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Study of genetic diversity using molecular markers in sunflower (*Helianthus annuus*)

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ABSTRACT

Considering the significance of sunflower (*Helianthus annuus* L.) cultivation under diverse agro-climatic conditions, present study was done to recognize assorted parental lines utilizing RAPD and ISSR markers to lessen environmental effects or test blunders at Rajasthan College of Agriculture, MPUA & T, Udaipur, Rajasthan during 2016. In the present study, RAPD primers produced 334 polymorphic band and ISSR primers produced 160 polymorphic bands. RAPD primers detected more polymorphic loci (56.88%) than the ISSR primers (52.24%). The dendrograms constructed by RAPD, ISSR primers and pooled data were associated with each other, indicating the efficacy of both marker systems in genetic diversity study of sunflower. Based on RAPD, ISSR and pooled data analysis, the restorer line EC 623023, R 16 and seed parental line CMS 234B, PET 2-7-1B were found to be genetically most diverse and fall into different groups. Therefore, their genetic origin seems to be different and could be efficiently used in sunflower breeding programs.

Keywords: Genetic diversity, ISSR, RAPD, Sunflower

The significance of sunflower (*Helianthus annuus* L.) as an oilseed crop in India is exceptionally recent and goes back to three decades. In any case, its commitment to self-sufficiency in consumable oil as well as to the "yellow revolution" in the country is remarkable (Rai 2002). In India, Seetharama (1981) developed the first sunflower hybrid by the use of well-defined CMS system which has generated a vast interest in the crop. In India, sunflower is cultivated over an area of 4.006 lakh ha with a production of 2.840 lakh tonnes and productivity of 709 kg/ha (DES 2018). With the expanding interest and demand for consumable oils, there is a need to develop new sunflower hybrids with an extensive level of heterosis for economic characteristics, which are likewise reasonable for various agro-climatic zones of India. Area under sunflower crop is negligible in Rajasthan state. However, there is a vast scope to bring the area under cultivation of sunflower in Rajasthan since agriculture in the state is primarily rainfed, receives much less annual rainfall and frequency of periodic drought is high.

Hybrids developed from genetically diverse parental lines are more heterotic and stable, diversity based on phenotypic values may not be the perfect representation of natural grouping of cultivars. Therefore, precisely diverse parental lines can be identified by employing suitable molecular markers thereby reducing environmental effects or experimental errors. As of late, impressive advancement has been made in the use of molecular markers for the characterization and assessment of plant genetic resources. Among a few productive techniques for uncovering genetic distinctness within and among plant populations, the absolute most ordinarily utilized strategies are RAPD and ISSR (Dudhe *et al.* 2019 and Maloo *et al.* 2020). Specifically, RAPD and ISSR are simpler to use than the other molecular marker systems, in light of the fact that in both of these techniques, earlier information on the target sequences is not needed (Reddy *et al.* 2002). Looking for the importance of sunflower crop under rainfed/famine situations, the present study was carried-out by crossing four diverse CMS lines with 10 testers.

MATERIALS AND METHODS

Fourteen phenotypically diverse parental lines for seed yield, oil content and maturity comprised 4 maintainers and 10 restores of this species having indigenous and exotic origin were selected for the present study. All the 14 genotypes of sunflower collected from NBPGR, New delhi, IIOR, Hyderabad, UAS, Bangalore and ORS, Latur (Maharashtra) were raised in pot experiment during 2016 at Rajasthan College of Agriculture, Udaipur.

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Table 1 Details of RAPD and ISSR primers used in molecular analysis of sunflower genotypes

Primers	Sequence (5'-3')	G:C content (%)
RAPD primers		
OPD-2	GGACCCAACC	70
OPM-13	GGTGGTCAAG	60
OPA-04	AAT CGG GCT G	60
OPA-05	AGG GGT CTT G	60
OPA-06	GGT CCC TGA C	70
OPA-09	GGG TAACGC C	70
OPT-10	GGCAGGCAGA	70
OPS-02	CCAAGTTCGC	60
OPW-04	CAGAAGCGGA	60
OPW-06	AGGCCCGATG	70
OPW-09	GTGACCGAGT	60
OPW-10	TCGCATCCCT	60
OPW-15	ACACCGGAAC	60
OPQ-09	GAACGGACTC	60
OPG-02	GGCACTGAGG	70
OPG-04	AGCGTGTCTG	60
OPG-09	CTGACGTCAC	60
OPH-20	GGGAGACATC	60
OPJ-06	TCGTTCCGCA	60
OPR-04	CCCGTAGCAC	80
ISSR primers		
UBC-820	GTGTGTGTGTGTGTGTC	17
UBC-810	GAGAGAGAGAGAGAGAT	17
UBC-807	AGAGAGAGAGAGAGAGT	17
UBC-836	AGAGAGAGAGAGAGAGYA	18
UBC-849	GTGTGTGTGTGTGTGTYA	18
UBC-873	GACAGACAGACAGACA	16
UBC-880	GGAGAGGAGAGGAGA	15
UBC-808	AGAGAGAGAGAGAGAGC	17
UBC-881	GGGTGGBGGTGGGGTG	16
UBC-845	CTCTCTCTCTCTCTCTRC	18
UBC-840	GAGAGAGAGAGAGAGAYC	18
IS-07	CACACACACACACAGT	16
HB-08	GAGAGAGAGAGAGG	14
UBC-827	TGTGTGTGTGTGTGTGA	17
HB-09	GTGTGTGTGTGTGG	14

Molecular marker evaluation: Genomic DNA of 14 genotypes of sunflower was extracted from fresh and young leaves employing 2% CTAB (Cetyl Trimethyl Ammonium Bromide) protocol of Saghai-Maroof *et al.* (1984) with slight alterations. RNase treatment was given to isolated genomic DNA for further purification to eliminate the RNA contamination. After purification, DNA quantification was done by 0.8% agarose gel with λ DNA (50 ng/µl) as standard. Intensity of the band of genomic DNA was compared with lambda (λ) for each diluted sample (20-25ng/µl). For DNA amplification of all 14 genotypes, a set of 20 random 10-mer RAPD and 15 ISSR primers were used (Table 2). PCR amplification was performed in a 25µl reaction volume having 20 ng genomic DNA, 1X PCR buffer, 1.5 mM $MgCl_2$, 0.25 mM dNTP mix, 1.5 U Taq polymerase (Sigma Chem. Co. USA) and 25ng primer (Operon Technologies Inc., USA) in a thermal cycler (Bio Rad T100, Thermal Cycler). After an initial denaturation cycle of five minutes at 94°C, PCR was programmed at 40 and 35 cycles for RAPD and ISSR primers, respectively. Each cycle comprised a denaturation step of four minutes at 94°C, one minute of variable annealing temperature (37^0C for RAPD and 27.0^0C - 47.4^0C for ISSR), and two minutes of extension step at 72°C, followed by five minutes of final extension at 72°C. The annealing temperature ranged from 27.0^0C to 47.4^0C for ISSR primers, as it is designed to anneal to a micro-satellite sequence, is longer than RAPD primers, and ISSR markers are therefore more reproducible than RAPD markers (Goulao and Oliveira 2001).

The amplified products were resolved on 2% agarose gel. The DNA ladder (100 bp) was also loaded in the gels for estimating the appropriate band size of amplified products. Gel was imaged under UV-light and recorded in the program for gel documentation (UPV, GelDoc-It Imaging System). Gels were scored for absence and presence of band as zero and one respectively in each genotype. To determine the genetic similarities and number of clusters among the sunflower genotypes, the RAPD and ISSR primer data was analyzed by NTSYSpc version 2.02 (Rohlf 1998). Based on Jaccard's similarity coefficient, a dendrogram was generated using Un-weighted Pair Group Method with Arithmetic Mean (UPGMA) for each primer.

RESULTS AND DISCUSSION

RAPD Polymorphism: The amplification products in terms of the percentage of PCR products, as generated by the RAPD primers are listed in Table 1. Primer, OPD 05 produced the highest polymorphism among the all markers in the genotypes studied. Ten out of 20 selected RAPD primers were found polymorphic thus produced large number of bands in 14 sunflower genotypes under evaluation. A total of 587 distinct, scorable bands were produced by 10 polymorphic RAPD primers with an average of 58.7 amplicons/primers. However, 334 out the total of 587 bands were polymorphic. The highest number of fragments (98 amplicons) were generated by the primer OPD 05, whereas, OPW 15 produced lowest number (38 amplicons). A high variation in the number of fragments could be attributed to the distinctions in the binding sites all through the genome of the included genotypes. While evaluating Indian bitter gourd (*Momordica charantia* L.) Behera *et al.* (2008) also reported such a high variation. In all the 14 genotypes average polymorphism was found to be 56.88% with 33.4 polymorphic amplicons for each of the 10 primers (Table 2).

Table 2 DNA amplification profile and polymorphism generated in sunflower using RAPD and ISSR markers

Marker	Size range (bp)	Total no. of bands	Monomorphic bands	Polymorphic bands	% polymorphism
RAPD markers					
OPA-5	300-1500	84	19	65	77.38
OPA-9	300-1500	70	45	25	35.71
OPD-5	300-1500	98	41	57	58.16
OPG-9	300-1500	45	9	36	80.00
OPQ-9	300-1500	70	24	46	65.71
OPW-6	300-1500	42	19	23	54.76
OPW-9	300-1500	42	20	22	52.38
OPW-4	300-1500	42	10	32	76.19
OPW-15	300-1500	38	16	22	57.89
OPA-4	300-1500	56	50	6	10.71
Total		587	253	334	56.88
ISSR markers					
UBC-820 di with 1	300-1500	55	28	27	49.09
UBC-810	300-1500	52	35	17	32.69
UBC-807	300-1500	37	28	9	24.32
UBC-836 di with 2	300-1500	46	7	39	84.78
UBC-849	300-1500	53	11	25	47.16
UBC-873 quadra	300-1500	57	19	43	75.43
Total		300	128	160	52.245

ISSR Polymorphism: In the present study, a total of 15 ISSR primers (Table 1) were investigated out of which 6 ISSR primers namely UBC-807, UBC-810, UBC-820, UBC-836, UBC-849 and UBC-873 showed positive results. A total of 160 polymorphic bands out of 300 amplified bands were obtained from 6 ISSR primers used. Primer UBC 873 (57) produced highest number of polymorphic bands, whereas UBC 807 (37) produced least (Table 2). Percent polymorphism ranged from 24.32% (UBC 807) to 84.78% (UBC 836) with an average of 52.24%. The size of PCR amplified products ranged from 300 bp – 1500 bp. Mahmoud and Abdel-Fatah (2012) and Garayalde (2011) additionally noticed comparative outcomes for (ISSR) markers in sunflower.

RAPD based cluster analysis: A dendrogram was constructed based on UPGMA cluster analysis using Jaccard's similarity coefficient matrix (RAPD) that grouped all 14 genotypes of sunflower into two main groups, viz. C-I and C-II at similarity coefficient of 0.51. Earlier, Ghany (2012) and Iqbal *et al.* (2008) observed the maximum similarity of 77.78% and the lowest similarity of 51.59% in sunflower. C-I comprised only one genotype (EC 623023). At similarity coefficient of 0.69, C-II divided into two subgroups, i.e. C-IIa and C-IIb. Subgroup C-IIa includes five genotypes, of which four were promising restorer lines (R 16, 6 D-1R, RHA 1-1 and RHA 138-2) and eight genotypes in subgroup C-IIb which were further subdivided into two subgroups at similarity coefficient of 0.74. Subgroup C-IIb1 contained seven genotypes including promising seed parental lines, viz. CMS 234B and ARM 249B, whereas C-IIb2 had only one genotype (PET 89-1B). The promising seed parental lines, viz. 234B, ARM 249B, PET 2-7-1B and restores, viz. R 16, EC 623023 and RHA 138-2 falls in distinct clusters owing to their genetic dissimilarities which in turn may result in the development of heterotic hybrids (Isaacs *et al.* 2003). The genotypes that were nearer to one another in the dendrogram were more like each other than those that were separated. The genetic distance between the studied genotypes is illustrated by dendrogram. On the basis of genetic distance, genotypes EC 623023, EC 601924, PET 89-1B, RHA 138-2 and RHA 1-1 were found the most diverse genotypes. Mostafa and Alfrmawy (2011) likewise utilized RAPD markers to survey hereditary relatedness in sunflower. The hybrids developed from CMS 234B with restorer EC 623023 and R16 were identified as highly heterotic and stable hybrids based on seed yield in consort with stability of component traits.

ISSR based cluster analysis: Jaccard's similarity coefficient values for 14 genotypes of sunflower were calculated which revealed the range of genetic similarity from a minimum of 0.50 to a maximum value of 0.90 with an average of 0.73. The dendrogram constructed by UPGMA cluster analysis of the data produced by ISSR primers, showed allocation of all the 14 genotypes in the two major groups with a similarity coefficient of 0.50. Likewise, RAPD dendrogram, C-I group comprised genotype EC 623023. The genotype EC 623023 was found to be the most diverse as it did not group with any other genotypes.

This indicates that their hereditary origin is by all accounts unique and could be proficiently utilized in superior hybrid development programs. Yang (2012) reported high hereditary distinctness in sunflower. The second major group (C-II) comprised rest of 13 genotypes, which was further divided into two subgroups with a similarity coefficient of 0.64. The promising restorer line R16 located in group C-IIb. The subgroup C-IIa further subdivided into two subgroups at similarity coefficient of 0.71. The genotypes RHA 1-1, ARM 249B and R 271-1 were grouped into subgroup C-IIa2. Of the three genotypes, two were promising restorer lines. Once again, the restorer line 6D-1R and EC 601924, located at separate place in subgroup C-IIa1. However, the promising seed parents namely, CMS 234B, PET 2-7-1B and PET 89-1B were found at separate place in subgroup C-IIa1. The highest similarity was observed between the genotype CMS 234B and 2-7-1B. The genotypes EC 623023, R16 and CMS 234B had high mean values for seed yield, and oil content with good GCA effect, and consequently could be further exploited. The vast majority of the crossing of these parental lines indicated significantly higher SCA effects, heterosis and high seed yield performance. Rest of the genotypes may have some hereditary/phylogenic relationship because of migration/inflow of genes.

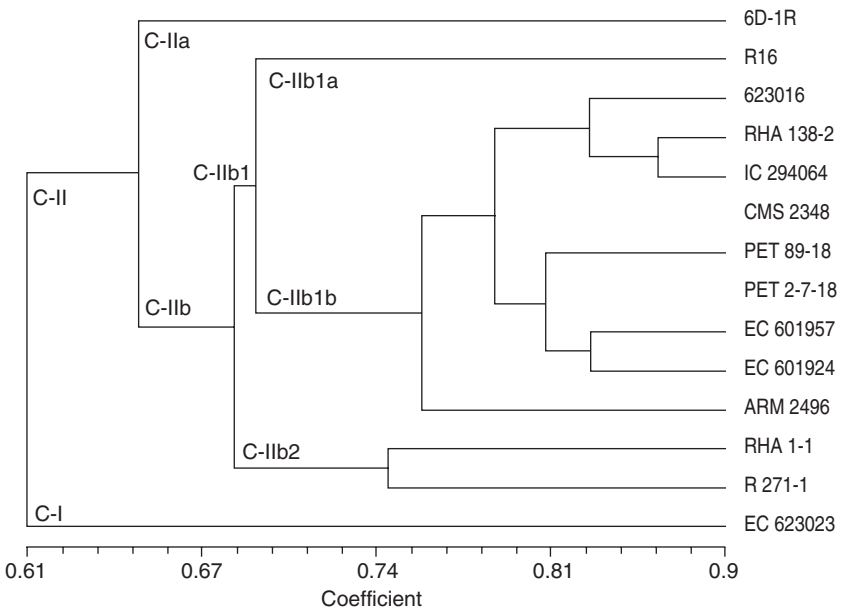

Fig 1 UPGMA cluster analysis based on pooled data (RAPD and ISSR) showing genetic relationship among sunflower genotypes.

RAPD and ISSR based cluster analysis (Pooled): A dendrogram based on pooled data of both markers, i.e. RAPD and ISSR, exhibited somewhat similar grouping pattern when used independently (Fig 1). Hence, both the marker systems either independently or collectively can be effectively used in determination of genetic relationships among sunflower genotypes. Similarity coefficient based on pooled data varied from 0.61 to 0.90. The dendrogram based on both marker system, grouped all the 14 genotypes into two distinct groups. In pooled analysis, once again the genotypes EC 623023 remained distinct (C-I) supporting individual result obtained by RAPD and ISSR marker system, indicates the efficacy of both marker system. The second group (C-II) was further divided into two subgroups. The promising restorer lines, viz. 6D-1R (C-Ia), R16 (C-IIb1a), RHA 1-1 (C-IIb2) and R 271-1 (C-IIb2) once again found a distinct place in dendrogram, demonstrating the more resemblance in grouping pattern with ISSR marker system than RAPD. Though, the construction of subgroups within the group varied among RAPD and ISSR. This indicates the ISSR marker system would be more informative than RAPD. Similarly, Galvan *et al.* (2003) reported that ISSR would be a better tool than RAPD for phylogenetic studies. The genetic similarity between promising seed parents namely, CMS 234B, PET 89-1B and PET 2-7-1B with both marker system could be described by the high degree of similarity coefficient (0.90) in these genotypes. This showed that ISSR data was closer to combined data. Further crossing of these parental lines with diverse restorer line may produce highly heterotic hybrids of sunflower. Several workers, viz. Verma *et al.* (2017), Dhudhe *et al.* (2019) and Maloo *et al.* (2020) applied combined RAPD and ISSR techniques for studying genetic divergence in field crops. The work on development and evaluation of new inbreds for their restorer, maintainer behavior, with an object to identify diverse maintainers or restorers in sunflower is going on in India. The identified diverse parental lines are being used to develop superior hybrids (Dudhe *et al.* 2019).

Conclusively, the restorer line EC 623023, R 16 and seed parental line CMS 234B, PET 2-7-1B were found to be genetically most diverse and fall into different groups. Therefore, their genetic origin seems to be different and could be efficiently used in sunflower breeding programs. Present study has shown the efficacy of RAPD and ISSR marker system in diversity analysis of sunflower genotypes. In view of this, we studied combining ability, heterosis, stability analysis for seed yield and its component traits in 3 environments (non-traditional areas in Rajasthan state of India) using 4 lines and 10 testers with different genetic background. It was observed that these genotypes per se possessed high performance for seed yield and its component traits with significant GCA effects. Most of the hybrids of these parental lines showed significant SCA effects, heterosis and high per se performance.

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