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Protocol for DNA extraction and molecular characterization in medicinal plant *Carica papaya* L. varieties

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Abstract
Carica papaya L. shows considerable phenotypic variation in many horticultural traits like, fruit size, fruit shape, flesh color and sweetness. In present study, six varieties were differentiated with the help of molecular marker technique. Genomic DNA was isolated from these six varieties using standardized DNA isolation protocol. For molecular characterization, total 30 different primers of ISSR were tested for all six varieties. The PCR amplification results obtained using ISSR primers for all six varieties which showed unique band patterns on agarose gel electrophoresis. A statistical analysis of the monomorphic and polymorphic bands of the all varieties was performed. Among 30 primers, 24 primers given high polymorphism and proved the best to distinguish six varieties from each other. It is observed that ISSR technique is a useful molecular marker to distinguish *Carica papaya* varieties for best medicinal formulations and for clonal selection.

Keywords: *Carica papaya* L, genomic DNA, ISSR technique, PCR amplification

Introduction
Carica papaya L. commonly called papaya is belongs to family Caricaceae and is one of the major fruit crops cultivated in tropical and subtropical zones for its important medicinal values. Papaya was introduced in India from Malaysia in the 16th century (Sharma and Mitra, 2014) [19], shows great genetic diversity among the cultivars. Although biodiversity in the family Caricaceae is spread over 6 genera and 35 species, *Carica papaya* L. is the only plant available in India.

Considerable phenotypic variations are observed in the cultivars for numbers of horticultural traits including fruit size, fruit shape, flesh color, flavor, sweetness, length of juvenile period, etc. (Kim *et al*., 2002) [13]. Papaya is a fruit tree model system as it possesses relatively small genome and high levels of phenotypic diversity, and is amenable to transformation (Kim *et al*., 2002) [13]. The high level of phenotypic diversity seen among papaya cultivars in the field does not correlate with the law levels of genotypic polymorphism thus far elucidated (Eustice *et al*., 2008) [9]. However, genetic variation is a pre requisite for any crop improvement program to be successful.

DNA based molecular markers are popularly use in breeding program of many crop species and the plantation crops. These markers are more reliable because the genetic information is unique for each species and is independent of age, physiological conditions, and environmental factors (Joshi *et al*., 2004) [11]. Many markers like ISSR, AFLP etc. are sensitive at variety levels identification and generally explore for fingerprinting, diversity analysis, gene mapping, variability tracking, individuals or lines carrying particular genes and for linkage maps (Kalendar *et al*., 2011) [12]. In comparison, ISSR markers are easy to use and economic than AFLP; and results in reproducible and reliable. ISSR primers are longer and highly polymorphic and are useful in studies of genetic diversity. Such amplifications do not require genome sequence information and leads to multilocus and highly polymorphous patterns (Bornrt and Branchard, 2012) [2].

For molecular marker study, extraction of highly purified DNA is prerequisite. Efficient protocol yield both qualitative and quantitative DNA for the downstream applications. A problem of DNA extraction is an inevitable issue in the field of plant molecular biology. Each plant posses its unique chemistry such as high levels of polysaccharides and other macro molecules, many types of secondary metabolites including phenols and flavanoids; which affecting DNA extraction procedure. Thus, it is essential to develop a DNA extraction method that can be applied to various plant tissues to give good quality DNA suitable for molecular applications such as PCR.

In this paper we aimed to develop an efficient extraction protocol for quality DNA from the cultivars of *C. papaya,* showing phenotypic variations. These cultivars are distinguished using ISSR markers.

Materials and Methods

Collection of plant materials

Healthy leaves of six cultivars of *C. papaya* L. were collected from the farmer's field near Porbandar (21.65°N, 69.63°E). These cultivars were designated as V1 to V6. The leaves were washed under running tap water and dried over filter papers. Leaves were cut to small pieces and crushed in liquid N. The crushed material was stored at -20˚ C till use.

Extraction of DNA

DNA was extracted using modified Mandaliya *et al*., (2010) [15] protocol. About one gram of leaf powder was taken in mortar pestle and crushed with 0.2gm PVPP. Then added 8ml pre-heated triton X buffer and crushed the material. It was incubated at 65°C in water bath and both mixed gently for 3 to 4 times. After incubation 2.68ml of 5M KAC was added and further incubated on ice for 30 min. The mixture was centrifuged for 20 min. at 13,000 g at 4°C. The supernatant was transformed to new tube and 5.32ml isopropyl alcohol was added. It was incubated for 1 h at room temperature. The mixture was centrifuged at 13,000g for 15min. After centrifugation supernatant was discarded and pellet was taken and rinsed with 70% ethanol. Then mixture was centrifuged at 10,000 g for 15 min. After the supernatant was discarded and pellet was air dried at 37°C until all ethanol residues was evaporated. The pellet containing DNA was then dissolved in 2ml TE buffer. The samples were gently shaken for 1 hr. till it completely dissolved.

The DNA was transferred to the microfuge tube and 2.5 µl RNase was added. The mixture was incubated for 90 min at room temperature and then 30 min on ice. After incubation the mixture was centrifuged at 12,000g for 10min. To the supernatant, phenol-chloroform (25 µl + 25 µl) to double the volume was added and mixed well. The mixture was centrifuged at 13,000 g for 10 min. After centrifugation supernatant was taken and equal volume of chloroform and isopropyl alcohol was added. Then mixture was centrifuged at 13,000 g for 10 min at 4°C. To the supernatant, 100µl of 2M NaAc and double the volume of 100% ethanol were added. The mixture was incubated for 20 to 30 min at RT and further centrifuged at 13,000 g for 10 min. The pellet washed with 70% ethanol and mixture was centrifuged at 12,000 g for 10 min. After extracting the pellet with 70% ethanol, pellet was air dried and then dissolved in 200 µl TE buffer and store at -20°C. Purity and presence of DNA can be detected through agarose gel electrophoresis. DNA samples were checked on 1% agarose gel.

ISSR primer amplification

A set of 30 ISSR primers were used for this study. DNA was amplified in a total volume 25 µl reaction mixture contained; 2.5 µl 10X buffer (10 mM Tris HCL pH 9, 50mM KCL, 0.1% Triton X 100), 2.5 µl 25mM $MgCl_2$, 1 µl 10mM dNTPs, 10 µM ISSR primer, 1 µl of Taq DNA polymerase, 2 µl DNA template. The PCR reaction was performed using the verity thermal cycler with 40 cycles of denaturation at 92°C temperature for 2 min, annealing 52°C for 1 min., and extension was done at 72°C for 1 min. Amplified DNA fragments were analyzed through 2 % agarose gel.

Results and Discussion

The isolation of high quality DNA is an important step in the field of plant molecular biology. For the molecular researches good quality of DNA is needed. The successful application of PCR based downstream applications requires efficient recovery of good quality and quantity of DNA. Plant produces secondary metabolites as well as other substances, that create problems while extraction of DNA. In present study, six different varieties of papaya were selected for ISSR marker analysis.

The method of Doyle and Doyle (1987) [8] have yielded good results for numbers of the plant materials (Chudasama *et al*., 2018, Monpara *et al*., 2017) [4, 16] however, DNA isolation of papaya showed very poor exaction and no PCR amplification was recorded. Papaya plant is known for its richness in flavanoids and phenols (Yogiraj *et al*., 2015) [23] and therefore we have used a modified protocol developed in our lab (Mandaliya *et al*., 2010) [15], for total genomic DNA isolation. In this method, the material was crushed with equal amount of PVPP. PVPP was used to purge polyphenols and it promoted precipitation of the phenolic compounds (Arif *et al*., 2010) [1]. PVPP forms complex hydrogen bonds with polyphenolic compounds which can be separated from DNA by centrifugation. The polyphenolic compounds were reduced by using PVPP in this DNA extraction procedure. The present method of DNA isolation was observed that suitable for various plant materials and the numbers of steps were taken into consideration to provide high quality of DNA. In present studies, the physical characterization of the final DNA pellet was transparent with no visible coloration, clear and sharp DNA bands were observed on agarose gel. Triton-X-100 extraction buffer was used in this method which shown proteins and polysaccharides become trapped in large complexes that are coated with dodecyl sulphate. Then addition of KAc participates in precipitation of this complex by replacing sodium ions with potassium ions. (Dellaporta *et al*., 1983; Zidani *et al*., 2005) [7, 24].

Variety -1

Variety -2

Variety -3

Variety -4

Variety -5

Variety -6

Fig 1: Six variety of *Carica papaya* L.

The removal of RNA by RNAse treatment is the generally accepted method; however degradation is often incomplete. The contaminated RNA that precipitates along with DNA causes many problems including inhibition of PCR amplification (Pikbart and Villeponteau, 1993) [18]. Nucleic acid solution often contains undesirable contaminant like cell debris, major protein and phenolic compounds. The phenol-chloroform extractions are needed to remove these contaminants (Doyal and Doyal, 1987) [8]. The nucleic acid precipitation is frequently performed using alcohol. Resultant DNA was diluted with monovalent salt sodium acetate followed by addition of alcohol yielded good precipitation. The nucleic acid was precipitated spontaneously and can be pelleted by centrifugation. The salt was removed by washing with 70% alcohol. After that the pellet was dissolved in TE buffer. The procedure has yielded high quantity DNA (Fig 2), but the time consumption was one of the most troublesome during nucleic acid recovery.

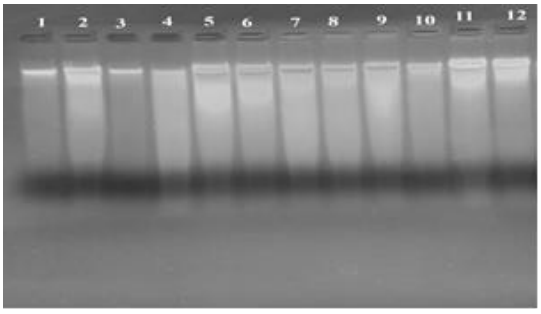

Fig 2: Agarose gel electrophoresis of DNA extracted from 6 varieties of papaya. [line 1-2 variety-1, 3-4 variety-2, 5-6 variety-3, 7-8 variety-4, 9-10 variety-5, 11-12 variety-6.]

ISSR analysis

Molecular marker techniques are preferentially used for discriminate plant species of variety levels (Chudasama *et al*., 2018, Brown *et al*., 2009, Uzun *et al*., 2010) [4, 3, 22]. DNA markers are closely linked to important agronomic traits; greatly contribute to practical crop improvements programs (Chundet *et al*., 2007, Dehesdtani *et al*., 2007) [5, 6]. There are a number of reports in the literature for the genetic diversity analysis of papaya using a range of different molecular markers (Kim *et al*., 2002) [13]. After isolation of DNA the molecular marker technique ISSR was performed. ISSR have high reproducibility possibility due to use of longer primers as compared to RAPD primers which permit the subsequent use of high annealing temperature (45-60°C) leading to higher stringency. The ISSR method provides an alternating choice to other systems for obtaining highly reproducible materials without any necessity for prior sequence information for various genetic analyses (Monpara *et al*., 2017) [16]. ISSR analysis is faster and it amplifies and detects a greater number of bands per primer (Godwin *et al*., 1997) [10].

The 30 ISSR primers including di, tri, tetra and penta nucleotides repeat were tested in this study. The products of ISSR were analyzed by 2% agarose gel electrophoresis. In table 1, list of primers used for ISSR is given. The annealing temperature of these primers is varying from 30°C to 60°C. In ISSR amplification of DNA polymorphic and monomorphic band pattern observed in electrophoresis (Fig 3-15). Clearly detectable amplified ISSR bands were obtained in size from 100 to 3000 bp for the six papaya cultivars. Among the 30 primers, 25 primers generated sharp, clear, and reproducible total 390 bands. The polymorphic 273 and monomorphic 117 bands were observed thus the percentage of average polymorphic bands observed 83.235% and of monomorphic 61.036%. The average 4.84 per primer were observed. The maximum number of amplified product was 10 (I-18) and minimum 1 (I-3, I-8, I-16).

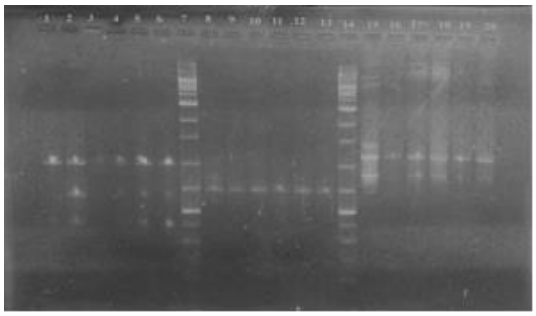

Fig 3: Amplification patterns of selected ISSR primers for 6 varieties of papaya. ISSR primers in these lanes are: lane 1-6 primer UBC826, lane 8-13 primer UBC814, lane 15-20 primer UBC822. Each six successive lane from 1-20 contains six varieties of papaya, while lane 7 and 14 are of BioLit ProxiO 1kb DNA Ladder.

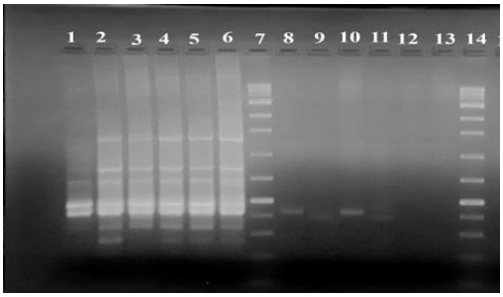

Fig 4: Amplification patterns of selected ISSR primers for 6 varieties of papaya. ISSR primers in these lanes are: lane 1-6 primer UBC862, lane 8-13 primer UBC867. Each six successive lane from 1-14 contains six varieties of papaya, while lane 7 and 14 are of Bio Lit ProxiO1kb DNA Ladder.

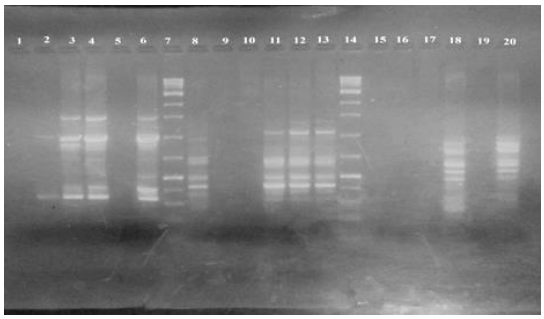

Fig 5: Amplification patterns of selected ISSR primers for 6 varieties of papaya. ISSR primers in these lanes are: lane 1-6 primer UBC864, lane 8-13 primer UBC886, lane 15-20 primer UBC888. Each six successive lane from 1-20 contains six varieties of papaya, while lane 7 and 14 are of BioLit ProxiO 1kb DNA Ladder.

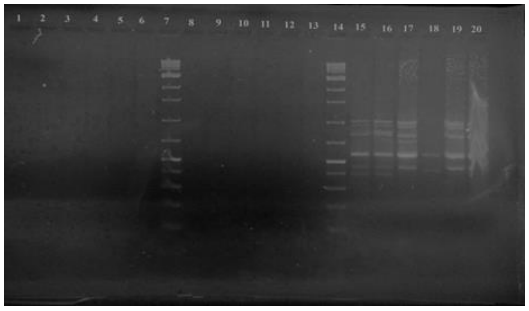

Fig 6: Amplification patterns of selected ISSR primers for 6 varieties of papaya. ISSR primers in these lanes are: lane 1-6 primer UBC868, lane 8-13 primer UBC872, lane 15-20 primer UBC881. Each six successive lane from 1-20 contains six varieties of papaya, while lane 7 and 14 are of BioLit ProxiO 1kb DNA Ladder.

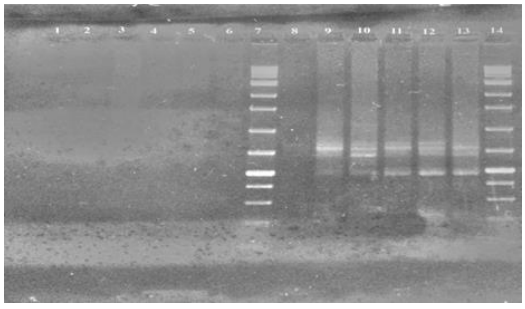

Fig 7: Amplification patterns of selected ISSR primers for 6 varieties of papaya. ISSR primers in these lanes are: lane 1-6 primer UBC868, lane 8-13 primer ISSR5. Each six successive lane from 1-14 contains six varieties of papaya, while lane 7 and 14 are of BioLit ProxiO 1kb DNA Ladder.

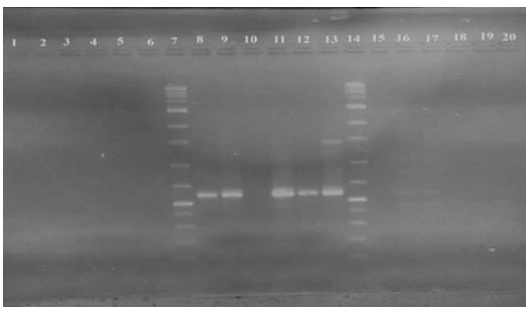

Fig 8: Amplification patterns of selected ISSR primers for 6 varieties of papaya. ISSR primers in these lanes are: lane 1-6 primer UBC872, lane 8-13 primer UBC8873, lane 15-20 primer UBC881. Each six successive lane from 1-20 contains six varieties of papaya, while lane 7 and 14 are of BioLit ProxiO 1kb DNA Ladder.

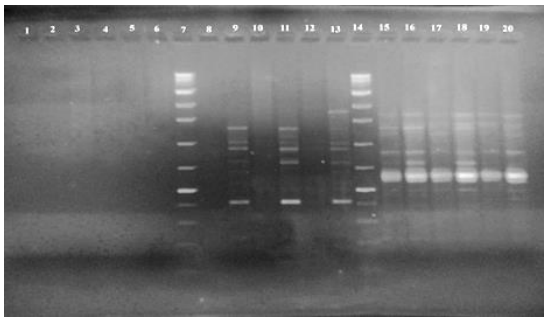

Fig 9: Amplification patterns of selected ISSR primers for 6 varieties of papaya. ISSR primers in these lanes are: lane 1-6 primer UBC872, lane 8-13 primer UBC8876, lane 15-20 primer UBC884. Each six successive lane from 1-20 contains six varieties of papaya, while lane 7 and 14 are of BioLit ProxiO 1kb DNA Ladder.

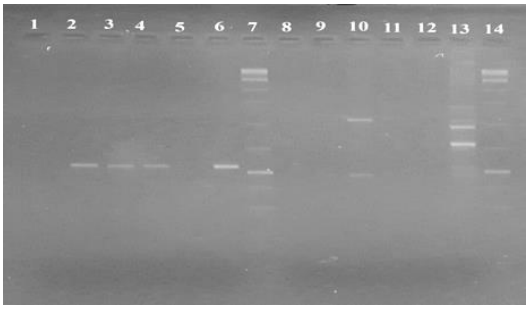

Fig 10: Amplification patterns of selected ISSR primers for 6 varieties of papaya. ISSR primers in these lanes are: lane 1-6 primer UBC880, lane 8-13 primer ISSR1, lane. Each six successive lane from 1-14 contains six varieties of papaya, while lane 7 and 14 are of BioLit ProxiO 1kb DNA Ladder.

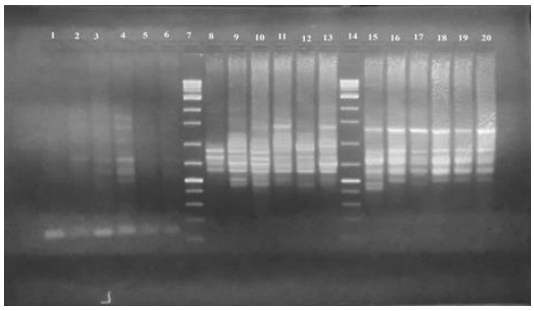

Fig 11: Amplification patterns of selected ISSR primers for 6 varieties of papaya. ISSR primers in these lanes are: lane 1-6 primer UBC885, lane 8-13 primer UBC889, lane 15-20 primer UBC891. Each six successive lane from 1-20 contains six varieties of papaya, while lane 7 and 14 are of BioLit ProxiO 1kb DNA Ladder.

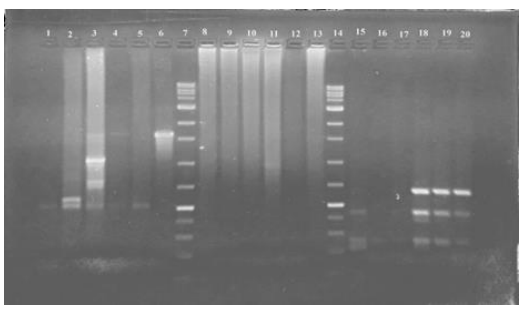

Fig 12: Amplification patterns of selected ISSR primers for 6 varieties of papaya. ISSR primers in these lanes are: lane 1-6 primer ISSR2, lane 8-13 primer ISSR3, lane 15-20 primer ISSR4. Each six successive lane from 1-20 contains six varieties of papaya, while lane 7 and 14 are of BioLit ProxiO 1kb DNA Ladder.

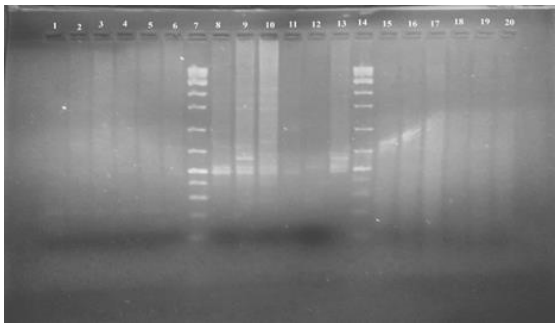

Fig 13: Amplification patterns of selected ISSR primers for 6 varieties of papaya. ISSR primers in these lanes are: lane 1-6 primer ISSR5, lane 8-13 primer ISSR6, lane 15-20 primer ISSR7. Each six successive lane from 1-20 contains six varieties of papaya, while lane 7 and 14 are of BioLit ProxiO 1kb DNA Ladder.

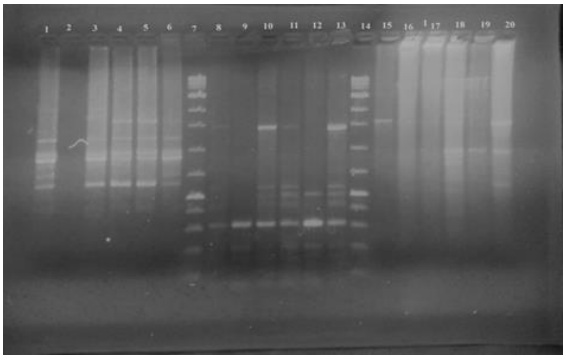

Fig 14: Amplification patterns of selected ISSR primers for 6 varieties of papaya. ISSR primers in these lanes are: lane 1-6 primer UBC817, lane 8-13 primer UBC834, lane 15-20 primer ISSR3. Each six successive lane from 1-20 contains six varieties of papaya, while lane 7 and 14 are of BioLit ProxiO 1kb DNA Ladder.

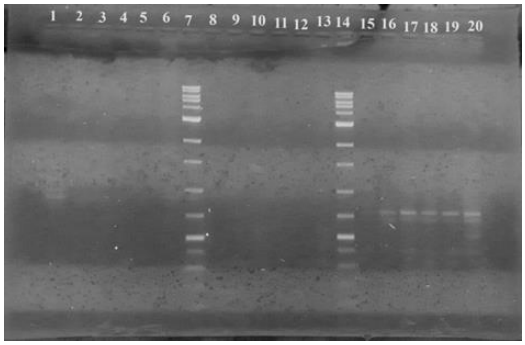

Fig 15: Amplification patterns of selected ISSR primers for 6 varieties of papaya. ISSR primers in these lanes are: lane 1-6 primer UBC817, lane 8-13 primer UBC834, lane 15-20 primer UBC840. Each six successive lane from 1-20 contains six varieties of papaya, while lane 7 and 14 are of BioLit ProxiO 1kb DNA Ladder.

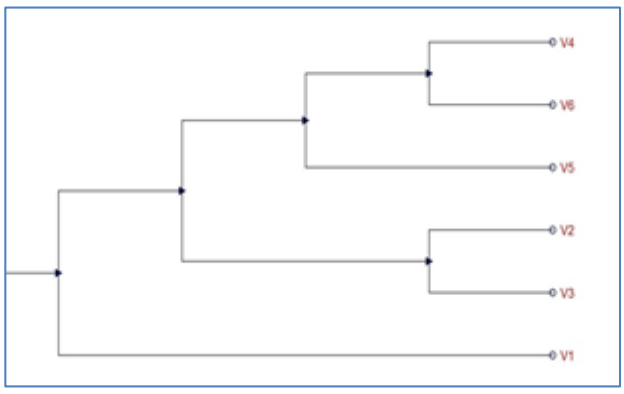

Fig 16: A dendrogram constructed from ISSR data in six variety of *Carica Papaya*, based on Jaccard's similarity coefficient and UPGMA clustering.

The total number of bands (TNB), number of monomorphic bands (NMB), number of polymorphic bands (NPB), the percentage of the monomorphic band (M %), the percentage of the polymorphic bands (P %), obtain per each primer are shown in Table 2. Among 30 primers, 17 primers were observed 100% polymorphism in UBC817, UBC826, UBC840, UBC864, UBC867, UBC873, UBC876, UBC880, UBC881, UBC886, UBC888, ISSR1, ISSR2, ISSR3, ISSR4, ISSR5, ISSR6, followed by 50% in UBC 822, 60.71% in UBC834, 51.35% in UBC862, 31.81% in UBC884, 40% in UBC885, 41.19% in UBC889, and 22.58% in UBC891. So here these primers gave high polymorphism and proves best to distinguish six varieties of papaya from each other; while, five primers I-1, I-2, I-12, I-13, I-30 were not given any polymorphism. The primer I-3 was given 100% monomorphism.

Table 1: ISSR primer set

Sr. No.	Primer name	Sequence	Ta (°C)
I-1	UBC803	(AT)8C	36
I-2	UBC808	(AG)8C	48
I-3	UBC814	(CT)8A	48
I-4	UBC817	(CA)8A	44.2
I-5	UBC822	(TC)8A	48
I-6	UBC826	(AC)8C	48
I-7	UBC834	(AG)8TT	44.2
I-8	UBC840	(GA)8TT	44.2
I-9	UBC862	(AGC)6	48
I-10	UBC864	(ATG)6	48
I-11	UBC867	(GGC)6	48
I-12	UBC868	(GAA)6	48
I-13	UBC872	(GATA)4	48
I-14	UBC873	(GACA)6	48
I-15	UBC876	(GATA)2(GACA)2	48
I-16	UBC880	(GGAGA)3	48
I-17	UBC881	(GGGGT)3G	48
I-18	UBC884	HBH(AG)7	48
I-19	UBC885	BHB(GA)7	48
I-20	UBC886	VDV(CT)7	48
I-21	UBC888	BDB(CA)7	48
I-22	UBC889	DBD(AC)7	48
I-23	UBC891	HVH(TG)7	48
I-24	ISSR1	(AGC)5GC	55
I-25	ISSR2	(CA)7AC	44.2
I-26	ISSR3	(GT)7AC	44.2
I-27	ISSR4	GCA(GA)7	47.2
I-28	ISSR5	(GA)9C	51.7
I-29	ISSR6	(GA)9A	49.5
I-30	ISSR7	(CG)8C	60

Table 2: ISSR profiles, % monomorphism and % polymorphism of six varieties of papaya.

Primer no.	Primer name	Total bands	V-1	V-2	V-3	V-4	V-5	V-6	Polymorphic bands	Monomorphic bands	% Polymorphism	% monomorphism
I-1	UBC803	0	-	-	-	-	-	-	-	-	-	-
I-2	UBC808	0	-	-	-	-	-	-	-	-	-	-
I-3	UBC814	6	1	1	1	1	1	1	-	6	-	100%
I-4	UBC817	24	5	0	4	5	5	5	24	-	100%	-
I-5	UBC822	12	4	1	2	2	1	2	6	6	50%	50%
I-6	UBC826	6	1	2	0	1	1	1	6	-	100%	
I-7	UBC834	23	2	2	4	6	3	6	17	6	60.71%	26%
I-8	UBC840	5	0	1	1	1	1	1	5	-	100%	
I-9	UBC862	37	3	6	5	6	6	6	19	18	51.35%	48.64%
I-10	UBC864	13	0	2	4	4	0	4	13	-	100%	-
I-11	UBC867	3	1	1	1	0	0	0	3	-	100%	-
I-12	UBC868	0	-	-	-	-		-	-	-	-	-
I-13	UBC872	0	-	-	-	-	-	-	-	-	-	-
I-14	UBC873	6	1	1	0	1	1	2	6	-	100%	-
I-15	UBC876	15	0	5	0	5	0	5	15	-	100%	-
I-16	UBC880	4	0	1	1	1	0	1	4	-	100%	-
I-17	UBC881	26	6	6	6	2	6	0	26	-	100%	-
I-18	UBC884	44	5	9	8	8	6	8	14	30	31.81%	68.18%
I-19	UBC885	10	1	2	2	3	1	1	4	6	40%	60%
I-20	UBC886	19	4	0	0	5	5	5	19	-	100%	-
I-21	UBC888	12	0	0	0	6	0	6	12	-	100%	-
I-22	UBC889	31	4	7	6	5	4	5	13	18	41.19%	58.06%
I-23	UBC891	31	7	5	4	5	5	5	7	24	22.58%	77.41%
I-24	ISSR1	7	0	0	2	0	0	5	7	-	100%	-
I-25	ISSR2	8	0	4	2	0	1	1	8	-	100%	-
I-26	ISSR3	7	1	0	0	2	1	3	7	-	100%	-
I-27	ISSR4	10	1	0	0	3	3	3	10	-	100%	-
I-28	ISSR5	21	0	4	4	4	5	4	21	-	100%	-
I-29	ISSR6	10	2	3	2	0	0	3	10	-	100%	-
I-30	ISSR7	0	-	-	-	-	-	-	-	-	-	-

Jaccard's similarity measure was used to carry out a UPGMA cluster analysis and to generate a dendrogram representing the relationship among six varieties of papaya. The jaccard's distance/similarity matrixes were calculated based on ISSR data. In ISSR analysis, highest similarity and distance observed between V-4 and V-6 (0.371) and V-1 and V-6 (0.750) respectively. The dendrogram was constructed based on UPGMA relationship of ISSR data. ISSR UPGMA analysis has shown 3 major clades. V-1 and V-5 were found in individual sub clades. V-2, V-3, V-4, V-6 were fallen together in another major clades, nevertheless, polymorphic band patterns has adequately distinguished the varieties.

In sum, the PCR based ISSR method has been used for detecting genetic relationship among six varieties of papaya. ISSR-PCR based method is easy, fast, and cost effective molecular marker technique which can be widely used to analyze plant diversity at genetic level. ISSR has been proposed as a more economical and comparatively simple DNA marker system. Recently several molecular marker methods such as random amplified polymorphic DNA (RAPD), simple sequence repeats (SSR), amplified fragment length polymorphism etc have been widely used for the variety of purposes relevant to crop improvement (Eustic *et al*., 2008, Sudha *et al*., 2013, Kore *et al*., 2018, Shivkumar *et al*., 2014). Compared with other molecular markers, ISSR has high polymorphism. Nagaoka and Ogihara (1997) reported that ISSR markers can provide more genetic information and the accuracy of the ISSR technique is equal to that of random fragment length polymorphism when investigating genetic relationship in a genus due to its high polymorphism.

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