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Molecular Characterization of Opium Poppy (*Papaver somniferum*) Germplasm

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Abstract: Problem statement: The medicinal value of opium poppy (2n = 22, *Papaver somniferum* L.) is due to presence of more than two dozen alkaloids. The genetic origin and diversity of cultivars used in the breeding collection is not fully known. To evaluate the genetic diversity of the 24 germplasm currently cultivated in the state to provide genetic information about heterogeneity, using RAPD and ISSR markers. **Approach:** Isolated genomic DNA was subjected to PCR amplification using either random or ISSR markers. The amplified fragments were scored and matrix was prepared. Jaccard similarity coefficients were calculated and dendogram was generated. **Results:** RAPD with 12 primers gave 46 scorable fragments out of which 32 bands were polymorphic (69.52%) with 2.6 polymorphic band per primer, while in ISSR analysis 9 primer gave good amplification with DNA of 24 opium poppy germplasm. They gave 27 scorable polymorphic with 3.0 bends per primer. Dendrogram constructed revealed that the based on RAPD the germplasm cultivated comprised of one major group A and one minor group B, while two clusters of two germplasm each stands apart, on the basis of ISSR they fall into one major group A and one minor groups B, while one germplasm clusters of UOP-60 stands apart. Based on combined RAPD and ISSR analysis these germplasm were divided into 1 major group A and 3 minor group B, C, D whereas one genotype UOP-6 stood apart. **Conclusion:** These results showed that the majority of cultivated germplasm fall in one cluster and have low genetic diversity.

Key words: RAPD, ISSR, molecular markers

INTRODUCTION

The opium poppy (2n = 22, *Papaver somniferum* L.) belonging to the family Papaveraceae, is an annual medicinal herb. The medicinal value of opium poppy is due to presence of numerous alkaloids out of which morphine, codeine, narcotine, thebaine and papaverine, are frequently used as pain killer, sedative, an analgesic, anti-tussive and anti spasmodic in modern medicine.

India is leading country in the production of opium poppy and is the world's largest producer of licit opium. India is the only country authorized by the United Nations single convention on narcotic drugs (1961) to produce gum opium. It is the largest producer of opium gum for the world's pharmaceutical industry. In 2002 India produced 820 Mt. of opium gum from 18447 ha. of cultivated area.

Plant breeders all over the country have developed various high yielding varieties, however most of them are yet to be characterized for their genetic diversity and gene pool. Molecular markers have been proved to be valuable tools in the characterization and evaluation of genetic diversity within and between species and populations. It has been shown that different markers might reveal different classes of variation[1,2]. 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 by each specific assay[3]. RAPD markers 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 program and germplasm collections[4]. RAPD markers are superior when simplicity and costs were considered[5]. RAPD has been used in analysis of genetic distance in different plant species[6-8]. ISSRs are the regions which lie within the microsatellite repeats and offer great potential to determine intergenomic diversity compared to other arbitrary primers, since they reveal variation within unique regions of the genome at several loci

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simultaneously. Several properties of microsatellites such a high variability among taxa, ubiquitous occurrence and high copy number in eukaryotic genome make ISSR extremely useful marker. They exhibit specificity of sequence-tagged site marker but need no sequence information for primer synthesis. ISSR usually detect a higher level of polymorphism then that detected with RFLP or RAPD analysis. In the present investigation we report genetic diversity in 24 poppy cultivars/lines from Rajasthan using RAPD and ISSR markers.

MATERIALS AND METHODS

The present investigation was conducted on leaves collected from opium poppy germplasm grown under AICRP project on opium poppy, Department of Plant Breeding and Genetics, RCA, Udaipur. Twenty four different germplasm of opium poppy from Rajasthan were taken Chemicals used in the present study were purchased from firms like BDH, E Merk, Himedia, SRL and Banglore Genei, India

The genomic DNA was isolated from powdered leaf tissue using CTAB and treated with RNase to eliminate RNA. DNA concentration and purity was measured by UV-absorbance. The integrity of the isolated DNA was verified by visualization of DNA on 0.8% Agarose gel with DNA standard uncut lambda DNA.

RAPD and ISSR analysis: RAPD and ISSR analysis was done by 15 random decamer primers and 16 ISSR primers obtained from Banglore Genei Pvt. Ltd. Banglore and Sigma Co. St luis respectively. PCR reactions were performed in final volume of 25 µL containing 10 × Assay Buffer, 1 units of Taq. DNA polymerase, 250 µM each of dNTPs, 10 pmols/reaction of primer and 25 ng of template DNA. The PCR was performed in 'Thermocycler' using the following cycling parameters.

Cycle 1:

- Initial denaturation (94°C) 4 min
- Denaturation (94°) 1 min
- Primer annealing (32°C) 1 min
- Primer Extension (72°C) 2 min

Cycle 2-34:

- Denaturation (94°C) 1 min
- Primer annealing (32°C) 1 min for RAPD primers
- Primer annealing 1.50min for ISSR primers
- Primer Extension (72°C) 2 min

Cycle 35:

- Denaturation (94°C) 1 min
- Primer annealing (32°C) 1 min
- Primer Extension (72°C) 7min

After amplification, the PCR products of RAPD were loaded on 1.6% Agarose gel while in case of ISSR they were loaded on 2.5% Agarose gel (Merk) prepared in 1 × TAE buffer containing 0.5 µg mL^{-1} of the ethidium bromide. The amplified products were electrophoresed for 2.5-3 h 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 the RAPD and ISSR products: In order to score and preserve banding pattern photograph of the gel was taken by a Gel Documentation System, under UV transilluminator. Bands were designated on the basis of their molecular sizes (length of polynucleotide amplified) using 100 bp DNA ladder (Hiper Himedia) as reference. The presence of each band was scored as '1' and while absence was marked as '0'. Faintly visible bands were not scored, but a major band corresponding to faint bands was considered for scoring. The matrix obtained by both RAPD and ISSR marker were analyzed by NTSY-pc software version 2.02 and dendrogram was generated by using UPGMA method.

RESULTS

RAPD: Out of fifteen decamer random primer used, 12 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. Electrophoresis pattern of RAPD amplified product on 1.6% Agarose gel is shown in Fig. 1 with primers OPAR 11 and OPP09. Table 1 shows the polymorphic amplicon generated by different primers, Primer OPP 12 gave 3 bands in the range of 600-1000 bp with 100% polymorphism, while 3 bands were produced by OPP-16 primer within the range of 400-1000 bp and showed 100% polymorphism. Two other primer named OPAP02 and OPF17 also showed 100% polymorphism with 4 and 3 bends respectively. However 50% polymorphism were showed by two primers OPF-19 and OPA-01 with 6 and 2 bands respectively,

Table 1: Polymorphism observed with RAPD primers

Primers code and Sequences (5'→ 3')	Total number of bands (a)	Base pair size of DNA bands	Total number of polymorphic bands (b)	Polymorphism % (b/a×100)
OPA-01, CAGGCCCTTC	2	800, 1000	1	50
OPP-09, GTGGTCCGCA	3	375, 600, 1000	2	66.67
OPP12, AAGGGCGAGT	3	600, 700, 1000	3	100
OPP16, CCAAGCTGCC	3	400, 600, 1000	3	100
OPD-12, CACCGTATCC	NA	NA	NA	NA
OPAT-08, TCCTCGTGGG	2	800, 1000	0	00
OPAR-11, GGGAAGACGG	7	250, 300, 400, 550, 600, 800, 950	6	85.71
OPAD-14, GAACGAGGGT	NA	NA	NA	NA
OPAP-02, CACCTGCTGA	4	500, 550, 600, 1000	4	100
OPP-05, CCCCGGTAAC	4	200, 500, 700, 1000	3	75
OPC-08, TGGACCGGTG	2	500, 700	0	00
OPD-05, TGAGCGGACA	NA	NA	NA	NA
OPE-03, CCAGATGCAC	7	300, 350, 400, 500, 600, 800, 1000	4	57.14
OPF-17, AACCCGGGAA	3	500, 700, 750	3	100
OPF-19, CCTCTAGACC	6	475, 550, 650, 800, 900, 1000	3	50
Total	46		32	-
Average	3.8		2.6	69.56

NA: Not Amplified

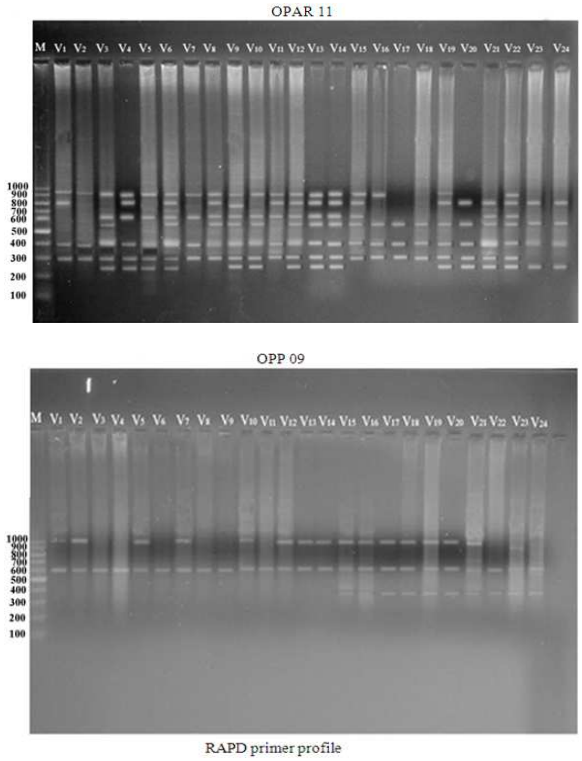

Fig. 1: Amplification of various genotypes by RAPD primers OPAR-11 and OPP-09. V1-V24 respectively being: UO-7982, UOP-1,UO-17682,UOP-4, UOP-5,UOP-6, UOP-57,UOP-83, UOP-74, UOP-34, UOP-43, UOP-44, UOP-53, UOP-60, UOP-78, MOP-9, CHETAK AFIM, MOP-518, MOP-506, MOP-1054, IC-19, IC-95, NBPGR-2, NBPGR-5

OPAR-11 and OPE-03 gave highest number of bands (7 bands) out of which 85.71% and 57.14% bands were polymorphic respectively, while OPP-05 gave 75% polymorphic bands and OPP-09 gave 66.67% polymorphic bands. Two primers (OPC-08, OPAT-08) gave all monomorphic bands.

Total 46 fragments were amplified in 24 genotypes with 12 primers giving an average of 3.8 fragment per primer. Out of these 46 bands 32 bands were polymorphic and the level of average polymorphism was 69.56% (Table 1.) with average 2.6 polymorphic bands per primer.

The amplified fragments obtained were in the range of 200-1000 bp. The maximum numbers of amplified bands were seen in genotype UOP-5, MOP-506 and IC-19 with 43 bands with 12 primers while genotype UOP-6 and UOP-83 had only 33 bands (data not given).

ISSR: Out of sixteen primers used 9 primers gave amplified products. Electrophoresis pattern of ISSR profile on 2.5% Agarose gel is shown in Fig. 2 with P-4 and P-1 primers. Table 2 shows the details about the polymorphic amplicon generated by different primers. Primer P-4 gave 6 bands in the range of 600 bp to 1000, 1000+1 and 1000+2 bp with 100% polymorphism, while 4 bands were produced by primer P-3 within the range of 700-1000 bp and showed 100% polymorphism. Three primer named P-1, P-8 and P-10 gave 3 polymorphic bends, while. Four primers P-5, P-11, P-13 and P-16 gave 2 polymorphic bands. Remaining 7 primers failed to amplify.

Table 2: Polymorphism observed with ISSR primers

Primer code and sequence (5'-3')	Annealing temperatures	Total number of bands (a)	Base pair size of DNA bands	Total number of polymorphic bands (b)	Polymorphism % (b/a×100)
P-1, A (GA)7 GC	45.3	3	300, 400, 550	3	100
P-2, (GTG)3 GC	NA	NA	NA	NA	NA
P-3, (GA)8 T	44.0	4	700, 750, 800, 1000	4	100
P-4, (CT)8 G	45.7	6	600, 800, 900, 1000, 1000+, 1000+2	6	100
P-5, (GA)8 TG	45.9	2	300, 450	2	100
P-6, A (GA)7 GTT	NA	NA	NA	NA	NA
P-7, (CT)6 G	NA	NA	NA	NA	NA
P-8, (CT)6 A	27.0	3	400, 500, 850	3	100
P-9, (GA)8 A	NA	NA	NA	NA	NA
P-10, A (GA)5 GT	27.5	3	500, 700,1000	3	100
P-11, (CT)8 TG	46.2	2	1000, 1000+	2	100
P-12, (CA)6 AC	NA	NA	NA	NA	NA
P-13, (CA)6 GT	40.2	2	500, 800	2	100
P-14, (CA)6 GG	NA	NA	NA	NA	NA
P-15, (CA)6 AG	NA	NA	NA	NA	NA
P-16, $(CA)_8GG$	41.6	2	400, 700	2	100

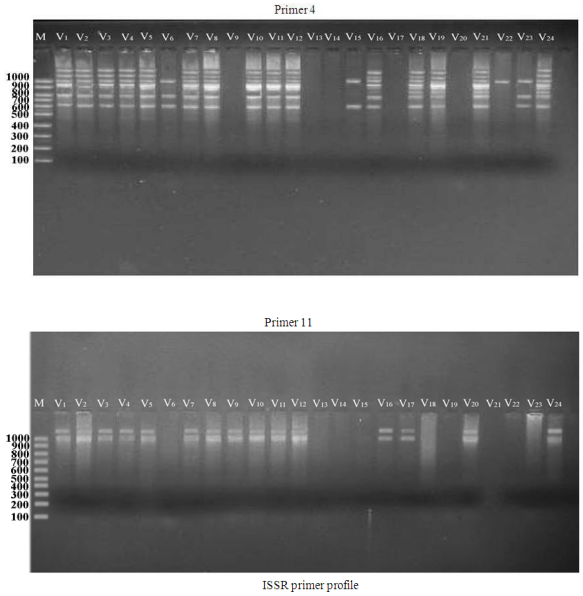

Fig. 2: Amplification of various genotypes by ISSR primer 4 and primer 11. V1-V24 respectively being: UO-7982, UOP-1, UO-17682, UOP-4, UOP-5, UOP-6, UOP-57, UOP-83, UOP-4, UOP-34, UOP-43, UOP-44, UOP-53, UOP-60, UOP-78, MOP-9, CHETAK AFIM, MOP-518, MOP-506, MOP-1054, IC-19, IC-95, NBPGR-2, NBPGR-5

Total 27 bands were amplified in 24 genotypes with 9 primers giving an average of 3.0 polymorphic bands per primer. The average size of fragments was obtained between 300-1000 bp. The maximum numbers of amplified bands were seen in genotype UOP-44 and UOP-33 with 25 bands while genotype MOP-1054 and IC-95 had only 8 bands (data not shown).

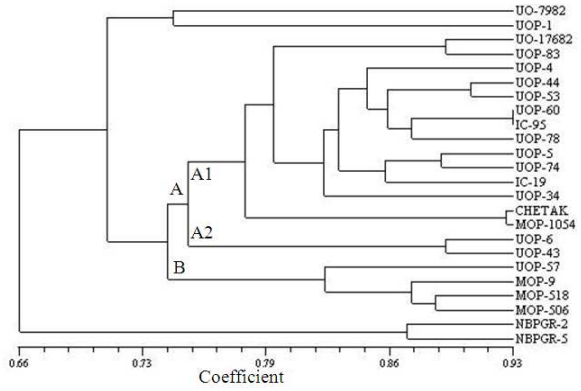

Fig. 3: Dendrogram showing relationship among 24 opium poppy accessions generated by UPGMA analysis based on RAPD

Genetic relationship among the Germplasm and cluster analysis:

RAPD: The dendrogram (Fig. 3) based on RAPD analysis has generated one major group A and one minor groups B, while two clusters of two genotypes each stands apart. The group A consists of two sub groups (A1 and A2) The sub group A1 consisted of 14 genotypes viz. UO-17682, UOP-83, UOP-4, UOP-44, UOP-53, UOP60, IC-95, UOP-78, UOP-5,UOP-78, UOP-74, IC-19, UOP-34, CHETAK and MOP-1054 , whereas sub group A2 consisted of 2 genotypes viz.UOP-6 and UOP-43. However the group within the sub group A1 consisted of 3 genotypes (UOP-60, IC-95 and UOP-78) had higher within group similarity of 87.4% while it is 77.2% for sub group (A1). The sub group A1 and A2 joined together at the similarity level of 75%, whereas minor groups B joined major group at the similarity level of 74.2 and 76.0% respectively.

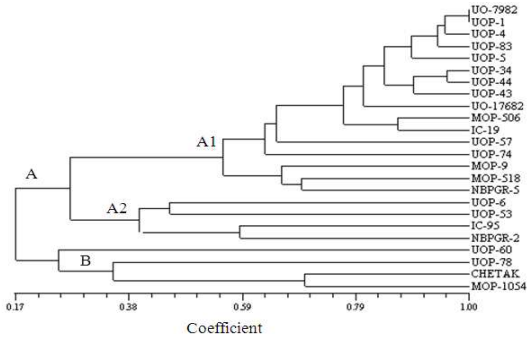

Fig. 4: Dendrogram showing relationship among 24 opium poppy accessions generated by UPGMA analysis based on ISSR

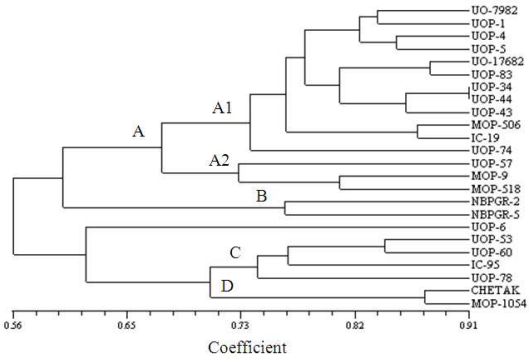

Fig. 5: Dendrogram showing relationship among 24 opium poppy accessions generated by UPGMA analysis based on RAPD and ISSR

However the average diversity estimated was very low 26.7% with a range from 7.5-44.45%.While group of UO-7982 and OUP-1 joined cluster at 70.9%, where as NBPGR group joined cluster at 66% similarity level. These results indicate that the majority of genotypes (sub-group A1) are close relativities.

ISSR: Genetic similarity estimation based on ISSR banding patterns were calculated using method of Jaccard's coefficient analysis. 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). The dendrogram showing relationships among various varieties was constructed using these clusters (Fig. 4).

The Jaccard's similarity coefficient values ranged from 0.100 (UOP-60 and MOP-518) to 0.96 (UOP-74 and UOP-44) with an average of 0.510. The dendrogram based on ISSR analysis has generated one major group A and one minor groups B, while one germplasm clusters of UOP-60 stands apart. The group A consists of two sub groups (A1 and A2) The sub group A1 consisted of 16 genotypes viz. UO-7982, UOP-1, UOP-4, UOP-83, UOP-5, UOP-34, UOP-44, UOP-43, UO17682, MOP-50, IC-19, UOP-57, UOP-74, MOP-9, MOP-518 and NBPGR-5, whereas sub group A2 consisted of 4 genotypes viz.UOP-6, UOP-53, IC-95 and NBPGR-2. However the group within the sub group A1 consisted of 2 genotypes (UO-7982 and UOP-1) had higher within group similarity of 100% while it is 55.2% for sub group (A1). The sub group A1 and A2 joined together at the similarity level of 26.7%, whereas minor groups B joined major group at the similarity level of 34 and 70% respectively. However the average diversity estimated was very low 26.7% with a range from 7.5-44.45%. While genotype UOP-60 joined cluster at 17% similarity level.

RAPD and ISSR: The matrix obtained by RAPD and ISSR analysis were pooled and evaluated. The Jaccard's similarity coefficient values ranged from 0.441 (UOP-57 and NBPGR-2) to 0.909 (UOP-34 and UOP-44) with an average of 0.704. The dendrogram (Fig. 5) showed one major group A and three minor groups B, C and D while one genotype clusters of UOP-6 stood apart. The group A consists of two sub groups (A1 and A2). The sub group A1 consisted of 12 genotypes viz. UO-7982, UOP-1, UOP-4, UOP-5, UO-17682, UOP-84, UOP-34, UOP-44, UOP-43, MOP-506, IC-19 and UOP-74, whereas sub group A2 consisted of 3 genotypes viz.UOP-57, MOP-9 and MOP-518. However the group within the sub group A1 consisted of 2 genotypes (UOP-34 and UOP-44) had higher within group similarity of 91% while it is 73.9% for sub group (A1). The sub group A1 and A2 joined together at the similarity level of 67.4%, whereas minor groups B joined major group at the similarity level of 60.1 and 75% respectively. The sub group C joined major group at the 62.3% with 4 genotypes (UOP-53, UOP-60, IC-95 and UOP-78) and where as group D joined major group at the similarity level of 70.8% with 2 genotypes (CHETAK and MOP-1054). However the average diversity estimated was very low 26.7% with a range from 7.5-44.45%, while genotype UOP-6 joined cluster at 62.3% similarity level.

DISCUSSION

Genetic similarity of *P. somniferum* genotypes estimated by a similarity matrix after RAPD analysis revealed maximum similarity between genotypes UOP-60 and IC-95. While genotypes UOP-57 with NBPGR-2 were most diverse. Fourteen genotypes fall in one cluster. ISSR marker analysis reveal that 16 genotypes fall in same cluster and that the genotypes UOP-60 and

MOP-518 are most diverse while genotypes UOP-74 and UOP-44 are similar. Combined RAPD-ISSR analysis also shows a cluster of 15 genotypes indicating that these genotypes have low genetic diversity.

CONCLUSION

These results suggest that the majority of germplasm cultivated in the state fall in one cluster and have low genetic diversity.

REFERENCES

1. Powell, W., M. Morgante and C. Andre, 1996. The comparison of RFLP, RAPD, AFLP an SSR (microsatellite) marker for germplasm analysis. Mol. Breed., 2: 225-238. DOI: 10.1007/BF00564200
2. Russell, J.R., 1997. Direct comparison of levels of genetic variation among barley accessions detected by RFLPs, AFLPs, SSRs and RAPDs. Theor. Applied Genet., 95: 714-722. DOI: 10.1007/s001220050617
3. Davila, J.A., Y. Loarce and E. Ferrer, 1999. Molecular characterization and genetic mapping of random amplified microsatellite polymorphism in barley. Theor. Applied Genet., 98: 265-273. DOI: 10.1007/s001220051067
4. Carvalho, V.P., F.C Ruas,, J.M Ferreira, M.P. Moreira and M.P. Ruas, 2004 Genetic diversity among maize (*Zea mays* L.) landraces assessed by RAPD markers. Genet. Mol. Biol., 27: 121-130. DOI: 10.1590/S1415-47572004000200017
5. Williams, J.G.K., A.R. Kubelik, K.J Livak, J.A. Rafalski and S.V. Tingey, 1990. DNA polymorphism amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res., 18: 653-65. DOI: 10.1016/S0006-3207(02)00041-1
6. Sharma, R.P. and T. Mahapatra, 1995. Molecular mapping and tagging of genes in crop plants. Genetica, 97: 313-320. DOI: 10.1007/BF00055317
7. Lashermes, P., P. Trouslot, F. Anthrony, M.C. Combes and A. Charrier, 1996. Genetic diversity for RAPD marker using cultivated and wild accessions of *Coffea arabica*. Euphytica, 87: 59-64. DOI: 10.1007/BF00022965
8. Colombo, C., G. Second and A. Charrier, 2000. Diversity within American cassava germplasm based on RAPD markers. Genet. Mol. Biol., 23: 189-199. http://www.scielo.br/pdf/gmb/v23n1/2331.pdf