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Efficiency of RAPD and ISSR Markers in Discriminating the Important Members of Cucurbitaceae Family

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Abstract: Cucurbits are vegetable crops of the family Cucurbitaceae which are cultivated in India for food throughout the country. Apart from its food yielding properties, some members of the family are well known for its medicinal values. There is a great diversity within this family. So, the objective of this study is to determine the genetic diversity among some economically important members of this family and also compare the efficiency of RAPD and ISSR marker to measure genetic diversity. The results showed that two markers have the similar potential for genetic diversity analysis and also indicate the great variability among five studied cucurbit species which can be useful for selection of a core collection to enhance the efficiency of germplasm management for use in cucurbit breeding and conservation.

Keywords: Cucurbits, DNA isolation, Jaccard's coefficient, RAPD, ISSR

Introduction

Cucurbits are vegetable crops belonging to family Cucurbitaceae, which primarily comprised species consumed as food worldwide. There is tremendous genetic diversity within the family, and the range of adaptation for Cucurbit species includes tropical and subtropical regions, arid deserts, and temperate regions [1-2]. A number of Cucurbit vegetables are exported from India. The genetic diversity in cucurbits extends to both vegetative and reproductive characteristics and considerable range in the monoploid (x) chromosome number [3] including 7 (*Cucumis sativus*), 11 (*Citrullus* spp., *Momordica* spp. etc.), 12 (*Benincasa hispida, Coccinia cordifolia, Cucumis* spp. etc), 13 (*Luffa* spp.), and 20 (*Cucurbita* spp.). Cucurbits are consumed in various forms i.e., salad (Cucumber, gherkins, long melon), sweet (ash gourd, pointed gourd), pickles (gherkins), deserts (melons) and culinary purpose [4]. Some of them are well known for their unique medicinal properties. ln India, a number of major and minor cucurbits are cultivated in several commercial cropping systems and also as popular kitchen garden crops. Cucurbits share about 5.6 % of the total vegetable production of India and according to FAO estimate; cucurbits were cultivated on about 4,290,000 ha with the productivity of 10.52 t/ha. According to an estimate, India will need to produce 215,000 t of vegetables by 2015 to provide food and nutritional security at individual level and, being a large group of vegetable; cucurbits provide better scope to enhance overall.

Cucurbitaceae family contains about 90 genera and over 700 species of economic importance. The family is distinct morphologically and biochemically from other families and is therefore considered monophyletic. As the plants of this family produce unisexual flowers, crosspollination is a regular feature. Few studies have been reported on the phylogeny of Cucurbitaceae [5]. The evaluation of genetic diversity and phylogenetic relationships among the members would promote the efficient use of genetic variations in the breeding programme. DNA markers provide

an opportunity to characterize genotypes and to measure genetic relationships more precisely than other markers. Information on genetic diversity and relationships within and among crop species and their wild relatives is essential for the efficient utilization or plant genetic resource collections. With the advent of polymerase chain reaction (PCR) technique, two quick and simple methods called random amplified polymorphic DNA, RAPD [6] and inter-simple sequence repeat, ISSR [7] are now widely used for the study of phylogeny and genetic diversity. RAPD markers have been used for the identification of cultivars and for assessing genetic diversity among cultivars of several crops like bean. ISSRs are semiarbitrary markers amplified by PCR in the presence of one primer complementary to a target microsatellite. Like RAPDs, ISSRs markers are also quick and easy to handle and have been successfully utilized for the phylogenetic analysis of *Oryza*, *Vigna* [8] and cultivar identification in strawberry.

The objectives of the present study are to determine the genetic diversity and phylogenetic relationships among some economically important members of Cucurbitaceae and to compare the resolving power of RAPD and ISSR for their applicability in the phylogenetic studies.

Materials and Methods

Materials

The plant of Cucurbitaceae family were grown and maintained in the experimental field on Department of Botany, University of Kalyani. The following species were taken (Table 1):

Table 1. List of Plant Materials undertaken in the present study and their Designation

Sl. No.	Species	Designation
1	*Momordica charantia*	S1
2	*Mukia scabrella*	S2
3	*Coccinia grandis*	S3
4	*Cucumis sativus*	S4
5	*Trichosanthes dioica*	S5

Preparation of materials

The young fresh leaves were taken from those field grown plants, washed thoroughly with 5% extran® and again with sterile distilled water for three minutes. The surface water on the leaflets was soaked by dry tissue paper. The leaflets were kept within the dry bottles in -20ºC freezer until use.

Methods

Genomic DNA is the blueprint of life. Isolation of high molecular weight genomic DNA free of protein and RNA contamination is the pre-requisite for molecular cloning experiments. The isolated DNA should also be amenable for PCR and restriction digestion. The plant tissue is notoriously difficult material for DNA isolation due to the presence of various secondary plant products. Plant g-DNA is usually isolated by lysing the nuclei of buffer containing detergents like SDS/CTAB in presence of EDTA and also by using benzyl chloride in suitable buffer. It is followed by phenol/ chloroform extraction to remove proteins and other contaminants. DNase free RNase is used to degrade RNA and subsequently proteinase-k is used to digest DNA bound protein and RNA to obtain plant g-DNA in purified form.

DNA isolation

DNA was isolated using the CTAB method [9] with minor modifications. 500 mg sample of fresh leaf was taken and grinded in a pre-chilled morter and pastle by adding 2.5 ml of extraction buffer (pre-warmed) and 1 ml of β-marcaptoethanol. Pour the sample in 2 ml of eppendrof tube. The tubes were then placed in a 65°C water bath for 1 hour with intermediate shaking after every 15 minutes. The samples were then cooled to room temperature and equal volume of chloroform: isopropanol (24:1) was added and vortexed briefly and gently to avoid shearing of the DNA. Then the tubes were inverted several times and spinned at 15000 rpm for 10 minutes at room temperature.

The aqueous top layer was removed and transferred to aqueous phase 2/3 volume of pre-chilled isopropanol was added and mixed by quick gentle inversion for 2 minutes. The tubes were then kept in deep freeze for overnight. The tubes were spinned at (5000-6000) rpm for 10 minutes. Isopropanol was decanted from the tubes and 70% chilled ethanol was added and mixed gently. After that the tubes were centrifuged at 10000 rpm for 5 minutes. The last two steps were repeated. Then the tubes were allowed to dry in laminar air flow. DNA was dissolved in 50 µl of dd water.

Phenol Equilibration

Phenol was removed from the freezer, and was allowed to warm to the room temperature. Then it was melted at 68ºC. Hydroxyquinoline was added to a final concentration of 0.1% this compound is an antioxidant, a partial inhibitor of Rnase, and a weak chelator of metal ion. In addition, its yellow color provides a convenient way to identify the organic phase. An equal volume of 0.5 M Tris-HCl (pH 8.0) was added. The mixture was stirred on a magnetic stirrer for 15 minutes. Stirrer was turned off. After the separation of two phases, the upper aqueous phase was aspirated as much as possible with the help of micropipette. Again, equal volume of 0.1 M Tris-HCl (pH 8.0) buffer was added to the phenol. The mixture was stirred on a magnetic stirrer for 15 minutes. The stirrer was turned off and the upper aqueous phase was removed with a use of micropipette. Extraction was repeated until the pH of the phenolic phase is>7.8 (measured by pH paper). In this way phenol was equilibrated and the final aqueous phase is removed. This equilibrated phenol was used for purification of DNA.

DNA purification

Major contaminants in crude DNA preparation are RNA, protein and polysaccharides. Inclusion of CTAB in DNA extraction buffers help in elimination of polysaccharides from DNA preparation to a large extent. The RNA is removed by treating the sample with RNase. Protein including RNase can be removed by treatment with protejnase K. Extraction with phenol: chloroform following RNase treatment is also employed for eliminating RNA and most of proteins. 10-15 µl of RNase was added in 1 ml of dissolved DNA sample. Then the tubes were kept at 37ºC for 1 hour in an incubator. After that equal volume of phenol: chloroform (1:1) was added to the tubes and mixed gently for 2 mins. The tubes are spinned at 13000 rpm for 10 mins at 4ºC. The upper aqueous phase was taken out in an eppendrof tube as much as possible. For elimination of phenol, chloroform: isoamyl alcohol (24:1) was added and extraction was carried out by centrifuging at 5000 rpm for 5 minutes at 4ºC. The supernatant was discarded and were washed with 70% alcohol, centrifuged at 10000 rpm for 5 minutes at 4ºC. This step was repeated for 3 times. After centrifuging, the pellet was air dried and dissolved in 50 µl of dd H_2O.

RAPD analysis

PCR DNA amplification was performed using 5 random decamer primers (Bangalore Genei, India). Amplifications were performed according to Williams et al. [6] in a 25 µl reaction volume containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgC1_2$, 0.2 mM each dNTP, 20 pmol RAPD primer, 50 ng genomic DNA, and 0.5 U Taq DNA polymerase (Bangalore Genei, Bangalore, India). Amplification conditions (thermocycler Model-2400, Perkin-Elmer, Boston, MA, USA) were initial denaturation at 94.8ºC for 4 min and 45 cycles at 94.8ºC for 1 min and then 38.8ºC for 1 min, a ramp to 72.8ºC for 2 min, followed by 7 min at 72.8ºC and indefinite soak at 4.8ºC. Amplicons were separated on 1.5% agarose gel in 1X TAE buffer (100 mM Tris-HCl, pH 8.3, 83 mM Acetic acid, 1 mM EDTA) at 50 V. Gels were then stained with 0.5 µl/ml ethidium bromide solution, visualized by illumination under UV light, and then documented using a gel documentation and image analysis system (Uvitek, UK). Low range of molecular marker (100bp to 3Kbp) (Bangalore Genei) was used to identify duplicate reaction.

ISSR analysis

Five ISSR primers were obtained from the Bangalore Genei, India, and PCR was carried out in 25µl volume containing 50 ng template DNA, 0.5 U Taq DNA polymerase, 10 mM dNTP, 10 mM primer in 1X reaction buffer that contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 2.5 mM $MgCl_2$ (Bangalore Genei, Bangalore, India). Amplification protocols were those stated for RAPD analysis. Amplified products were loaded on 1.5% agarose gel

and separated in 1X TBE buffer at 50 V, and then gels were stained with ethidium bromide, and visualized under UV by image analyses.

Data analysis

Only consistent, bright, reproducible (i.e., band absence was randomly verified) RAPD and ISSR bands were scored as present (1) or absent (0), where each character state was treated independently. Genetic similarity and cluster analyses were performed by subjecting character state data to empirical examination using the NTSYS-PC software version 2.0 (Exeter Software, NY, USA; Rohlf, 1998 [10]), where the SIMQUAL program was used to calculate Jaccard's coefficients of similarity as follows:

$$\text{Jaccard's coefficient} = \frac{\text{NAB}}{\text{NAB} + \text{NA} + \text{NB}}$$

Results and Discussion

A total of 33 amplicons were produced by examining 5 RAPD primers (Table 2). Amplicon numbers per primer ranged from 4 (OPS-2) to 11 (OPS-3) and varied in size between 150 bp and 2600 bp. Of the 33 amplified bands, 27 were polymorphic, with an average of 5.4 polymorphic fragments per primer.

Table 2. List of RAPD Primers

Sl. No.	Sequence (5'-3')	Primer Code	Amplified Loci	Amplification range (bp)
1	GAATTCCTTA	OPS-1	7	275bp-1800bp
2	AATTACTAAG	OPS-2	4	300bp-2600bp
3	GGCCTTTTAA	OPS-3	11	200bp-2000bp
4	ACCCTTAGAG	OPS-4	5	300bp-2600bp
5	TTTATCTTAC	OPS-5	6	150bp-2200bp

Table 3. List of ISSR Primers

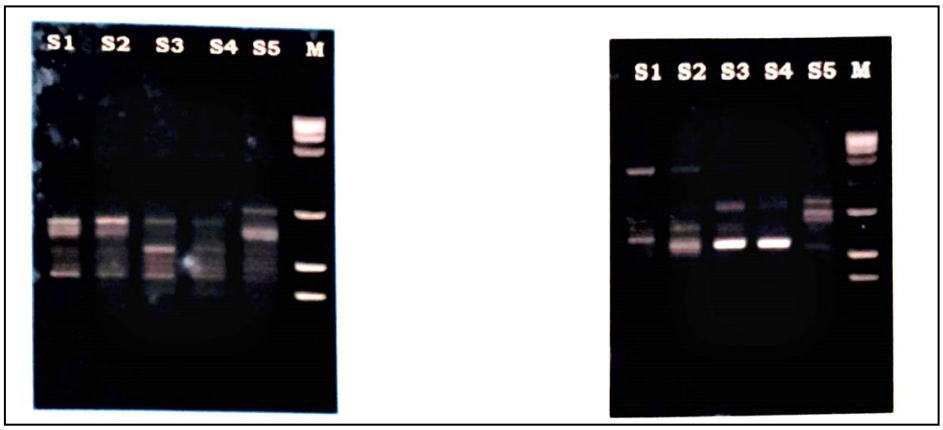
Sl. No.	Sequence (5'-3')	Primer Code	Amplified Loci	Amplification range (bp)
1	$(GA)_8GT$	BG-1	9	200bp-1800bp
2	$(GACA)_4$	BG-2	10	375bp-2600bp
3	$(CT)_8AC$	BG-3	6	200bp-2000bp
4	$(GAG)_6AG$	BG-4	5	265bp-2400bp
5	$(GT)_6GC$	BG-5	4	300bp-2700bp

Table 4. Similarity matrix based on RAPD data

Designation	S1	S2	S3	S4	S5
S1	1.000				
S2	0.710	1.000			
S3	0.667	0.667	1.000		
S4	0.594	0.484	0.500	1.000	
S5	0.767	0.548	0.563	0.438	1.000

Table 5. Similarity matrix based on ISSR data

Designation	S1	S2	S3	S4	S5
S1	1.000				
S2	0.750	1.000			
S3	0.424	0.536	1.000		
S4	0.484	0.400	0.222	1.000	
S5	0.697	0.677	0.483	0.313	1.000



(a) (b)

Figure 1. Amplification pattern generated by the (a) RAPD primer OPS-3 and (b) ISSR primer BG-2

The polymorphism percentage ranged from 25% (OPS-1) to a maximum of 72.8% (OPS-3), with an average of 81.81% polymorphisms when all primers were taken collectively. Jaccard's similarity coefficient ranged from 0.438 to 0.71(Table 4). Figure 1a shows the banding pattern obtained with the primer OPS-3. Five ISSR primers produced, on average, 37 bands in the accessions examined, of which 30 (81.08%) were polymorphic (Table 3). The number of amplicons per accession varied from 4 (BG-5) to 10 (BG-2), where sizes ranged between 200 bp and 2700 bp. The average number of bands observed per primer and those that were polymorphic was 6. Jaccard's similarity coefficient ranged from 0.313 to 0.75 (Table 5). Figure 1b shows the banding pattern obtained with the primer BG-2.

RAPD and ISSR studies have been widely used for population genetic studies in both wild [11] and cultivated plants [12]. By contrast, previous reports on ISSR analysis mainly focused on cultivated species [13-14]. Here, we have compared the discriminating power of the two marker systems: RAPD and ISSR. Our results suggest that two markers have the similar potential for genetic diversity analysis in five Cucurbita species. However, moderate amount of similarity exists among the two marker systems. However, relationships among the different genera differ to some extent with the marker type. For RAPD and ISSR markers, a high reproducibility in dendrogram topologies was obtained, with a few differences. Genetic variation among cucurbits based on RAPD and ISSR analysis could be useful to select parents.

Conclusion

Our results indicate the presence of great genetic variability among cucurbit species and are useful in the assessment of cucurbit diversity, and the selection of a core collection to enhance the efficiency of germplasm management for use in cucurbit breeding and conservation.

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