ISSN 2278- 4136
ZDB-Number: 2668735-5
IC Journal No: 8192
Volume 2 Issue 1
Online Available at www.phytojournal.com

Journal of Pharmacognosy and Phytochemistry

Rapd Analysis of the Seven Cultivated Varieties of *Capsicum annuum* L.

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Randomly Amplified Polymorphic DNA (RAPD) is a novel procedure for the identification of polymorphism in plants based on PCR. It does not require prior knowledge of a DNA sequence. RAPD markers are based on the amplification of unknown DNA sequences using single, short, random oligonucleotide primers. Many of the technical limitations of RFLPs have been overcome by RAPD.

In the present study seven varieties of *C. annuum* L. were analysed for RAPD polymorphism using 5 random primers. The study shows that the varieties show remarkable genetic variation. RAPD primers showed different DNA fingerprints for different varieties of *Capsicum* L. studied. Variation was observed at varietal level in *C. annuum* L. with respect to the morphological traits, phytoconstituents estimated (capsaicin, sugar and vitamin C) and karyotype analysis also. Thus all the seven can be distinguished at varietal level. Therefore they can be used as a suitable source material for future breeding, genetic and other experiments. The use of other molecular markers like SSRs, AFLP, etc. would provide more precise estimates of genetic variability in the varieties.

Keyword: RAPD, *Capsicum annuum* L., Polymorphism.

1. Introduction

RAPD is a fast and sensitive method and is able to provide reproducible and characteristic fingerprints of complex genomes without prior sequence information. The use of short primers of arbitrary sequence during PCR results in amplification of different segments of genomic DNA, which after gel electrophoresis gives characteristic band patterns. Most informative DNA bands on RAPD are usually of the 300-3000bp range. RAPD provides a cost-effective method for the precise and routine evaluation of variability. It may also be used to identify areas of maximum diversity. Thus molecular technique provides a powerful tool for the study of plant population genetics.

RAPD has been used for genetic fingerprinting[16], creating linkage maps[12]; locating disease resistance genes[7,8]; identifying chromosome-specific markers[11] and characterization of somatic hybrids[3].

In the past few years, the RAPD technique has been widely accepted in labs throughout the world. Many agronomically important species have been analysed by this technique. This technique helps to identify a large number of markers which can be used for estimating genetic variation[10].

2. Materials and methods:

Seven cultivated varieties of *C. annuum* L. were selected for the present work. They were Achari (ACH), G4, Pusa Jwala (JW), Phule Jyoti (JY),

Phule Kirti (KR), Pusa Sadabahar (SADA), and Sanyogita Special (SS). Green and fresh young leaves from ten plants used for the morphological studies of each variety were harvested at the same plant growth stage and used for the DNA extraction.

2.1 DNA extraction:

Weigh approximately 50 – 80 mg of fresh leaves. Cut into fine pieces and grind. Grind the material in total of 2 ml extraction buffer and 100 µl of β-mercaptoethanol by adding buffer in parts while grinding. Take in a microfuge tube and deep freeze for 5min. Centrifuge at 10000 rpm for 5mins. Discard the supernatant. Add 700 µl of suspension buffer and 100 µl of SDS to the pellet. Incubate at 65 °C for 30 minutes. Add 160 µl ammonium acetate and incubate for 15 minutes at RT. Add 1ml chloroform - isoamyl alcohol mixture. Mix by several inversions. Centrifuge at 10000 rpm for 8 minutes. Collect the top aqueous layer in a new microfuge tube. Add equal volume of absolute ethanol. Deepfreeze for 1 hour. Centrifuge at 10000 rpm for 15 minutes. Discard ethanol. Dissolve the pellet in elution buffer. Store at 4 °C. Perform Agarose gel electrophoresis with 0.8% Agarose to check for presence of DNA.

2.2 Oligonucleotide Primers:

The RAPD Primers used and their sequences are shown in Table 1. Five primers were used in the study namely OPM-01, OPN-05, OPP-01, OPQ-01 and OPT-04.

2.3 RAPD analysis:

The genomic DNA isolated from different chili samples (leaves) was subjected to polymerase chain reaction (PCR) amplification with 5 random 10-mer primers (originally designed by Operon Technologies, Calif. and synthesized by MWG Biotech, Bangalore) Amplification of genomic DNA was carried out in 50 µL reaction mixture containing 1µl (10–20 ng) genomic DNA as template, 25µl PCR master mix (Fermentas # K0171), 2 µl primer and 22 µl of nuclease free water. DNA amplification was performed in a DNA thermal cycler (Biometra, Germany). PCR conditions were as follows. First cycle of 5min at 94 °C for template denaturation, followed by 40 cycles of 1 min at 94 °C, 1 min at 37 °C, 2 min at 72 °C. An additional cycle of 7 min at 72 °C was used for final primer extension. All amplifications were repeated three times to select reproducible and intensive bands which were considered in the subsequent analyses. Amplification products were analysed by electrophoresis on 1.2% agarose gel.

Table: PCR reaction mixture:

Component	Volume
PCR Master mix	25 µl
DNA (diluted original DNA to 1:3)	1 µl
RAPD primer	2 µl
Nuclease free water	22 µl
Total volume	50 µl

Table: PCR conditions:

Temperature	Duration	Number of cycles
94 °C	5 min	1
94 °C	1 min	34
37 °C	1 min	
72 °C	2 min	
72 °C	7 min	1
4 °C	Over night	Over night

2.4 Agarose gel electrophoresis:

Agarose gels were prepared using an agarose concentration appropriate to the size of DNA fragments to be separated. For genomic DNA 0.8% concentration of agarose gel was used whereas for PCR product 1.2% agarose gel was used. Agarose was added to a 1X TAE buffer and melted in a microwave oven. Care was taken to ensure even mixing and complete dissolving of the agarose. A gel insert tray was sealed with tape and a gel comb inserted. The agarose was cooled to 55 °C before pouring onto the gel insert tray. Care was taken to ensure that all bubbles were removed before the gel set. The tape was removed from the gel insert tray and the gel comb was carefully removed. Electrophoresis buffer was added in order to cover the gel and to load the DNA samples. DNA samples or PCR products were added to the sample wells in the agarose gel using a micropipette. Care was taken

not to damage the sample wells with the micropipette during the procedure. Loading dye as well as DNA markers of different sizes were used from Fermentas Company. A small gel was used for 1 hour at 60 V.

Reagents used: 100bp DNA ladder ready to use Fermentas # SM1143, GeneRuler 1kb Fermentas # SM0311 and 6X Loading dye solution Fermentas # R0611

The resulting patterns obtained were consistently reproducible under strictly the same working conditions.

2.5 Statistical Analysis:

The RAPD products from the seven genotypes of *C. annuum* were scored as band presence (1) and absence (0). The specific distinguishable bands of the seven varieties studied are shown in Table 2. The marker OPM-05(650 bp) was unique to SADA. The marker OPQ-01(200 bp) was absent only in SADA and the marker OPT-04(700 bp) was also absent only in SADA. The minor bands (circled in Figs. 5.1-5.7) were not considered for data analysis because they are not reliable as markers. A binary matrix (Table 3) was thus generated which was used to compute pair-wise similarity coefficients based on Jaccard's[5]. UPGMA (Unweighted Pair Group Method based on Arithmetic Averages)[14] by SAHN clustering was utilized to create a dendrogram (Fig. 1) based on the genetic distance matrix. Data analysis was carried out using computer package program Numerical Taxonomy and Multivariate Analysis System i.e. NTSYS-pc, Version 1.80[13].

Table 1: Details of RAPD primers used in the study

Name of Primer	Primer sequence	Range of amplifications (bp)
OPM-01	GTTGGTGGCT	900-950
OPN-05	ACTGAACGCC	500-650
OPP-01	GTAGCACTCC	550-600
OPQ-01	GGGACGATGG	200–750
OPT-04	CACAGAGGGA	700-1000

Table 2: Specific distinguishable bands of the seven cultivated varieties of *C. annuum* L.

Variety	Specific primer and measurement range (bp) of DNA
ACH	OPM-01(900), OPN-05(550), OPP-01(600),OPQ-01(200), OPT-04(700,1000).
G4	OPM-01(900), OPP-01(600), OPQ-01(200), OPT-04(700).
JW	OPM-01(900), OPN-05(550), OPP-01(600), OPQ-01(200), OPT-04(700,1000).
JY	OPM-01(900), OPP-01(600), OPQ-01(200), OPT-04(700),
KR	OPM-01(900), OPN-05(500,550), OPP-01(600), OPQ-01(200,500), OPT-04(700).
SADA	OPM-01(900), OPN-05(500,650), OPP-01(600).
SS	OPM-01(900), OPN-05(500,550), OPP-01(600), OPQ- 01(200,500), OPT-04(700).

N.B.: Underlined shows band present only in variety SADA

Table 3: Similarity matrix of the seven cultivated varieties of *C. annuum* L. generated using Jaccard's estimate of similarity.

	ACH	G4	JW	JY	KR	SADA	SS
ACH	1.00						
G4	0.66	1.00					
JW	1.00	0.66	1.00				
JY	0.66	1.00	0.66	1.00			
KR	0.63	0.57	0.63	0.57	1.00		
SADA	0.25	0.33	0.25	0.33	0.37	1.00	
SS	0.63	0.57	0.62	0.57	1.00	0.37	1.00

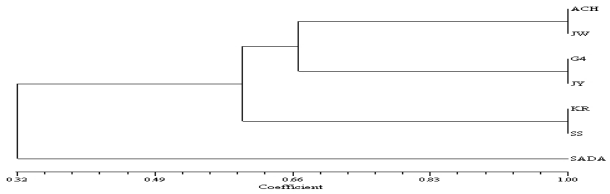
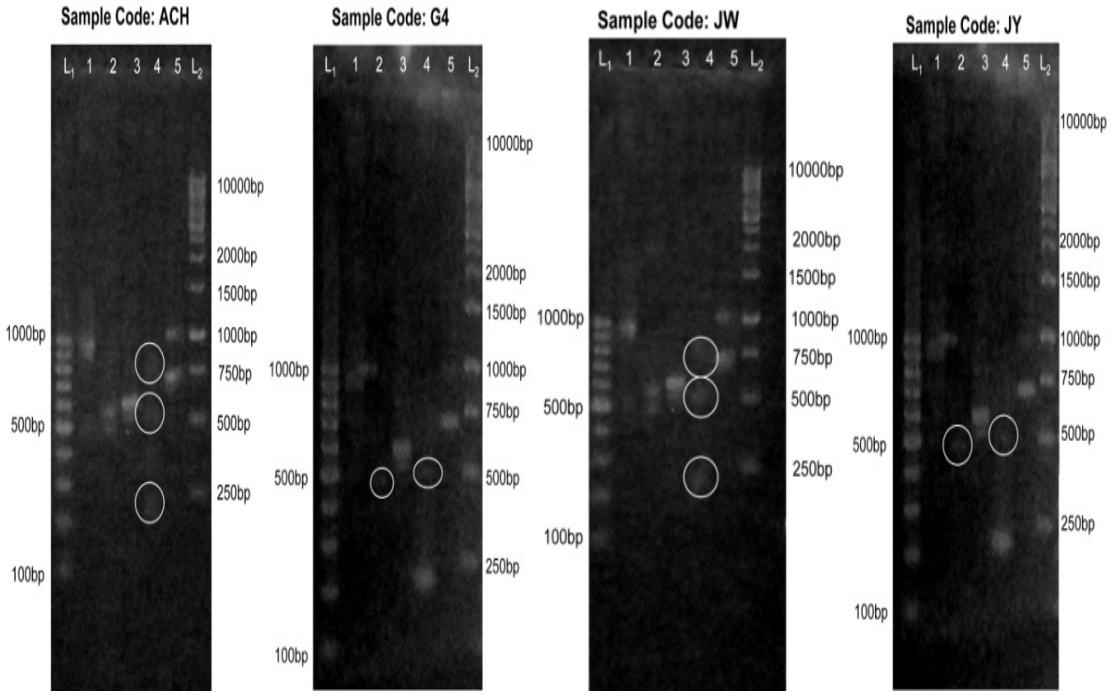
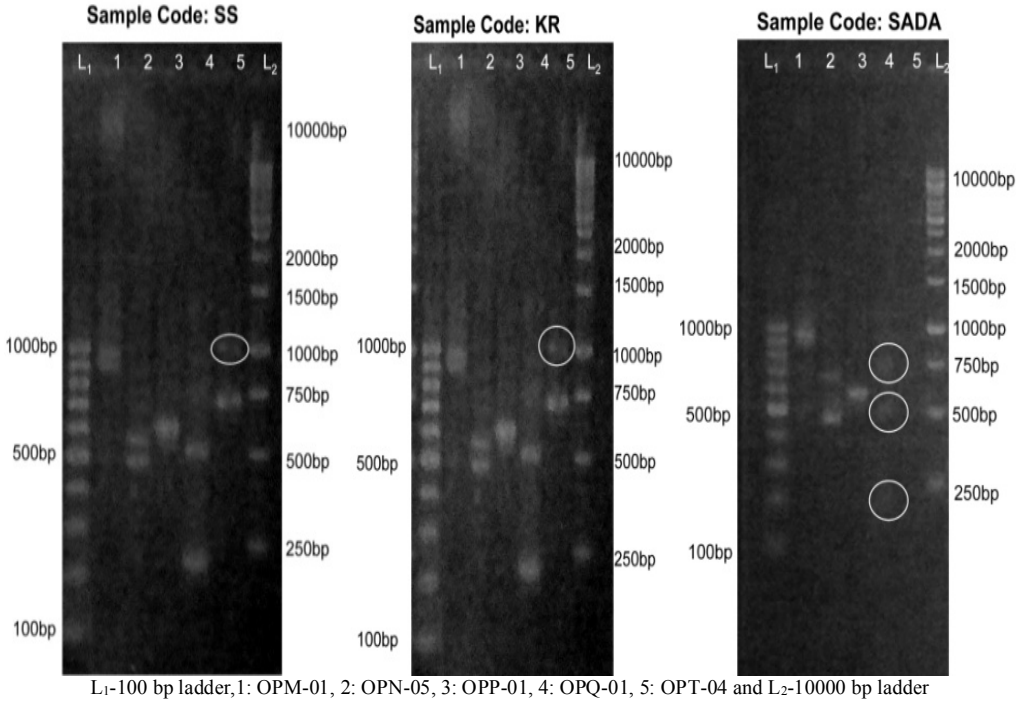


Fig 1: Dendrogram of the seven cultivated varieties of *C. annuum* L. by UPGMA cluster analysis.





L1-100 bp ladder,1: OPM-01, 2: OPN-05, 3: OPP-01, 4: OPQ-01, 5: OPT-04 and L2-10000 bp ladder

Fig 2: RAPD profile for the seven cultivated varieties of *C. annuum* L. generated by the five primers.

3. Results

The total number of primers tested were eight. Of these five primers showed polymorphism. A total of nine bands amplified. Of these seven bands were polymorphic and two were monomorphic. Therefore 77.77% of the bands showed polymorphism.

The five primers used for the RAPD analysis showed the following results:

1) Primer OPM-01 and OPP-01: Amplified products of 900 bp and 600 bp respectively from each primer showed the presence of a single band. Thus both of them were monomorphic.
2) Primer OPN-05: It showed the presence of three polymorphic bands i.e. 500 bp, 550 bp and 650 bp. The 500 bp fragment was absent in variety ACH, G4,JW and JY. The 550bp was present in variety ACH,JW,KR and SS. The 650 bp amplified product observed in one variety i.e. SADA.
3) Primer OPQ-01: Two polymorphic bands i.e. 200 bp and 500 bp were observed. The 200 bp fragment was present in six varieties and absent in one variety i.e. SADA. The 500 bp fragment was present in KR and SS discriminating these two varieties from the rest five varieties.
4) Primer OPT-04: Two amplified products of 700 bp and 1000 bp were observed with respect to this primer. The 700 bp fragment was present all the varieties except variety SADA. The 1000 bp amplified product was present in varieties ACH and JW.

The marker OPM-05 (650 bp) was unique to SADA. The markers OPQ-01 (200 bp) and OPT-04 (700 bp) were absent only in SADA. Thus the markers showed polymorphism among the varieties. This indicates that the varieties are genetically distinct. The Jaccard's estimate of similarity was used to construct a similarity matrix. The similarity values ranged from 0.25 for SADA–ACH and SADA-JW to o.66 for JW-G4 and JW-JY

5. Discussion:

The dendrogram analysis (using Jaccard coefficient) separated the seven genotypes of *C.annuum* L. into three clusters. The first group was further categorised into two sub-groups

including ACH-JW and G4-JY. The second group consisted of KR-SS. One variety i.e. SADA joined the three clusters separately.

When the cluster analysis of RAPD patterns was associated with morphological characteristics of the seven cultivated varieties of *C. annuum* L. it was found that the varieties could be classified into three major groups. The first group included two sub-groups. The first sub-group was ACH with medium fruits and JW with large fruits. Both showed similar calyx length. The second sub-group included G4 and JY with medium fruits. They showed similar style lengths. The second group included KR with small fruits and SS with long fruits. The third group had only SADA with medium fruits. Other morphological characteristics also classified SADA into a separate group because it had maximum value for the calyx length, corolla length, style length and leaf area. It also showed maximum number of flowers with style protruding out of the stamina tube. The pericarp weight per fruit was minimum and the fruits were found to grow erect in this variety. Thus it appears to be genetically distinct from the other varieties. Most of these characteristics point towards an advanced nature of this variety.

Previous studies by Lefebvre *et al*.[6] and Paran *et al*.[9] revealed that the small-fruited cultivars of *C. annuum* formed a more divergent group than the large-fruited cultivars. In the present study also the small and medium-fruited varieties like ACH, G4, JY, KR and SADA appear to be more divergent than the large-fruited varieties like JW and SS. Wang *et al*.[15] distinguished 8 accessions of *Opuntia* on the basis of phenotypic and molecular analysis. A clear separation was obtained among the species and accessions of *Passiflora* by Crochemore *et al*.[4] by using RAPD markers. Azeez *et al*.[2] used RAPD technique to access the genetic diversity among 30 accessions of *Santalum album*. Cluster analysis using UPGMA separated the accessions into two major groups. Abd El-Samad[1] estimated genetic diversity in wild and cultivated forms of Beet using RAPD and AFLP markers. RAPD data provided a more accurate picture to discriminate wild Beet accessions from other cultivated forms.

6. Conclusion:

Thus various results promote the use of RAPD markers in discrimination of the genotypes which can be exploited in the improvement of the varieties. It can be shown from these studies that RAPD analysis in association with morphological characteristics can be used as a more specific and reliable method in classifying the different cultivars having closely related genotype. Therefore RAPD can be a valuable tool for genetic and breeding programmes.

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