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Diversity analysis by RAPD among the selected sunflower (*Helianthus annus* L.) parental lines

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Abstract
PCR amplifications from the 10 RAPD primers gave an average of 58.7 selected markers /primer. Amplification products were obtained in the range of 38-98. For OPD 05 primer, the highest number of fragments (98 amplicons) was detected, while OPW 15 primer revealed the least number (38 amplicons). A total of 587 amplicons were generated by the tested primers with an average of 58.7 amplicons/primer. Based on RAPD similarity matrix data (Table 2), the value of similarity coefficient ranged from 0.44 to 0.97 i.e., 44-97% or the genetic diversity ranged from 3-56%. The average similarity across all the genotypes was 0.73 showing that the genotypes were moderately similar genetically. Maximum similarity value of 0.97 was observed between genotypes 234B and 2-7-1B whereas, EC 623023 & EC 601957 were found to be genetically diverse with minimum similarity value of 0.44. Genotype EC 623023 showed low similarity values with most of the genotypes and was thus most diverse from rest of the genotypes. The RAPD cluster tree analysis of 14 Helianthus annus genotypes showed that they were mainly divided into two major clusters. The genotypes EC 623023, R 16, EC 601957 (testers) and CMS 234B and ARM 249B (lines) were spotted as genetically diverse since they are falling in different clusters based over RAPD marker analysis. Therefore could be efficiently utilized in improvement programmes.

Keywords: Sunflower, RAPD, diversity

Introduction
The importance of sunflower as an oilseed crop in India is of very recent origin and date backs to three decades. But its contribution towards attaining self-sufficiency in edible oil as well as to "yellow revolution" in the country is note-worthy [1]. In India, first ever sunflower hybrid developed by Seetharam [2], gave a fillip and renewed the interest in crop because of well-defined CMS system. Hybrids are preferred over varietal populations because of their high productivity in terms of seed and oil yield. With the increase in demand for edible oils, there is a need to develop new sunflower hybrids with considerable magnitude of heterosis for economic traits and also suited to different Agro-climatic zones of India.
Significant progress has been made in recent years in the application of molecular markers to plant genetic resources characterization and evaluation. Among several efficient methods for revealing genetic variability within and among plant populations, some of the most widely applied methods are RAPD [3, 4, 5] and ISSR p [6, 7]. Many researchers [8, 9] have pointed out that DNA-based markers were superior to isozyme in detecting genetic diversity. Particularly RAPD are simpler to used than the simple sequence repeats (SSR) technique as prior knowledge of the target sequences of flanking of the repeat regions is not required [10].

Material and Methods
Fourteen phenotypically diverse parental lines for seed yield, oil content and maturity comprised 4 maintainer and 10 restores of this species having indigenous and exotic origin were selected for the present study. A set of 20 random 10-mer primers were used for detecting the polymorphism among 14 genotypes of *H. annus* L.

Table 1: Description of parents

Parents	Origin	Important traits
CMS 234A	UAS, Bangalore	Early maturity and high oil content
CMS PET 89-1A	IIOR, Hyderabad	High seed yield.
CMS PET 2-7-1A	IIOR, Hyderabad	High seed yield.
ARM 249A	IIOR, Hyderabad	High seed yield.
R-16	IIOR, Hyderabad	Early maturity and High seed yield.
RHA 1-1	ORS, Latur	Downy mildew resistant.
RHA 138-2	ORS, Latur	High seed yield and black colour seed.
R-271-1	ORS, Latur	Early maturity and High seed yield.
IC-294064	NBPGR, New Delhi	Early maturity.
EC-623023	IIOR, Hyderabad	High seed yield.
EC-623016	IIOR, Hyderabad	High seed yield.
EC-601957	IIOR, Hyderabad	High seed yield.
EC-601924	IIOR, Hyderabad	High oil content and Downy mildew resistant.
6-D-1R	UAS, Bangalore	High oil content.

Table 2: Details of RAPD primers used in molecular analysis of sunflower genotypes

S. No.	Primers	Sequence (5'-3')	G:C Content (%)
1.	OPD-2	GGACCCAACC	70
2.	OPM-13	GGTGGTCAAG	60
3.	OPA-04	AAT CGG GCT G	60
4.	OPA-05	AGG GGT CTT G	60
5.	OPA-06	GGT CCC TGA C	70
6.	OPA-09	GGG TAACGC C	70
7.	OPT-10	GGCAGGCAGA	70
8.	OPS-02	CCAAGTTCGC	60
9.	OPW-04	CAGAAGCGGA	60
10.	OPW-06	AGGCCCGATG	70
11.	OPW-09	GTGACCGAGT	60
12.	OPW-10	TCGCATCCCT	60
13.	OPW-15	ACACCGGAAC	60
14.	OPQ-09	GAACGGACTC	60
15.	OPG-02	GGCACTGAGG	70
16.	OPG-04	AGCGTGTCTG	60
17.	OPG-09	CTGACGTCAC	60
18.	OPH-20	GGGAGACATC	60
19.	OPJ-06	TCGTTCCGCA	60
20.	OPR-04	CCCGTAGCAC	80

DNA amplification conditions and gel electrophoresis

For RAPD markers, the amplification reaction was carried out in 25 µL reaction volume containing 1xPCR buffer, 1.5 mM MgCl2, 0.25 mM dNTPs, 25 ng primer (Operon Technologies Inc. USA), 1.5 unit of Taq DNA polymerase and 20 ng template DNA. PCR amplification was performed in a T-gradient thermal cycler (Bio-Rad; T Gradient). It was programmed to fulfill 40 cycles (for RAPD analysis) or 35 cycles (for ISSR analysis) after an initial denaturation cycle for 5 min at 94 ºC. Each cycle consisted of a denaturation step 45 sec at 94 ºC, an annealing step for 1 min at 36 ºC (for RAPD analysis) and an extension step at 72 ºC for to 1 min, followed by extension cycle for 5 min at 72 ºC in the final cycle. The PCR products were separated on a 1.5% (for RAPD analysis) ethidium bromide- stained agarose (Bio-Rad) in 1xTBE buffer. Electrophoresis was performed for 2.5 h at 100V and visualized with a UV transilluminator.

RAPD data analysis:: The presence or absence of each size class was scored as 1 or 0, respectively. The percent disagreement value (PDVs) found were used to generate a matrix via the Unweighted Pair Group Mean Arithmetic average (UPGMA) using Statistica program (NTSYSpc version 2.02 [11]. This matrix was used to calculate similarity/genetic distance [12].

Genetic analysis and cluster tree analysis: The data obtained using RAPD primers were further used to construct similarity matrices of the *Helianthus annus* genotypes using 'Simqual' programme of software NTSYS-pc. Dendograms were constructed using similarity matrix values as determined from RAPD data for 14 *Helianthus annus* genotypes using unweighted pair group method with arithmetic average (UPGMA) sun programme of NTSYS-pc software.

Results and Discussion

Polymorphism in sunflower (Helianthus annus L.) using RAPD primers

Twenty RAPD primers having 60% or more G+C content were used for the present investigation. The DNA amplification and polymorphism generated among various genotypes of *Helianthus annus* using RAPD primers are presented in Table 1. Out of these 20 primers, 10 primers showed polymorphism. Primer OPG-9 generated 45 bands in the range of 300 bp to 1500 bp with 80% polymorphism. A total of 587 amplified bands were obtained of which 334 were polymorphic (Figure 1).

The amplification products produced from RAPD primers are listed in Table 1 in terms of the percentage of PCR products appeared in the genotypes studied. Most of markers were obtained with the OPD 05 primer. The RAPD analysis carried out on the 14 genotypes of *H. annus* produced a large number of distinct fragments for 10 primers only. Ten out of 20 selected arbitrary primers generated a total of 587 scorable bands of which 334 were polymorphic, with an average of 58.7 amplicons/primer. OPD 05 gave the highest number of fragments (98 amplicons), while OPW 15 revealed the least number (38 amplicons). Average polymorphism across all the 14 genotypes of *Helianthus annus* was found to be 56.88% and average number of polymorphic amplicons per primer was 33.4. Fig. 1 shows the RAPD profile for the 14 genotypes yielded by OPA-5, OPA-9, OPD-5, OPG-9, OPQ-9, OPW-6, OPW-9, OPW-4, OPW-15and OPA-4.

Behera [13] reported an average of 7.17 markers/ primer in Indian bitter gourd (*Momordica charantia* L.). Such a high variation in the number of fragments produced by these arbitrary primers could be attributed to the differences in the binding sites throughout genome of the genotypes included. The total number of polymorphic observed bands was 334 with an average of 33.4 polymorphic amplicons/ primer. This represented a level of polymorphism of 56.88% (Table 1).

Similarity matrices based on RAPD data

Based on RAPD similarity matrix data (Table 2), the value of similarity coefficient ranged from 0.44 to 0.97 i.e., 44-97% or

the genetic diversity ranged from 3-56%. The average similarity across all the genotypes was 0.73 showing that the genotypes were moderately similar genetically. Maximum similarity value of 0.97 was observed between genotypes 234B and 2-7-1B whereas, EC 623023 & EC 601957 were found to be genetically diverse with minimum similarity value of 0.44. Genotype EC 623023 showed low similarity values with most of the genotypes and was thus most diverse from rest of the genotypes. Crossing between genotypes with low similarity coefficient manifest high heterosis [14]. In the present study hybrids developed from parents with low or moderate similarity coefficient *viz*. CMS 234B (line), EC 623023, R16 (testers) also manifested high magnitude of heterobeltiosis and economic heterosis (Table 2).

Ghany [15] from a study on molecular markers analysis revealed that the genetic similarity ranged from 9% to 77% for the RAPD. Earlier, Iqbal [16] through the estimation of the random amplified polymorphic DNA method observed the maximum similarity of 77.78% between R-SIN-82 and RN-46. The lowest similarity, 51.59%, was observed between the exotic lines CM-612 and HA-27 of sunflower.

Cluster tree analysis

The average linkage between *Helianthus annus* genotypes was used for constructing phylogenetic tree depicting the relationship among the 14 *Helianthus annus* parents used.

RAPD marker based cluster tree analysis

The RAPD cluster tree analysis of 14 *Helianthus annus* genotypes showed that they were mainly divided into two major clusters at a 50% similarity (Figure 2). Genotype 6D-1R was out-grouped from all other genotypes at 50% similarity and formed the first cluster. At a 64% similarity was the second cluster having all remaining genotypes. Genotype R 16 was again out-grouped and formed another solitary cluster at 64% similarity. The second sub cluster of cluster II consisted RHA 1-1, ARM 249B, R 271-1. The sub cluster of cluster II was further divided into two groups - group I and group II. Group I consisted of 2 genotypes namely EC 623023 and EC 601957, whereas group II consisted of rest of the 7 genotypes namely EC 623016, 234B, PET 2-7-1B, PET 89-1B, EC 601957, RHA 138-2 and IC 294064.

The cluster tree again revealed similar results based over similarity matrices. The association amongst different genotypes is presented in the form of dendogram, the genotypes which lay nearer to each other in dendogram were more similar to one another than those laying apart. The dendogram also showed the relative magnitude of resemblance among different genotypes of *Helianthus annus* used in current investigation. Mostafa and Altrmawy [17] and Issacs [19] also used RAPD marker to evaluate genetic relationship in sunflower.

Table 3: DNA amplification profile and polymorphism generated in sunflower using RAPD markers

S. N.	RAPD Marker	Size range (bp)	Total no. of Bands	Monomorphic bands	Polymorphic bands	% polymorphism
1.	OPA-5	300-1500	84	19	65	77.38
2.	OPA-9	300-1500	70	45	25	35.71
3.	OPD-5	300-1500	98	41	57	58.16
4.	OPG-9	300-1500	45	9	36	80.00
5.	OPQ-9	300-1500	70	24	46	65.71
6.	OPW-6	300-1500	42	19	23	54.76
7.	OPW-9	300-1500	42	20	22	52.38
8.	OPW-4	300-1500	42	10	32	76.19
9.	OPW-15	300-1500	38	16	22	57.89
10.	OPA-4	300-1500	56	50	6	10.71
Mean	Total		587	253	334	56.88

Table 4: Jaccards similarity coefficient for RAPD profile generated by Agarose gel electrophoresis

Genotypes	6D-1R	R-16	EC 623016	RHA1-1	RHA 138-2	R-271-1	CMS 234B	IC 294064	EC 623023	PET 89-1B	PET 2-7-1B	ARM 249B	EC 601957	EC 601924
6D-1R	1													
R-16	0.74	1												
EC 623016	0.82	0.71	1											
RHA1-1	0.64	0.58	0.71	1										
RHA138-2	0.84	0.74	0.87	0.74	1									
R-271-1	0.69	0.58	0.71	0.74	0.74	1								
CMS234B	0.82	0.61	0.89	0.71	0.82	0.71	1							
IC 294064	0.76	0.66	0.84	0.76	0.87	0.71	0.84	1						
EC 623023	0.46	0.61	0.48	0.41	0.56	0.41	0.48	0.53	1					
PET 89-1B	0.84	0.64	0.87	0.69	0.74	0.74	0.87	0.76	0.46	1				
PET 2-7-1B	0.84	0.64	0.92	0.69	0.84	0.74	0.97	0.87	0.51	0.89	1			
ARM 249B	0.71	0.56	0.79	0.76	0.76	0.71	0.79	0.74	0.53	0.82	0.82	1		
EC 601957	0.76	0.61	0.89	0.61	0.82	0.61	0.84	0.79	0.58	0.76	0.87	0.74	1	
EC 601924	0.82	0.61	0.84	0.66	0.82	0.61	0.79	0.79	0.43	0.76	0.82	0.64	0.79	1

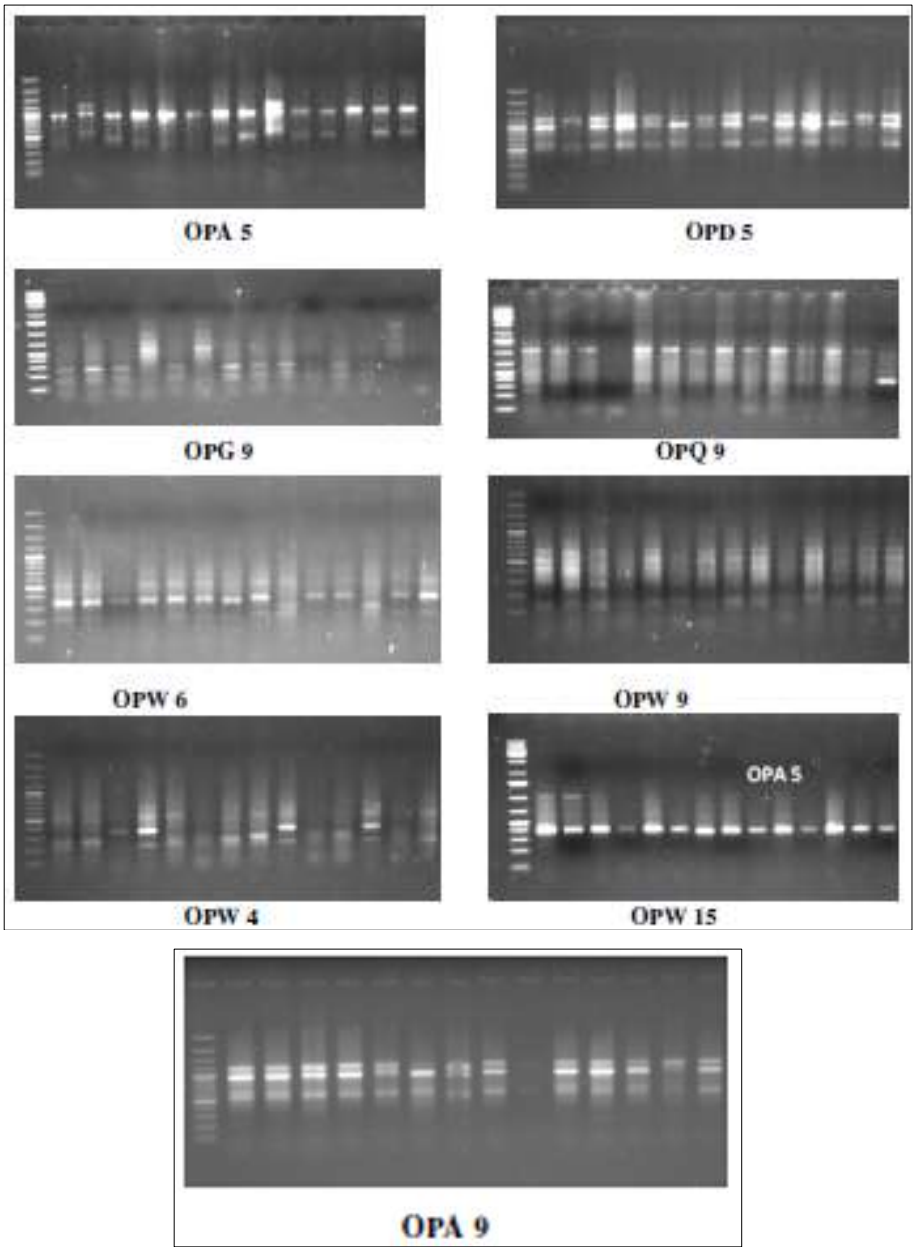

Fig 1: Polymorphism resultant from the use of RAPD primers for *H. annus* genotype

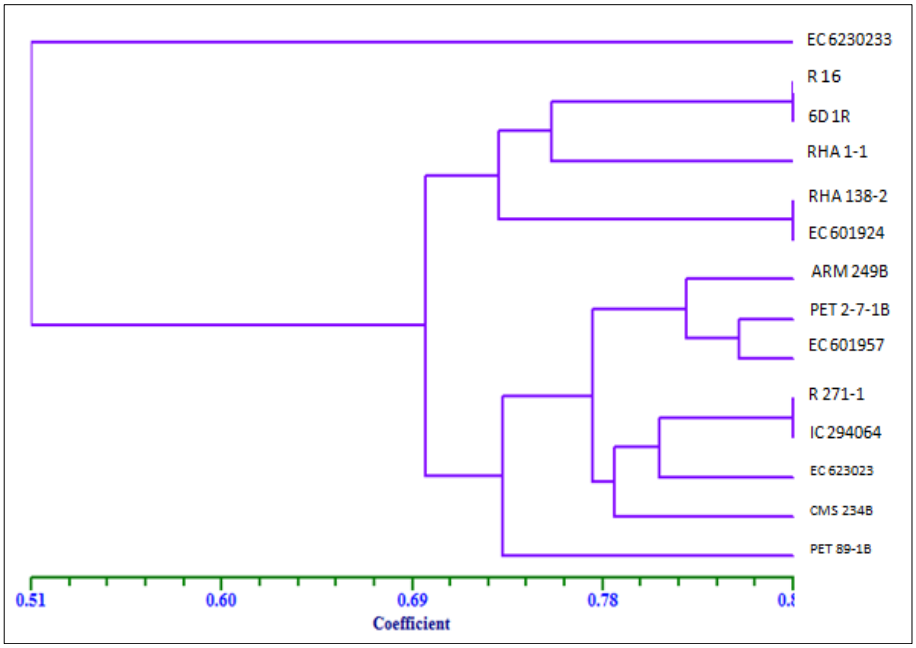

Fig 2: Dendogram constructed with UPGMA clustering method among 14 genotypes of sunflower using RAPD primers

Conclusion

PCR amplifications using 10 RAPD primers resulted in an average of 58.7 selected markers per primer, with amplification products ranging from 38 to 98. Among the primers, OPD 05 produced the highest number of fragments (98), while OPW 15 yielded the least (38). In total, 587 amplicons were generated, averaging 58.7 amplicons per primer. The RAPD similarity matrix showed similarity coefficients ranging from 0.44 to 0.97, indicating genetic diversity ranging from 3% to 56% among the 14 Helianthus annuus genotypes studied. The average similarity coefficient of 0.73 suggested moderate genetic similarity across all genotypes. Genotypes EC 623023 and EC 601957 exhibited the lowest similarity coefficient of 0.44, indicating their genetic distinctiveness. Cluster analysis grouped the genotypes into two major clusters, highlighting genetic relationships and potential for heterosis exploitation in sunflower breeding programs.

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