Electronic Journal of Plant Breeding, 7(4): 1216-1223 (December 2016)
ISSN 0975-928X
DOI: 10.5958/0975-928X.2016.00169.1

Research Note

Morphological and molecular genetic diversity analyses in *Helianthus annuus* (L.)

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(Received: 16 Feb 2016; Accepted: 12 Dec 2016)

Abstract
The knowledge of genetic diversity among parental lines is a prerequisite for selecting parents for hybridization in any heterosis breeding programme. Evaluation of 13 parental lines using D^2 analysis was undertaken during spring 2014 at PAU, Ludhiana. The genotypes were grouped into four clusters. Cluster II comprised of maximum number of genotypes (5) *viz.*, *CMS*-47A, P 93R, P145R, P103R, P 167R followed by cluster I (4 genotypes i.e. *CMS*-11A, *CMS*-67A, *CMS*-234A, 95-C-1); cluster III and IV having two genotypes each *i.e. CMS*-68A, RCR 8297 and P 124R, P 134R respectively. Maximum inter cluster distance of 578.187 was recorded between genotypes of cluster II and IV while minimum inter cluster distance (175.195) was observed between genotypes present in cluster III and IV. Thirty SSR markers were used for molecular markers analysis. Total number of alleles amplified by 26 polymorphic primers was 51 with an average of 1.96 alleles per locus. The average value of polymorphic information content (PIC) for all the 26 polymorphic markers was 0.47. A polygenetic tree generated, based on Dice dissimilarity matrix and cluster analysis, led to the clustering of the sunflower genotypes into two major groups (one group comprising *R* lines while other comprising *A* lines) while one genotype (CMS 234A) formed separate independent cluster. The result of dissimilarity matrix revealed high genetic diversity among all the inbreds lines. Overall, the values for genetic distances ranged from 0.13 to 0.71. The highest genetic distance (0.71) was observed between the genotypes i.e. CMS 67A and P 93R followed by 0.70 for CMS 67A and P124R. Minimum genetic distance 0.13 was observed between P 103R and P 134R, P 134R and P 145R which was indicative of common parentage of these genotypes in the pedigree tree. The principle coordinated analysis along with pattern of clustering of dissimilarity matrix separated the lines into two groups; one having B-lines and other for *R*-lines. The analysis of molecular variance (AMOVA) showed greater variation within genotypes (68%) as compared to between genotypes (32%).

Keywords
Sunflower, inbreds, SSR, morphological diversity, genetic diversity

Sunflower (*Helianthus annuus* L.) one of the important oilseed crops (Leclereq, 1969), is a model system for the genomic studies of family Asteraceae (Paniego *et al* 1999). Genetic diversity analysis in breeding material is of paramount importance because crosses attempted between lines of diverse origin generally display greater heterosis and generate more useful recombinants than crosses made between closely related lines. Genetic diversity between populations indicates the differences in gene frequencies. In addition to estimates of variability, knowledge of genetic diversity among genotypes is essential for selecting diverse parents for hybridization programme. The knowledge of genetic diversity allows the plant breeder to better understand the evolutionary relationship among the inbred lines. Future breeding program is dependent on the availability of genetic variability to increase the productivity. Genetic diversity information also permits the classification of germplasm into heterotic groups, which is important for hybrid breeding program. Even though the genetic diversity mechanisms that explain heterosis are not fully understood, it is well documented that crosses between unrelated and consequently genetically distant parents shows greater hybrid vigor than crosses attempted between closely related parents (Stuber, 1994).

In the past decades different morphological and molecular markers have been used for estimation of genetic diversity. Traditionally, assessment of genetic diversity has been based on the differences in morphological and agronomic traits or pedigree information on different crops. Diversity analysis based on morphological data gives actual performance of the germplasm, however, it may fluctuate with the environment while, molecular analysis are stable and not affected by the environmental conditions. The genetic variation in sunflower germplasm and the identification of heterotic groups based on both morphological traits (Kholghi *et al.*, 2011) and molecular markers such as SSR (Darvishzadeh *et al.*, 2010), ISSR (Garayalde *et al.*, 2011) and AFLP (Hongtrakul *et al*., 1997) has been carried out. Genetic variability among the cultivated sunflower has been reported to be low.

 DOI: 10.5958/0975-928X.2016.00169.1

The present study was planned to assess the genetic diversity of different genotypes of sunflower using both morpo-physiogical traits and SSR markers. The main objective of this study was identification of the most diverse lines from the available germplasm. The highly diverse lines selected as a result of this study will be used in future breeding programs to exploit their heterotic potential.

Field experiment: The materials for the present study consisted of 13 sunflower genotypes developed at PAU, Ludhiana comprising eight *B* lines and five *R* lines selected for development of experimental hybrids. All the 13 lines were evaluated in a Randomized Block Design with three replications in field area of the oilseeds section, Punjab Agricultural University, Ludhiana during spring 2014. Each genotype was sown in two rows of four meter length, with inter and intra row spacing of 60 cm and 30 cm, respectively. Two to three treated seeds with Imidacloprid (Gauch) @ 5 g/kg of seeds were dibbled per hill in the rows, after applying half the recommended N and entire dose of P and K as basal. The remaining N was top dressed followed by interculture, when the crop was around 35 days old. Thinning was done to maintain one healthy plant per hill attended 15 days after complete emergence of the experimental crop. All the recommended agronomic practices for Punjab state were followed to raise a successful crop. The observations from all genotypes were recorded from ten random but competitive plants for 19 characters viz., days to emergence, flower initiation, days to 50 per cent flowering, days to maturity, plant height (cm), head diameter (cm), stem girth (cm), number of leaves per plant, chlorophyll content (SPAD), internodal distance (cm), petiole length (cm), number of filled seeds per head, seed yield per plant (g), test weight (g/100 seeds), volume weight (g/ 100ml), hull content (%) and oil content (%). After computing means, the data were subjected to Mahalanobis (1936) D^2 statistics as described by Rao (1952). The genotypes were grouped into different clusters according to Toucher's method (Rao, 1952) while inter and intra cluster distances were calculated as per Singh and Chaudhary (1977).

DNA extraction and PCR procedures: DNA extraction and polymerase chain reaction (PCR). DNA sample was extracted by using modified CTAB method by Doyle and Doyle (1987) from the lyophilized tissues of 15 days older sunflower young leaves. The quantification of the DNA was done using a Nanodrop ND-1000 Spectrophotometer. The DNA samples were diluted to a working concentration of 10 ng/µL. A set of 30 SSR primers (selected from NCBI and published source table 2) were used for the finger printing of collected leaf sample of 13 inbred lines. Each PCR amplifications was performed in 20µl solution containing 2.5µM of each SSR primer, 0.4 Units *Taq* DNA polymerase (SRL Technologies), 100µM of each dNTP (Promega), 2µl 10X PCR buffer, 2.5 mM $MgCl_2$ (Promega), 0.20µl of stabilizer (1% W-1 (v/v), SRL Technologies), ddH_2O and 25 ng template DNA in a 96-well Eppendorf Mastercycler Gradient (Type 5331, Eppendorf AG, Hamburg, Germany) (Darvishzadeh *et al* 2010). Thermocycler programmed for an initial denatuaration at 95^0 C 3 min, 10 cycles of 1 min denaturation at 94^0 C, 1 min annealing at 35^0 C and 2 min extension at 72^0 C followed by final extension for 10 min at 72^0 C.

The reaction products were then mixed with an equal volume of formamide dye [98% (v/v) formamide, 10 mM EDTA, bromophenol blue and xylene cyanol] and resolved in a 2.5% (w/v) agarose gel in 0.5X TBE, stained with ethidium bromide (1.0 (gml-1) and photographed under UV light.

Statistical analysis: The PCR amplification products were scored for the presence (1) or absence (0) of each marker band across all 13 genotypes and the data used to construct a binary data matrix (Mohammadi, 2006). This was further used to measure the genetic diversity and distance between different genotypes. For each primer pair the total number of polymorphic alleles and polymorphism information content (PIC) were calculated for all the accessions. The software package DARwin5 [Perrier and Jacquemond 2006] was used or estimation of genetic diversity of the sunflower genotypes which has been assessed by clustering analysis in which a Dice similarity matrix was generated using SSR data and dendrogram was constructed using unweighted neighbor joining method (UPGMA). Booststrap analysis was performed to test the robustness of the tree with 1000 bootstraps.

Euclidean distance graph (Fig.1) based on Mahalanobis D^2 statistics grouped the genotypes into four clusters. The critical examination of clusters indicated the presence of high level of genetic diversity in the material. The distribution of these parental lines in each of the four clusters is presented in table 1.

Cluster composition: Cluster II comprised of maximum number of genotypes (5), followed by cluster I (4), cluster III and cluster IV each having two genotypes (Table 1, Fig. 1). Similar clustering pattern of genotypes among clusters (clusters having only one or two genotypes) has also been observed by Shamshad *et al* (2014). The genotypes grouped within a cluster exhibit a narrow range of genetic variability, whereas those grouped in different clusters represent wider variability. Depending on their inter cluster distances the

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genotypes included in the different clusters are considered genetically dissimilar with respect to the aggregate effect of the characters examined; the hybridization attempted between these parental lines is expected to yield higher and desirable recombinants. Therefore, putative parents for crossing programme should belong to different clusters characterized by large inter-cluster distance. Taking this into consideration it was observed that the genotypes belonging to cluster II had maximum Euclidean distance value of 578.187 from the genotypes belonging to cluster IV, for other genotypes this distance varied from 404.297 (II and III) to 192.708 (IV and III). The study indicates a lot of diversity between these parental lines which can be exploited in hybrids based on the contribution of different character towards diversity. Based on these observations the genotypes CMS-47A, P 93R, P145R, P103R, P 167R of cluster II and P 124R, P 134R of cluster IV are expected to give maximum heterotic combinations for various traits upon hybridization.

Mean values of clusters: The cluster II with the largest number of genotypes was characterized by the lowest mean value for days to flower initiation (72.47), days to maturity (101.00) and highest mean value for head diameter (15.55). The lowest average for plant height, highest average harvest index (6.78) and test weight (3.10) were recorded by cluster III and IV comprising two genotypes each. Minimum plant height (128.27), maximum number of filled seeds (918.83) and high harvest index (6.78) was the characteristic feature of cluster IV (P 124R, P 134R). Members of cluster I were recorded best w.r.t. number of leaves per plant, photosynthetic efficiency and oil content. Plant height was highest (143.27 cm) for cluster I and lowest (18.96 g) for cluster IV. Maximum (5.34 g) and minimum mean value (3.01 g) for test weight was observed for cluster II and cluster III respectively. For oil content, cluster II and cluster I recorded lowest (32.58%) and highest (37.20%) mean values respectively. For, important economic trait like seeds yield (g/plant) and number of filled seed per head, highest value was recorded for cluster IV (34.63g, 918.83), while these were lowest (18.96 g, 341.33) for cluster II. The genotypes in cluster II were early in flowering, moderate in yield potential with high head diameter, plant height, test weight and average in hull content (Table 3), whereas, genotypes in cluster IV were high yielding with good oil content. This indicates that, most of the genotypes from these two clusters are likely to produce the recombinants combining high yield potential with desirable yield contributing character. Cluster mean analysis is indicative of extent of diversity among different clusters which can be of practical value in sunflower breeding as earlier discussed by Mohan and Seetharam (2005) and Arshad *et al* (2007). Thus, the *CMS* and restorer analogues with outstanding mean performance from these clusters may be identified as potential parents and could be utilized in the synthesizing new hybrids.

Molecular characterization of genotypes using SSR markers: A total of 30 SSR primer pairs were used for the estimation of genetic diversity among 13 parental lines (five male sterile line and eight restorer lines). Out of which only 26 primers were found to be polymorphic (Table 4). Total numbers of alleles amplified by 26 polymorphic primers were 51 with an average of 1.96 alleles per locus. The polymorphic banding pattern of locus is presented in Fig 2. These results were closer to those of Darvishzadeh *et al* (2010). As sunflower is a highly cross pollinated crop therefore, a high number of alleles per locus could be a result of the natural out-crossing among the parental material and also due to having a broad genetic base. The number of amplified products varied from two (ORS 58, ORS 488, HA 1604) to three (ORS718 and ORS423). The results depicted that total polymorphism was 75.19% and it ranged from 25% to 100%. The discrimination power of each SSR markers was estimated by PIC. PIC values (expected heterozygosity) for polymorphic primers ranged from 0.07 (ORS718) to 0.89 (ORS1265). The average value of polymorphic information content (PIC) for all the 26 polymorphic was 0.47. The PIC values estimate the discriminatory power of a marker and is defined as the probability that given marker genotypes of an affected parent's offspring will permit the deduction of the parent genotype at the marker locus (Botstein *et al* 1980). This average PIC value was slightly lesser than that reported by Gedil (1999) and Yu *et al* (2002) with a PIC score of 0.49 and 0.55 respectively for polymorphic SSR markers in sunflower.

Genetic dissimilarity: The results of dismilarity matrix revealed a high genetic diversity among all the parental lines. Overall, the values for genetic distances ranged from 0.13 to 0.71. The highest genetic distance (0.71) was observed between the genotypes i.e. *CMS* 67A and P 93R followed by 0.70 among genotypes *CMS* 67A and P 124R. Minimum genetic distance (0.13) was observed between P 103R and P 134R, P 134R and P 145R (table 4.4), which is indicative of common parentage in the pedigree tree.

Cluster Analysis: Unweighted pair-group method using arithmetic averages (UPGMA) cluster analysis was used to construct the dendrogram (Fig. 2). The constructed dendrogram divided 13 genotypes into three main clusters (A, B and C). Cluster A had two sub clusters i.e. 1 and 2, cluster B had one sub clusters i.e. 3, whereas, cluster C

 DOI: 10.5958/0975-928X.2016.00169.1

had one sub cluster i.e. 4. Two lines fell in sub cluster 1 (*CMS* 67A and *CMS* 68A), three in sub cluster 2 (RCR 8297, *CMS* 47A and *CMS* 11A), only one in sub cluster 3 (*CMS* 234A), seven in sub cluster 4 (95-C-1, P 93R, P 124R, P 103R, P 134R and P145R). Overall the genetic distances observed among the accessions were low which shows that the genetic material under study is closely related and similar kind of trend is also reflected by dendrogram (Fig. 2) and the principle coordinate analysis (Fig. 3 & Table 6) The principle coordinate analysis showed no definite clustering among the parental material and hence confirming the results that the lines under study are somewhat closely related and most probably belong to the same geographical region. Further analysis of molecular variance (Table 5) shows a little variation among the sterile lines and restorer lines, hence confirming the results that the lines used in the study are closely related and of same geographical origin. The analysis of molecular variance (AMOVA) shows greater variation amongst maintainer and restorer genotypes (68%) as compared to between these *B* and *R* line genotypes (32%). Therefore these genotypes may be used for developing B × B and R × R gene pools for further derivation of new diverse and improved inbred lines followed by synthesis of new hybrids.

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Electronic Journal of Plant Breeding, 7(4): 1216-1223 (December 2016)
ISSN 0975-928X
DOI: 10.5958/0975-928X.2016.00169.1

Table 1. Grouping of parental lines into clusters on the basis of D^2 analysis for morpho-physiological, yield and quality traits

Cluster No.	No. of genotypes in each cluster	Name of genotypes
Cluster I	4	*CMS*-11A, *CMS*-67A, *CMS*-234A, 95-C-1
Cluster II	5	*CMS*-47A, P 93R, P145R, P103R, P 167R
Cluster III	2	*CMS*-68A, RCR 8297
Cluster IV	2	P 124R, P 134R

Table 2. Inter and intra cluster distance values for 4 clusters formed with morpho-physiological, yield and quality traits

	Cluster I	Cluster II	Cluster III	Cluster IV
Cluster I	**89.755**	192.708	217.728	391.081
Cluster II		**73.146**	404.297	578.187
Cluster III			**58.616**	175.195
Cluster IV				**16.990**

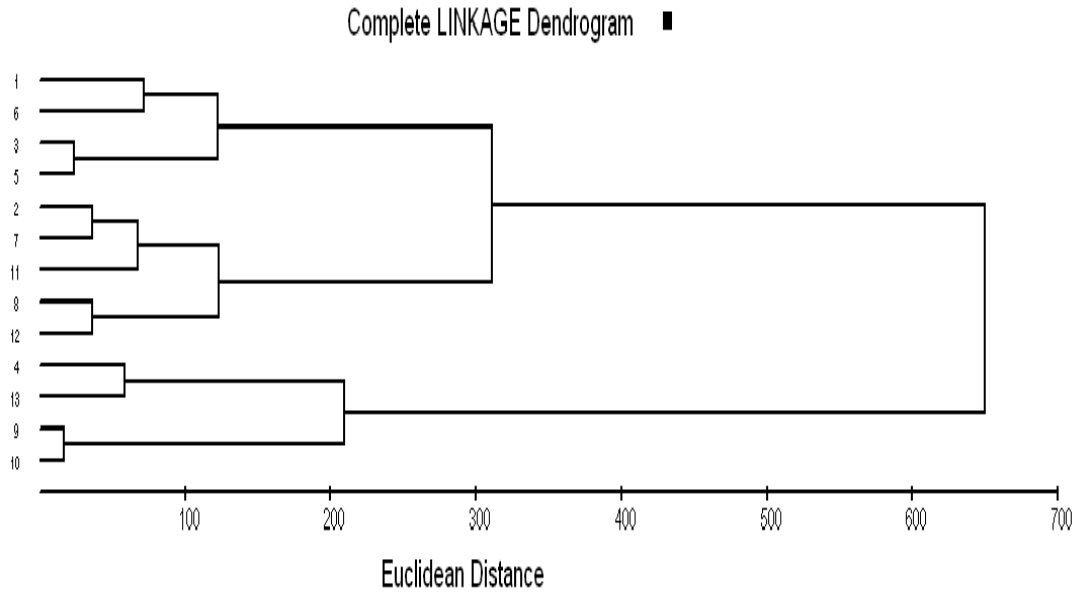

Fig.1 Dendrogram showing cluster formation for 13 genotypes

 DOI: 10.5958/0975-928X.2016.00169.1

Table 3. Mean performance of parental lines with respect to different morpho-physiological and yield traits

	DE	FI	DF	DM	PH	HD	SG	NL	PE	PL
Cluster I	12.83	75.08	77.53	104.83	143.27	14.11	4.84	28.08	33.70	12.00
Cluster II	17.07	72.47	75.33	101.00	134.60	15.55	4.80	25.33	32.29	11.05
Cluster III	15.83	73.00	75.67	104.00	139.20	12.94	4.65	26.83	36.68	9.47
Cluster IV	17.50	73.67	77.17	106.50	128.27	14.82	4.85	26.17	30.75	12.60

	ID	SY	FS	TW	HC	VW	BY	HI	OC
Cluster I	6.13	27.32	529.17	5.05	1.40	35.25	0.50	5.87	37.20
Cluster II	5.39	18.96	341.33	5.34	1.53	29.10	0.47	4.58	32.58
Cluster III	6.02	23.42	744.83	3.10	1.56	36.92	0.37	6.47	36.43
Cluster IV	5.60	34.63	918.83	4.23	1.16	33.00	0.61	6.78	33.16

Days to emergence (DE), Days to flower initiation (FI), Days to 50% flowering (DF), Days to maturity (DM), Plant height (PH), Head diameter (HD), Stem girth (SG), No. of leaves/plant (NL), Photosynthetic efficiency (PE), Petiole length (PL), Internodal distance (ID), Seed yield/ plant (SY), No. of filled seeds/ head (NF), Test weight (TW), Hull content (HC), Volume weight (VW), Biological yield (BY), Harvest Index (HI), Oil content (OC)

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Table 4. PIC values of the SSR primers used for diversity analysis of sunflower genotypes

S. No.	Primer	Forward Primer (5'-3')	Reverse Primer (5'-3')	PIC value
1.	ORS 58	TGTACCAAGGGTCGTTGTCA	CGACCCCGAGTTTTGTTG	0.14
2.	ORS 488	CCCATTCACTCCTGTTTCCA	CTCCGGTGAGGATTTGGATT	0.40
3.	ORS 844	ACGATGCAAAGAATATACTGCAC	CATGTTTAATAGGTTTTAATTCTAGGG	0.49
4.	ORS1265	GGGTTTAGCAAATAATAGGCACA	ACCCTTGGAGTTTAGGGATCA	0.89
5.	ORS 878	TGCAAGGTATCCATATTCCACAA	TATACGCACCGGAAAGAAAGTC	0.38
6.	ORS 988	TTGATTTGGTGAAAGTGTGAAGC	CGAACATTATTTACATCGCTTTGTC	0.40
7.	HA 1604	GCAAATGCACTAAAGGCCCC	CCCTACTCAAACCTTACCTC	0.40
8.	ORS 920	CGTTGGACGAAGAACTTGATTT	ACTTCCGTTTGTTCCGAGCTT	0.15
9.	ORS 880	AAGTAGCTTTGCTTTCCTTCGTC	CGAAACGCGGATTATTGTCTTAT	0.28
10.	ORS 718	CACTTTACGCACACCAAACC	ATGCAACACCCGAATCAAAG	0.07
11.	ORS 423	TCATATGGAGGGATCTGTTGG	AAGCAACCATAATGCATCAGAA	0.11
12.	ORS 160	TCCCTTCCTTTCATCGTCTGCT	TGGCAATTTGCCAAGGACC	0.28
13.	ORS 996	CGGTGAGAATAACCTCGGAAGA	ATCAGTCCTTCAACGCCATTAGT	1.00
14.	ORS 1068	AATTTGTCGACGGTGACGATAG	TTTTGTCATTTCATTACCCAAGG	0.14
15.	ORS 345	GCACTTGGAATGGCAAGACT	CGAGACGACTTAGATCCGTTG	0.28
16.	ORS 437	GACGTCTTCACAGTTCAAATAACG	GCATCGACTCTGTTCTTCTCG	0.52
17.	HA 3691	GAATGAAGCATGTGGAAGGCGG	GTGGAGGTGATGATGGTATGAG	0.57
18.	ORS 47	TGAGATTCCTCGTACTTCATCTG	CCATAGGATGCATAGGAAGAGG	0.62
19.	ORS 149	GCTCTCTATCTCCCTTGACTCG	TGCTCTAAGATCTCAGGCGTGC	0.59
20.	HA 3638	GACATAATCACTAGTTGTTGGTGC	CTCCTCCCACCTCAACAATTTC	0.60
21.	HA 3639	GCAACATGCAGTTCCTAATCAAAC	TCACCGAACTTCAATATCACCAC	0.57
22.	ORS 518	CGCCATATCAGCAAGGAAAT	GGTGTTTGTGGAAAACTTACCC	0.71
23.	ORS 16	GAGGAAATAAATCTCCGATTCA	GCAAGGACTGCAATTTAGGG	0.64
24.	ORS 345	GCACTTGGAATGGCAAGACT	CGAGACGACTTAGATCCGTTG	0.95
25.	ORS 899	GCCACGTATAACTGACTATGACCA	CGAATACAGACTCGATAAACGACA	0.62
26.	HA 3651	GGAATTATCCATTGTAGGTTTGG	GGATGATTGATTAATTGAGGG	0.57

Electronic Journal of Plant Breeding, 7(4): 1216-1223 (December 2016)
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Table 5. Genetic distances (GD) between different genotypes of sunflower

Genotypes	11A	47A	67A	68A	234A	95C-1	93 R	103R	124R	134R	145R	167R
47 A	0.24											
67 A	0.40	0.36										
68 A	0.36	0.40	0.28									
234 A	0.42	0.56	0.56	0.43								
95-C-1	0.42	0.42	0.58	0.51	0.62							
P 93 R	0.50	0.50	**0.71**	0.64	0.58	0.20						
P 103 R	0.50	0.50	0.65	0.63	0.49	0.22	0.20					
P 124 R	0.49	0.62	0.70	0.59	0.52	0.31	0.29	0.18				
P 134 R	0.41	0.41	0.65	0.54	0.43	0.28	0.22	**0.13**	0.17			
P 145 R	0.54	0.50	0.62	0.55	0.49	0.30	0.24	0.15	0.18	**0.13**		
P 167 R	0.57	0.48	0.67	0.64	0.46	0.36	0.30	0.36	0.44	0.35	0.27	
RCR 8297	0.33	0.33	0.54	0.51	0.63	0.24	0.35	0.36	0.33	0.39	0.36	0.45