Journal of Medicinal and Aromatic Plant Sciences 38(1-4) (2016) 43-49

RAPD Analysis in Opium Poppy (*Papaver somniferum* L.)

CL KHATIK* • SP SHARMA[1] • SR MALOO[1] • NS DODIYA[1] • A JOSHI[2] • RK JAIN[2]

Article History

Received: 13th July, 2016
Accepted: 30th December, 2016

Key words
Genetic diversity
Opium poppy
Papaver somniferum
RAPD

ABSTRACT

The RAPD analysis was carried out with 28 crosses, 8 parents and 2 checks of opium poppy. Purified and isolated DNA was subjected to PCR based marker analysis (RAPD) for assessment of genetic diversity. The quality of DNA was determined by calculating ratio between A_{260} and A_{280} observed between 1.857 to 2.167 which indicated a good quality of plant DNA. The concentration of DNA ranged between 123 µg/µl (UOP-60 x UOP- 99) to 750 µg/µl (UOP-79 x UOP- 80). In the RAPD analysis 12 primers gave good amplified products with template DNA. Polymorphism shown by 12 primers ranged between 50 per cent (OPP-02) to 100 per cent (OPA-01, OPA-08, OPB-06 and OPD-05). Average polymorphism was found to be 84.80 per cent. From RAPD profiling similarly matrix was obtained and Jaccard's similarity coefficient lies between 0.57 (UOP-69 x UOP-80) to 0.95 (UOP-53 x UOP-79) with an average of 0.79. On this basis a dendrogram was constructed using UPGMA method. Dendrogram differentiated 28 crosses, 8 parents and 2 checks of opium poppy into one major and four minor groups. Further genetically diverse parents and crosses can be alternatively used for accumulating favourable genes so as to finally improve the productivity.

INTRODUCATION

The Opium Poppy (*Papaver somniferum* L., 2n=22) belonging to the family *Papaveraceae*, is an annual medicinal herb. The medicinal value of Opium Poppy is due to presence of more than two dozen alkaloids out of which morphine, codeine, narcotine, thebaine and papaverine, are frequently used as analgesic, sedative, anti-tussive and anti spasmodic drugs in modern pharmacy.

Molecular markers are valuable tools in the characterization and evaluation of genetic diversity within and between species and populations. It has been shown that different markers reveal different classes of variation [1,6,20]. It is correlated with the genome fraction surveyed by each kind of marker, their distribution throughout the genome and the extent of the DNA target which is analyzed through each specific assay [2]. RAPD markers

*Corresponding author, Email: champalalkhatik@gmail.com, Department of Plant Breeding and Genetics, Rajasthan College of Agriculture, Maharana Pratap University of Agriculture and Technology, Udaipur (Rajasthan) -313 001
[1]Department of Plant Breeding and Genetics, Rajasthan College of Agriculture, MPUAT, Udaipur.
[2]Department of Molecular Biology and Biotechnology, Rajasthan College of Agriculture, MPUAT, Udaipur.

are commonly used because they are quick and simple to obtain enabling genetic diversity analysis in several types of plant material such as natural populations, population in breeding programmes and germplasm collections [5,9,16]. RAPD markers are superior when simplicity and costs are taken into considered [8,14,18]. RAPD [19] has been used in analysis of genetic distance in different plant species [7,13,15,17]. In the present investigation efforts were made on 28 crosses, 8 parents and 2 checks of poppy to assess their genetic diversity using RAPD analysis.

MATERIALS AND METHODS

The present investigation was conducted on opium poppy grown under AICRP project on Medicinal and Aromatic Plants, Department of Plant Breeding and Genetics, RCA, Udaipur. In all 28 crosses, 8 parents and 2 checks of opium poppy were characterized in the present study.

DNA isolation, purification and quantification

5 gram of leaf tissues was collected from a single plot of each cross, parent and check. The leaf tissues was frozen in liquid nitrogen and ground to a fine powder in a pestle and mortar. The genomic DNA was isolated from powdered leaf tissue using CTAB method [3] and treated with RNase to eliminate RNA. DNA concentration was measured by UV-absorbance method. The concentration of DNA preparation varied from 123 µg/µl (UOP-60 x UOP- 99) to 750 µg/µl (UOP-79 x UOP- 80). The integrity of the isolated DNA was verified by visualization of DNA on 0.8% Agarose gel with DNA standard uncut lambda DNA. The quality of DNA was determined as the ratio of A_{260}/ A_{280}, which ranged from 1.857 to 2.167, that indicated a good quality plant DNA (Table-1).

RAPD analysis

Amplification of polymorphic DNA was done by using 30 primers obtained from Department of Molecular Biology and Biotechnology, Rajasthan college of agriculture, Udaipur. The details of PCR reaction mixture is given in Table 2.

Table-1: Concentration of DNA in Opium poppy crosses, parents and checks.

S. No.	Crosses)/ Parents/Checks	Ratio of A_{260}/A_{280}	Conc. of DNA (µg/µl)
1	UOP-53 x UOP-69	1.909	360.0
2	UOP-53 x UOP-79	1.966	284.0
3	UOP-53 x UOP-80	1.948	277.0
4	UOP-53 x UOP-20	1.926	321.0
5	UOP-53 x UOP-1185	1.891	297.0
6	UOP-53 x UOP-60	2.052	387.0
7	UOP-53 x UOP-99	1.947	635.0
8	UOP-69 x UOP-79	1.894	218.0
9	UOP-69 x UOP-80	1.958	566.0
10	UOP-69 x UOP-20	1.900	140.0
11	UOP-69 x UOP-1185	1.962	250.0
12	UOP-69 x UOP-60	1.902	238.0
13	UOP-69 x UOP-99	2.133	314.0
14	UOP-79 x UOP-80	2.110	750.0
15	UOP-79 x UOP-20	2.082	373.0
16	UOP-79 x UOP-1185	2.080	382.0
17	UOP-79 x UOP-60	1.989	458.0
18	UOP-79 x UOP-99	2.096	375.0
19	UOP-80 x UOP-20	2.044	225.0
20	UOP-80 x UOP-1185	2.000	162.0
21	UOP-80 x UOP-60	2.019	257.0
22	UOP-80 x UOP-99	2.000	186.0
23	UOP-20 x UOP-1185	2.118	188.2
24	UOP-20 x UOP-60	2.154	168.6
25	UOP-20 x UOP-99	2.062	162.0
26	UOP-1185 x UOP-60	2.081	189.0
27	UOP-1185 x UOP-99	2.167	131.9
28	UOP-60 x UOP-99	1.923	123.0
29	UOP-53 (P1)	1.957	225.0
30	UOP-69 (P2)	1.862	132.0
31	UOP-79 (P3)	1.872	358.0
32	UOP-80 (P4)	2.000	230.0
33	UOP-20 (P5)	1.872	179.0
34	UOP-1185 (P6)	1.888	495.0
35	UOP-60 (P7)	1.857	159.0
36	UOP-99(P8)	1.929	331.0
37	CHETAK APHIM (C_1)	1.902	148.5
38	MOP-540 (C_2)	1.940	243.0

The Polymerase Chain Reaction (PCR) was performed in PCR machine (Thermo cycler) using the cycling parameters as detailed in Table 3.

Following the amplification, the PCR products were loaded on 0.8% Agarose gel which was prepared in 1 x TAE buffer containing 0.5 mg/ml of

Table 2: PCR reaction mixture used for molecular analysis of opium poppy

S. No.	Component	Final Concentration	Single tube (20µl)
1	DNA template 50 ng/ µl	50ng	1.00 µl
2	**Master mixture**		
	1.dNTP mix	200 µM	1.6 µl
	2.Taq DNA polymerase	1U	0.33 µl
	3.Reaction buffer(10X)	1X	2.00 µl
	4.Primer	0.5 µM	2.00 µl
	5.dd H_2O		13.07 µl

Table 3: Different Cycles of PCR Amplification

Cycle	Denaturation		Annealing		Extension	
First cycle	94°C	5 min	-	-	-	-
2-45 cycles	94°C	1 min	35°C	1 min	72°C	2 min
Last cycle	-	-	-	-	72°C	5 min

the ethidium bromide. The Amplified products were electrophoresed for 2.5-3 hrs at 50 V with cooling. After separation the gel was viewed under UV transilluminator and photographed with the help of gel documentation system (Alpha DG DOC).

Scoring of RAPD products

In order to score the DNA banding patterns photograph of the gel was taken on a Gel Documentation System, under UV transilluminator. RAPD bands were designated on the basis of their molecular sizes (length of polynucleotide amplified). 100bp DNA ladder (hiper himedia 50 lane) loaded simultaneously with primer products in the gel was used to estimate the molecular sizes. The distance run by amplified fragments from the well was translated to molecular sizes with reference to molecular weight marker. The presence of each band was scored as '1' and its absence as '0'. Faintly visible bands were not scored, but a major band corresponding to faint bands was considered for scoring.

Statistical analysis for similarity coefficient

The scores (0 or 1) for each band obtained from photograph were entered in the form of a rectangular data matrix (qualitative data matrix). The pair-wise association coefficients were calculated from qualitative data matrix using Jaccard's similarity coefficient. The equation for calculating Jaccard's similarity coefficients 'F' between two samples A and B is:

$$f = n_{x\,y} / (n_1 - n_z)$$

n_{xy} = Number of bands common to sample A and sample B.

n_1 = Total number of bands present in all samples.

n_z = Number of bands not present in sample A or B but found in other samples.

Cluster analysis for the genetic distance was then carried out using UPGMA (Unweighted Pair Group Method with Arithmetic Mean) clustering method. The genetic distances obtained from cluster analysis through UPGMA were used to construct the dendrogram, depicting the relationships of the clones using computer program NTSYS pc version 2.02 [11].

RESULTS AND DISCUSSION

RAPD analysis

Genetic diversity assessment can increase the effectiveness of plant breeding programmes. The 28 opium poppy crosses, 8 parents and 2 checks were successfully discriminated on the basis of their RAPD patterns. Out of 30 primers used in this study 12 primers gave amplified products. Each RAPD products was assumed to represent a single locus and data were scored as (1) and (0) for presence and absence, respectively. Result are presented in Table 3 Only those fragments which consistently amplified were considered for analysis. Polymorphism shown by 12 primers ranged between 50 per cent (OPP-02) to 100 per cent (OPA-01, OPA-08, OPB-06 and OPD-05). Average polymorphism was found to be 84.80 per cent (Table 4). Electrophoresis pattern of RAPD profile with OPA-1 primers gave 10 bands in the range of 300 bp to 1300 bp with 100% polymorphism. Primer OPA-08 gave 8 bands in the range of 200 bp to 1400 bp with 100%

polymorphism, while 7 bands were produced by OPA-09 primer within the range of 300 bp to 1200 bp and showed 85.71 % polymorphism. The primer OPA-14 had given 5 scorable bands from which 3 were polymorphic and showed 60% polymorphism. However, OPB-06 primers gave 10 bands with all polymorphic bands and 100% polymorphism while OPC-08 had 6 bands out of which 4 were polymorphic and polymorphism was 6.66 %. The primer OPD-05 gave 9 bands in the range of 100 bp to 1500 bp with 100% polymorphism, while 9 bands were produced by OPP-01 primer within the range of 250 bp to 1800 bp and showed 88.88 % polymorphism. The primer OPP-02 had given 4 scorable bands from which 2 were polymorphic and showed 50% polymorphism. However, OPP-03 primers gave 6 bands with 5 polymorphic bands and 83.33% polymorphism while OPP-04 had 5 bands out of which 3 were polymorphic and polymorphism was 60.00 % and OPB-11 primers gave 6 bands in the range of 450 bp to 3200 bp with 66.66% polymorphism. The representative photographs of electrophoresis gels showing RAPD profiles after amplification with RAPD primers are depicted in Fig. 1&2.

The12 RAPD primers on 28 opium poppy crosses, 8 parents and 2 checks generated total 85 bands, out of which 72 were polymorphic. The number of DNA amplified fragments per primer ranged from 0 to 8. The most informative primers were found to be OPA-01, OPA-08,OPB-06 and OPD-05 with 10 polymorphic bands respectively while least informative was OPP-02 with only 2 polymorphic bands out of 4 bands. The average size of fragments obtained was between 100-3200 bp. The maximum numbers of amplified bands were seen in crosses UOP-53 x UOP- 80, UOP-69 x UOP- 20 and UOP-20 x UOP- 99 with 57 bands while parents UOP-60 had 56 bands. This type of results were also reported by [8,10,12,18].

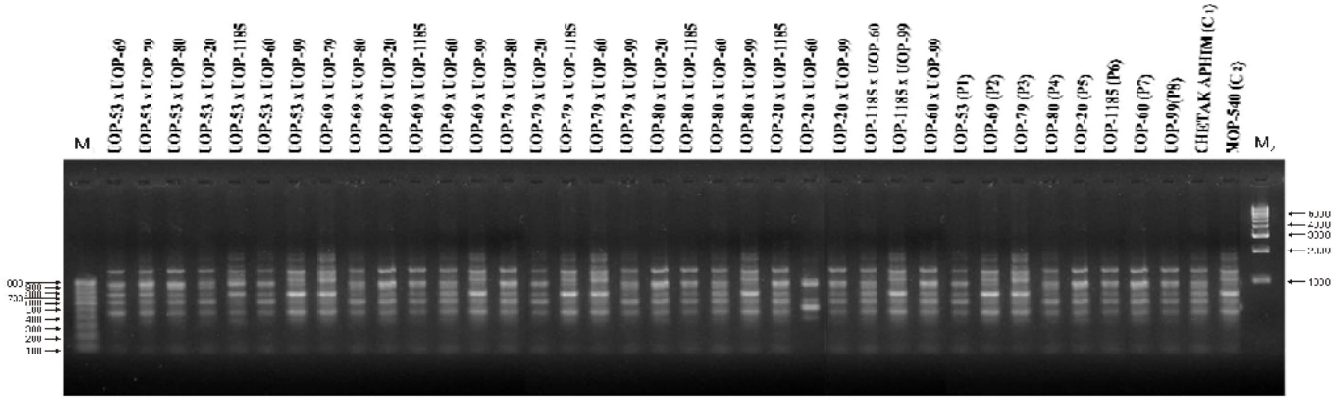
Genetic relationship among the Germplasm and cluster analysis

The banding pattern generated and polymorphism reflected in these patterns was used to calculate the diversity among accessions included in the present study. Genetic similarity estimates based on RAPD banding patterns were calculated using method of Jaccard's coefficient analysis [3]. The similarity coefficient matrix generated for the primers was subjected to algorithm UPGMA (Unweighted Pair Group Method with Arithmetic Mean) and clusters were generated using NTSYS 2.02 pc program [11]. The dendrogram showing relationships among various varieties was constructed using these clusters (Figure-3).The Jaccard's similarity coefficient values ranged from 0.57 (UOP-69 x UOP-80) to 0.95 (UOP-53 x UOP-79) with an average of 0.79.

Table-4:Polymorphism information based on RAPD primers analyzed.

S. No.	Primers code	Sequences (5'→3')	Total No. of bands (a)	Total No. of polymorphic bands (b)	Polymorphism % (b/a × 100)
1	OPA-01	CAGGCCCTTC	10	10	100.00
2	OPA-05	AGGGGTCTTG	NA	NA	NA
3	OPA-07	GAAACGGGTG	NA	NA	NA
4	OPA-08	GTGACGTAGG	8	8	100.00
5	OPA-09	GGGTAACGCC	7	6	85.71
6	OPA-10	GTGATCGCAG	NA	NA	NA
7	OPA-11	CAATCGCCGT	NA	NA	NA
8	OPA-14	TCTGTGCTGG	5	3	60.00
9	OPA-15	TTCCGAACCC	NA	NA	NA
10	OPA-16	AGCCAGCGAA	NA	NA	NA
11	OPB-02	TGATCCCTGG	NA	NA	NA
12	OPB-03	CATCCCCCTG	NA	NA	NA
13	OPB-04	GGACTGGAGT	NA	NA	NA
14	OPB-05	TGCGCCCTTC	NA	NA	NA
15	OPB-06	TGCTCTGCCC	10	10	100.00
16	OPB-07	GGTGACGCAG	NA	NA	NA
17	OPB-08	GTCCACACGG	NA	NA	NA
18	OPB-10	CTGCTGGGAC	NA	NA	NA
19	OPB-11	GTAGACCCGT	6	4	66.66
20	OPC-08	TGGACCGGTG	6	4	66.66
21	OPD-05	TGAGCGGACA	9	9	100.00
22	OPD-12	CACCGTATCC	NA	NA	NA
23	OPD-16	CCAAGCTGCC	NA	NA	NA
24	OPE-03	CCAGATGCAC	NA	NA	NA
25	OPF-13	CCTCTAGACC	NA	NA	NA
26	OPJ-04	CCGAACACCG	NA	NA	NA
27	OPP-01	GTAGCACTCC	9	8	88.88
28	OPP-02	TCGGCACGCA	4	2	50.00
29	OPP-03	CTGATACGCC	6	5	83.33
30	OPP-04	GTGTCTCAGG	5	3	60.00
	Total		85	72	-
	Average		2.83	2.40	84.80

NA - Not amplified

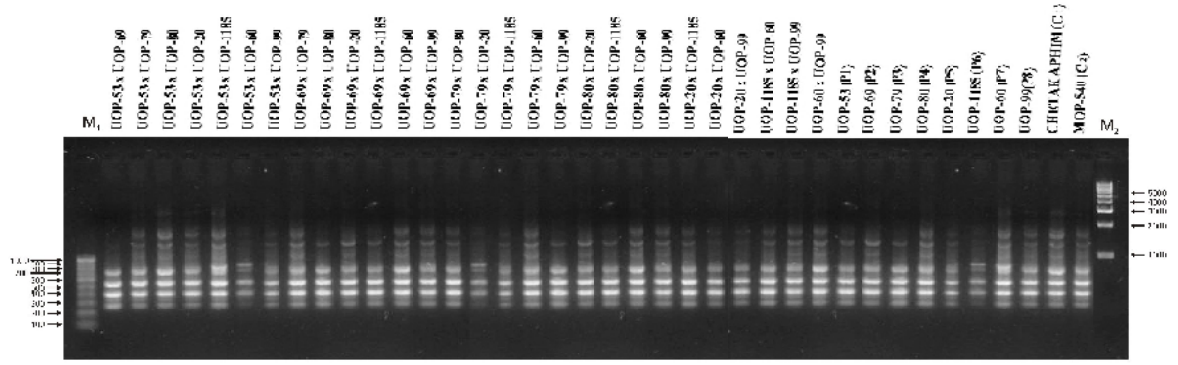


OPD-05

Each lane is labelled with Crosses (1 to 28), Parents (P1 to P8) and Checks (C1 & C2) at the top of the plate.

M1- 100 bp DNA Ladder, M2- 1000 bp DNA Ladder

Fig 1: RAPD Profile of Opium poppy Crosses, Parents and Checks generated with Primer OPD-05



OPP-01

Each lane is labelled with Crosses (1 to 28), Parents (P1 to P8) and Checks (C1 & C2) at the top of the plate.

M1- 100 bp DNA Ladder, M2- 1000 bp DNA Ladder

Fig 2: RAPD Profile of Opium poppy Crosses, parents and Checks generated with Primer OPP-01.

The cross UOP-69 x UOP-80 was most dissimilar and scored values of lower order with other crosses (average similarity co-efficiency being 0.57 over rest of the crosses and hence, maximum diverse from the rest) followed by UOP-53 x UOP-99, UOP-20 x UOP-99 and UOP-53 x UOP-80 with average similarity co-efficiency of 0.58,0.58 and 0.59. The cross UOP-53 x UOP-79 was most similar and score higher values with other crosses (average similarity co-efficiency being 0.95 over rest of the crosses and hence, minimum diverse from the rest) followed by UOP-80 x UOP-20, UOP-53 x UOP-80, UOP-69 x UOP-60 and UOP-80 x UOP-1185 with average similarity co-efficiency of 0.93, 0.92,0.92 and 0.92.

The dendrogram based on RAPD analysis has generated one major group A and four minor groups B, C, D and E, respectively. The major group A consists of two sub groups (A1 and A2). The sub group A1 consists of two sub group (A-I and A-II). The sub group A-I consisted of 11 crosses, 3 parents and 2 checks viz., UOP-53 x UOP-69,

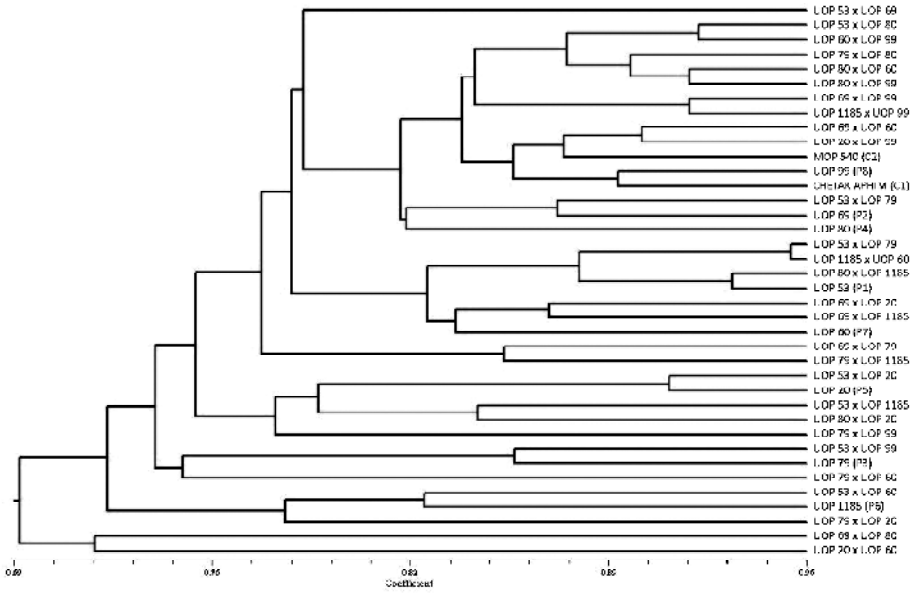


Figure-3: Dendrogram generated for opium poppy crosses, parents and checks using UPGMA cluster based on Jaccard similarity coefficient (RAPD analysis)

UOP-53 x UOP-80, UOP-60 x UOP-99, UOP-79 X UOP-80, UOP-80 x UOP-60, UOP-80 x UOP-99, UOP-69 x UOP-99, UOP-1185 x UOP-99,UOP-69 x UOP-60,UOP-20 x UOP-99,UOP-53 x UOP-79, UOP-99,UOP-69,UOP-80,MOP-540 and Chetak Aphim whereas sub group A-II consisted of 5 crosses and 2 parents viz., UOP-53 x UOP-79, UOP-1185 x UOP-60, UOP-80 x UOP-1185, UOP-69 x UOP-20, UOP-69 x UOP-1185, UOP-53 and UOP-60. However the class within the sub group A2 consisted of two crosses (UOP-69 x UOP-79 and UOP-79 x UOP-1185) had higher within group similarity of 89.4% while it is 89.2% for sub group A1. The sub group A1 and A2 joined together at the similarity level of 76.1% whereas four minor groups B, C, D and E. The B minor group consisted of 4 crosses and 1 parent viz., UOP-53 x UOP-20, UOP-53 x UOP-1185, UOP-80 x UOP-20, UOP-79 x UOP-99 and UOP-20 had higher within group similarity of 89.1% for group B. However the group C consisted of 2 crosses and one parent viz., UOP-53 x UOP-99, UOP-79 x UOP-60 and UOP-79 had higher within group similarity of 82.2% for group C. The minor group D consisted of 2 crosses and 1 parent viz., UOP-53 x UOP-60, UOP-79 x UOP-20 and UOP-1185 the similarity level of 82.0% respectively. The minor group E consisted of only 2 crosses viz., UOP-69 x UOP-80 and UOP-20 x UOP-60, joined major group (A) at the similarity level of 76.1% and 69.2% respectively.

The results of the present investigation could be used as a stepping stone for evolving a well defined approach based on evaluation and characterization of genetic variation in opium poppy, which is one of the important medicinal crop. On the basis of present study it may be concluded that RAPD profile of opium poppy *viz.* UOP-20 x UOP-60, UOP-69 x UOP-80, UOP-79 x UOP-60, UOP-79 x UOP-99, UOP-53 x UOP-69, UOP-80 and UOP-60 can be used for the diversity studies. Further the groups/clusters obtained by dandrogram could also be distinguished by similarity for the morphological characteristics within each group/cluster.

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