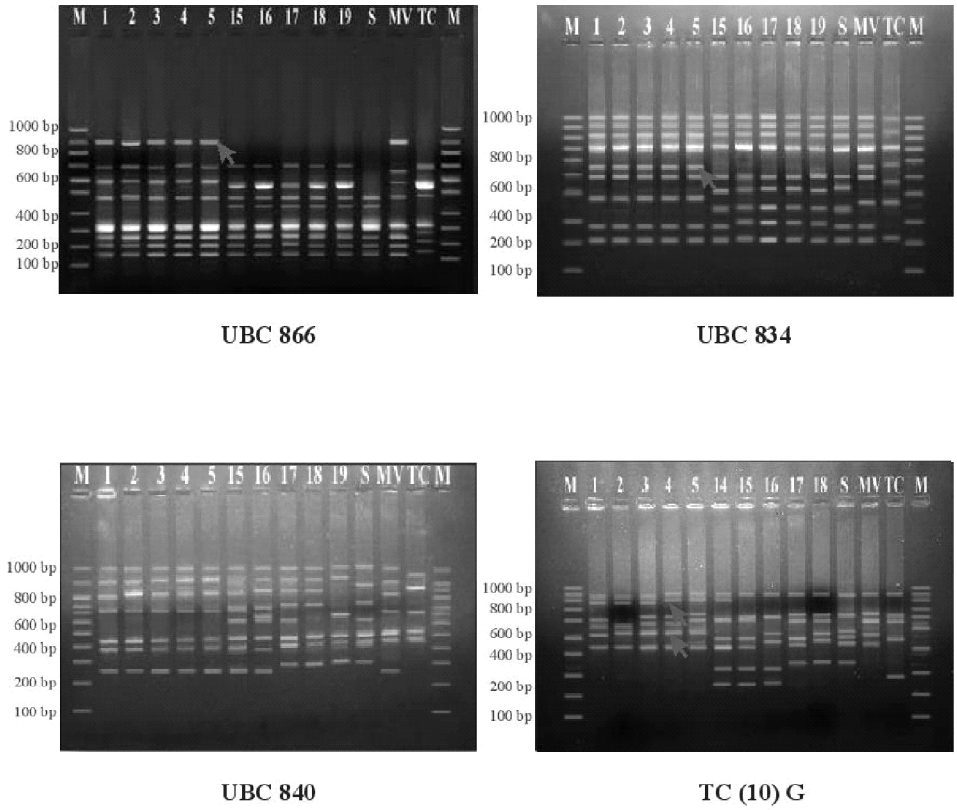


Plate 4: Amplification patterns of jackfruit genotypes with ISSR primers - UBC 866, UBC 834, UBC 840 and TC (10) G

Table 5
Cluster wise listing of accessions according to molecular characterization

Clusters				
I	*II*	*III*	*IV*	*V*
Acc. 1	Sindoor	Thamarachakka	Acc. 2	Acc. 15
Acc. 3			Muttom Varikka	Acc. 16
Acc. 4				Acc. 17
Acc. 5				Acc. 18
				Acc. 19

consists of five accessions (Acc 15, Acc 17, Acc 18, Acc 19, Acc 16).

In the present ISSR assay, total numbers of markers observed among the accessions and varieties with ten ISSR primers were 86 with average of 8.6 bands per primer. And out of them 48 were polymorphic which gave a percentage of polymorphism of 55.01 per cent with an average of 4.8 polymorphic bands per primer. ChunHai *et al.* (2009) evaluated 76 accessions of jackfruit using 24 ISSR primers and found 477 bands out of which 427 bands were polymorphic accounting to 89.52 per cent polymorphism.

Dendrogram for ISSR cluster analysis was generated by the unweighed pair group method with arithmetic mean (UPGMA) using the software package NTsys pc version 2.02i (Rohlf, 2005). Jaccard's similarity coefficient ranged from 70 to 100. Five main clusters were formed at 77 per cent similarity. The first cluster grouped 4 accessions (Acc 1, Acc 3, Acc 5, Acc4). The second cluster consists of Sindoor. Third cluster consist of Thamarachakka, fourth cluster consist of Acc 2 and MuttomVarikka and the fifth cluster consist of 5 accessions (Acc 15, Acc 17, Acc 18, Acc 19, Acc 16). Dendrogram was generated using NTsys (figure 1). First cluster included the firm fleshed jackfruit accessions whereas Cluster V included all the soft fleshed jackfruit accessions. Thus molecular data could be effectively utilized in identifying soft fleshed and firm fleshed accessions from a germplasm. Contrary to this, Ying-zhi *et al.* (2010) reported that soft and firm fleshed types were not clustered into distinct groups while characterizing 50 accessions of jackfruits with AFLP markers. Characterization using ISSR markers in this study also gave 55 per cent polymorphism which shows the existence of a good amount of genetic variability.

Genetic diversity of plants are determined by their morphological characteristics. But these traits are influenced by environmental factors and many of the qualitative characters which are of polygenic inheritance and expressed only after flowering or fruiting. Hence such types of characterization are not cent per cent reliable and also time consuming. In this context biochemical markers can be employed for a more accurate assessment. Scientists used isozymes and molecular markers to measure the genetic diversity and genetic relatedness in jackfruit (Schnell *et al.,* 2001 and Azad *et al.,* 2007).

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