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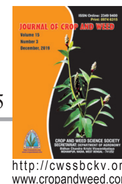

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Molecular characterization of sunflower advanced inbred lines derived from powdery mildew resistance breeding programme

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ABSTRACT

Sunflower, an important oilseed crop ranks second as a source of edible oil at the global level. In order to derive hybrids producing high oil and achene yield, potential and distant inbred lines need to be produced to take advantage of commercial heterosis. Evaluation of genetic diversity of such parental lines and assessment of zygosity in hybrids using various molecular markers is very important in sunflower hybrid breeding programs. In the present study, 121 sunflower genotypes including 117 advanced inbred lines derived for powdery mildew resistance and 4 checks were genotyped using 30 microsatellite markers. The number of alleles determined varied from 2 to 3 alleles/locus. Average number of alleles per locus were 2.1. The most powerful markers on the basis of PIC value were ORS938 (0.5) and ORS 1088 (0.5) whereas the least powerful marker was ORS309. The molecular diversity analysis grouped 121 sunflower inbred lines into sixteen main clusters indicating moderate amount of diversity among them at 0.72 Jaccard's coefficient of similarity. Careful selection of diverse inbred lines would help in the hybrid development programs by exploiting commercial heterosis.

Keywords: Advanced inbred line, clustering, genetic divergence, microsatellites, PIC value

Sunflower (*Helianthus annuus* L.) as an oilseed crop, is known for its valued and healthy vegetable oil in the oilseed scenario of India. It is widely known as "Golden Girl of American Agriculture," belonging to the family *Asteraceae* and ranks second (the first being soybean) as an edible oilseed crop at the global level. Sunflower cultivation in India was started in 1972, when OPVs (open-pollinated varieties) EC68414 (Peredovick), EC68415 (Armavirski), EC664131 and EC69874 were introduced from Russia. The multidisciplinary research activity in sunflower also was started in 1972-73 under AICRP (All India Coordinated Research Project) on Oilseeds. However, sunflower production in the country did not increase as expected until the late 70's. The popularization of an early maturing sunflower variety 'Morden' (in 1978) and the first sunflower hybrid BSH-1 in 1980 revived the interest in sunflower cultivation and the crop registered impressive growth in acreage and production in the country (Seetharam and Virupakshappa, 1994).

Recently, the development of sunflower hybrids derived from new CMS sources of male sterility and corresponding fertility restorers, has gained a special emphasis in sunflower breeding. Sunflower breeders' goal is towards developing hybrids with high oil and achene yield coupled with resistance for diseases and insect pests. In order to derive hybrids producing high oil and achene yield, potential, unique and distinct inbred lines need to be developed to take benefit of commercial heterosis. Genetic evaluation of diversity of such inbred lines to be used as parents and assessment of hybrid purification using various DNA markers is prerequisite in sunflower hybrid developmental programs. Use of morphological markers in identifying sunflower parental lines could be impossible as they are limited and influenced by environment. The DNA markers are abundant, unlimited, distributed throughout the genome and are unaffected by environments or epistasis.

DNA markers such as RAPD (Random Amplified Polymorphic DNA), AFLP (Amplified Fragment Length Polymorphism) and SSR (Simple Sequence Repeats) are PCR (Polymerase Chain Reaction) based and currently are being used in various crop breeding platforms. The SSRs are widely used markers for genotyping the crop germplasm collections. Microsatellite marker is mostly used in comparison to other molecular markers in genomic studies because it is highly polymorphic, co-dominant, highly abundant, analytically simple and reproducible (Zeinalzadeh Tabrizi*,* 2014). Most of the SSR markers are single locus and exhibit Mendelian inheritance. Various genotyping platforms such as PCR based DNA fragment analysis, construction of genomic libraries and semi-automated genotyping using SSR markers can be easily performed (Cregan *et al.,* 1999). Zia *et al*. (2014) suggested that SSR is powerful and reliable tool for accounting the diversity at molecular level.

The present study was planned to understand the molecular diversity in sunflower advanced inbred lines, which were derived for powdery mildew resistance.The inbred lines were clustered into sixteen clusters based on the genetic relationships among them. A careful

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selection of these inbred lines would help in exploiting heterosis, developing heterotic groups and deriving powdery mildew resistant hybrids in sunflower crop improvement programs.

MATERIALS AND METHODS

Experimental materials

The present study on assessing molecular diversity included 121 sunflower genotypes consisting of 117 advanced inbred lines derived for powdery mildew resistance for 4-5 generations of self-pollination' and 4 commercial checks *viz.,* DRSF 108, DRSF 113, RSFV 901and Morden as a susceptible check. The advanced inbred lines included resistant, moderately resistant, susceptible and highly susceptible inbred lines for the powdery mildew disease (Table 1). The experiment for assessment of molecular diversity in these advanced inbred lines was carried out during *rabi* 2017 at Main Agricultural Research Station, University of Agricultural Sciences, Raichur. Five to seven seeds of each genotype were sown in pots separately during *rabi* 2017.

DNA isolation and Polymerase chain reaction

Leaf samples from twenty day old healthy seedlings were collected for DNA isolation. The extraction of plant genomic DNA was carried out according to the CTAB method of DNA extraction (Zeinalzadeh Tabrizi *et al*., 2015). Nanodrop ND1000 Spectrophotometer was used to quantify the DNA. The samples were diluted using double distilled water to get a working concentration of 50-60ng/µL.Thirty SSR primers were used in the present study. The PCR was carried out in a volume of 10 µl reaction mixture containing 2 µl of 2mM 10x Taq buffer (Sisco Research Laboratories Pvt. Ltd.), 2 µl of 2.5mM $MgCl_2$ (Sisco Research Laboratories Pvt. Ltd.), 2µl of 2.5mM dNTPs (Thermo Fisher Scientific), 0.2 µl of 1unit Taq DNA Polymerase (Thermo Fisher Scientific), 1µl each of the forward and reverse primer (10 Pico Mol.), 1µl DNA template (50-60ng/µL) and 0.8 µl distilled water. Conditions for the PCR were as follows;

(a) initial denaturation at 94 °C for 4 mins

(b) 30 cycles of run, each with denaturation at 94 °C for 30 seconds, primer annealing temperature as per the primer used for 1 min and extension at 72 °C for 30 seconds

(c) final extension at 72 °C for 10 min

(d) final hold at 4°C for 10 min. The amplification products were separated on 3 % agarose (3 g agarose in 100 ml of 1X TAE buffer) stained with ethidium bromide. The bands were visualized under ultraviolet light. The approximate size of the DNA fragments was determined with the help of 100 bp DNA ladder (New England BioLabs).

Scoring of SSR generated bands

The binary method was followed for scoring the presence or absence of bands. Score 1 was assigned to the presence of band while 0 for the absence of band. The binary data was then used for further calculations. The ability of a marker for assessing polymorphism or to differentiate the genotypes within a given germplasm, depending on the allelic frequency and the number of alleles amplified was expressed in terms of polymorphism information content. PIC value of markers was calculated using the following formula (Anderson *et al.,* 1993).

$$PIC=1-\sum_{i=1}^{k} P_i^2$$

where,

k is the sum total of alleles amplified for a given marker locus

P_i is the frequency of the ith allele in the lines analysed

Statistical analysis

NTSYS-pc was used to know the genetic similarity among the sunflower genotypes. The similarity coefficient was computed using Dice coefficient (Dice, 1945) to create similarity matrix. Dice coefficient was computed by

$$S_D=\frac{2_{n_{ik}}}{n_i+n_k}$$

where,

n_{ik} : Number of identical bands detected in both variety i and variety k

n_i : Number of bands detected in variety i but absent in variety k

n_k : Number of bands detected in variety k but absent in variety i

The coefficients of genetic similarity or relatedness were estimated to quantify the genetic relationship among 121 sunflower genotypes. The information was then used for constructing a dendrogram using unweighted pair group method and using arithmetic averages (UPGMA) sequential agglomerative hierarchal nested (SHAN) cluster.

RESULTS AND DISCUSSION

Understanding the magnitude of molecular diversity and nature of relationships among genotypes in a population is helpful for their characterization and documentation. It also assists for the effective selection as parents for distant hybridization to develop hybrids in crops like sunflower (Senior *et al.,* 1998; Sun *et al.,* 2001). Studies reported that SSRs are the most frequently used markers for characterizing sunflower genetic resources (Zeinalzadeh Tabrizi *et al*., 2015; Filippi *et al.,* 2015).

Table 1: List of advanced inbred lines used in the present study including their differential host reaction for powdery mildew disease

Sl. No.	Host reaction	No. of genotypes	Genotypes
1	Resistant	4	PM-2, 66, 81 & 82
2	Moderately resistant	21	PM-5, 14, 15, 16, 17, 18, 19, 20, 27, 41, 67, 72, 73, 75, 76, 83, 111, 117, 118, 132, 156,
3	Susceptible	74	PM-3, 4, 6, 7, 8, 11, 12, 13, 21, 33, 34, 30, 44, 45, 35, 36, 37, 38, 39, 40, 42, 43, 46, 49, 50, 52, 53, 54, 56, 57, 62, 63, 64, 65, 78, 84, 87, 92, 94, 96, 97, 106, 107, 108, 109, 110, 121, 123, 128, 129, 130, 131, 133, 134, 137, 138, 139, 146, 152, 155, 157, 158, 159, 160, 161, 163, 164, 165, R-GM-39, R-GM-49, R-GM-41, RSFV-901, DRSF-108, DRSF-113
4	Highly susceptible	22	PM-26, 31, 32, 68, 69, 71, 79, 91, 95, 98, 99, 100, 102, 103, 112, 113, 126, 135, 147, 151, R-GM-27, MORDEN

Table 2: Sequences and characteristics of investigated polymorphic SSR loci

Sl. No.	Markers	Annealing temperature	Forward Primer (5'-3')	Reverse primer (5'-3')	No. of alleles produced	Polymorphic information content
1	ORS309	50.1	CATTTGGATGGAGCCACTTT	GATGAAGATGGGGAATTTGTG	2	0.06
2	ORS310	49.75	AATTCCCACGCAAACTTCAA	GGGTAAAGGGGCAACCTAT	2	0.42
3	ORS316	48.8	TGGCGTCTTCATAGCATCAG	GAGATTTGAGCTTCGTGTTGC	2	0.20
4	ORS318	50.75	TCCATGAGTTGGTCGTATGC	CCGCATATTGAAACTGCATC	2	0.17
5	ORS321	52.8	TGTCGAAGAGTTGTCGGAAC	GGGAAGGTGAAACCCTAACC	2	0.36
6	ORS938	53.85	ACCAACTCCCATGCAACCTAA	GCGTTCTCACCGTTCTAACACTT	2	0.50
7	ORS1088	53.25	ACTATCGAACCTCCCTCCAAAC	GGATTTCTTTCATCTTTGTGGTG	2	0.50
8	ORS1159	51.7	TTTCGTGATGGTGATTGATGATT	CAGCAACTCTGACCGTTTCATTA	2	0.19
9	ORS1220	52.95	ACAATAGCCTTCAGTTGCCTTGT	GTTTAGTTGAAACCCGGATCG	2	0.40
10	ORS1245	52.35	GAAGTGGAGCAATGTTGGTGA	CGCCAAGATATTAGTGTGATGATT	3	0.37
Total	**21**	**3.179**				
Mean	**2.1**	**0.32**				

Table 3: Clustering of 121 sunflower inbred lines based on SSR markers analysis (0.72 coefficient of similarity)

Clusters	No. of genotypes	Genotypes
I	18	PM-2, 31, 151, 3, 5, 14, 15, 34, 81, 44, 97, 8, 165, 16, 17, 18, 37, R-GM-27.
II	4	PM-67, 66, 69, 117.
III	21	PM-92, 100 102, 20, 35, 42, 78, 106, 118, 72, 95, 96, 107, 45, 41, 75, 103, 76, 155, 163, DRSF-113
IV	38	PM-6, 21, 52, 54, 64, 73, 94, 129, 139, 156, 49, 68, 126, 11, 12, 71, 53, 113, 32, 38, 108, 33, 134, 146, 147, 91, 132, 133, 7, 27, 46, 50, 128, 43, 159, R-GM-39, DRSF-108, R-GM-41.
V	9	PM-30, 26, 135, 161, 121, 125, 138, Morden, RSFV-901.
VI	6	PM-4, 130, 137, 131, 157, 151.
VII	2	PM-13, 160.
VIII	1	PM 158
IX	5	PM-79, 82, 98, 99, R-GM-49.
X	3	PM-19, 39, 87.
XI	8	PM-56, 57, 62, 40, 109, 152, 63, 84.
XII	1	PM-111
XIII	2	PM-110, 112.
XIV	1	PM-83
XV	1	PM-65
XVI	1	PM-36

A total of 10 primers were found polymorphic out of thirty.The number of detectable alleles varied from 2 to 3 alleles/locus. Nine markers (ORS321, ORS318R, ORS1245, ORS1220, ORS1159, ORS938, ORS309, ORS310, ORS316) amplified 2 alleles and marker ORS1245 amplified maximum of 3 alleles. The number of alleles generated by 10 polymorphic primers were 21 (Table 2). Average alleles per locus were 2.1, which is in unison with the results of Hvarleva *et al.* (2007). The present study reported much lower mean number of alleles per marker compared to other studies conducted by Yu *et al.* (2002) and Tang and Knapp (2003) in sunflower. The lower mean value of alleles detected per marker could be because of smaller number of markers taken here as compared to previous studies. Tang and Knapp (2003) detected 3.5 average allele per locus using 122 microsatellite markers while genotyping for inbred lines of confectionery and oilseed types in sunflower.

The strength and reliability of a marker can be determined by its PIC value. Greater the PIC value stronger will be the marker for discrimination of genotypes. SSR markers have the highest polymorphic information content (PIC) because of high mutation rate at microsatellites (Zhang *et al*., 2005). Maximum PIC values were recorded by ORS938 (0.5) and ORS 1088 (0.5) whereas the least PIC value was recorded by ORS309 (0.06) (Table 2). The average PIC value in the present study was 0.32, which is less than that reported by Filippi *et al*. (2015). Out of 30 primer pairs used, 20 were found to be monomorphic. These monomorphic primers may show polymorphism among other elite wild sunflower germplasm.The study by Yu *et al.* (2002) suggested that the markers which are not polymorphic among potential inbred lines were found to be polymorphic among elite, exotic and wild germplasm. Hence it could be concluded to reuse the monomorphic primers identified in the present study in the genomic studies of such kind of germplasm.

Genetic diversity in terms of genetic distance among parents is an important criterion while planning for hybridization for exploiting maximum heterosis. Genetic distance is directly related to the magnitude of heterosis. Greater the genetic distance between any two genotypes, more will be the heterosis upon crossing those two genotypes.The construction of the dendrogram was done based on unweighted pair-group method using arithmetic averages (UPGMA) cluster analysis (Fig. 2). The genotypes were clustered into 16 clusters at 0.72 coefficient of similarity. These 16 clusters again divided into sub-clusters.Cluster IV which contains maximum of 38 inbred lines sharing high similarity among them. The cluster IV was followed by Cluster III and cluster I with 21 and 18 inbred lines, respectively. Cluster V

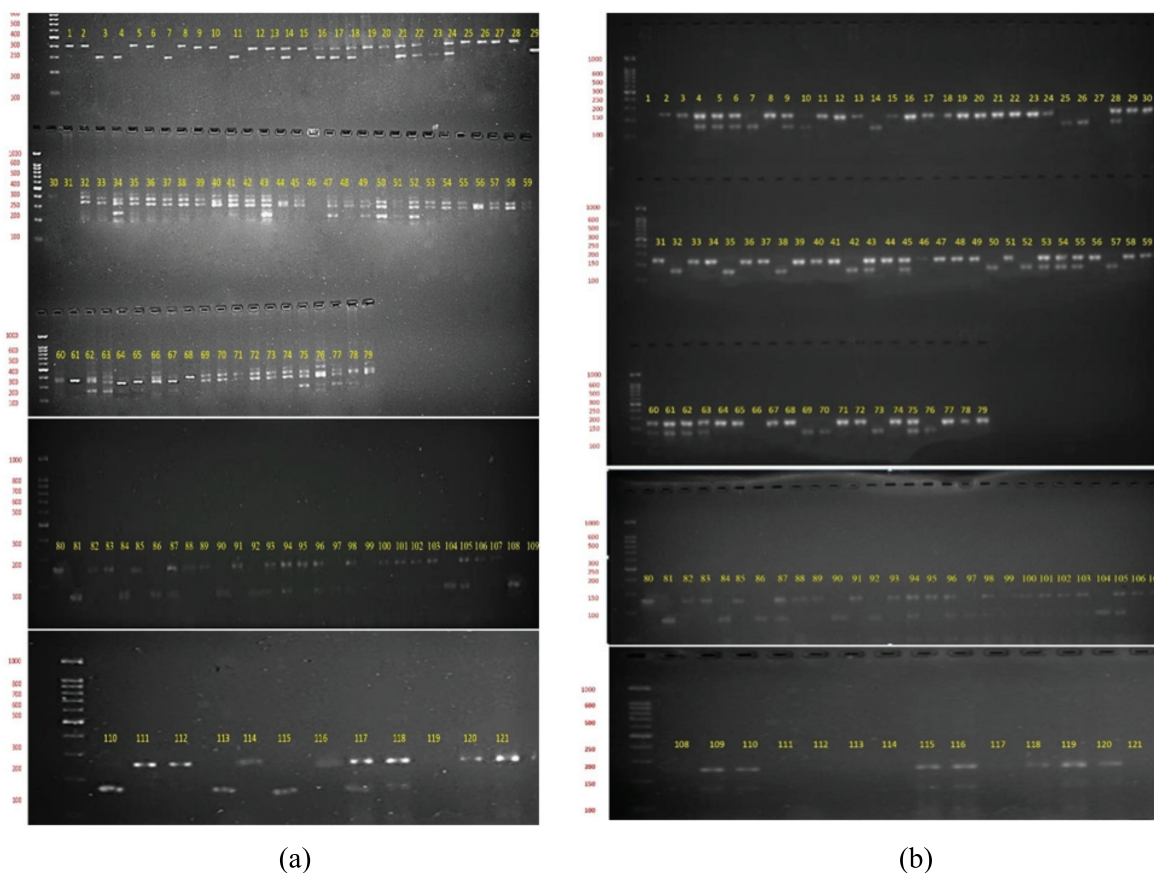

Fig. 1: Molecular profiling of (a) ORS 938 and (b) ORS 1088

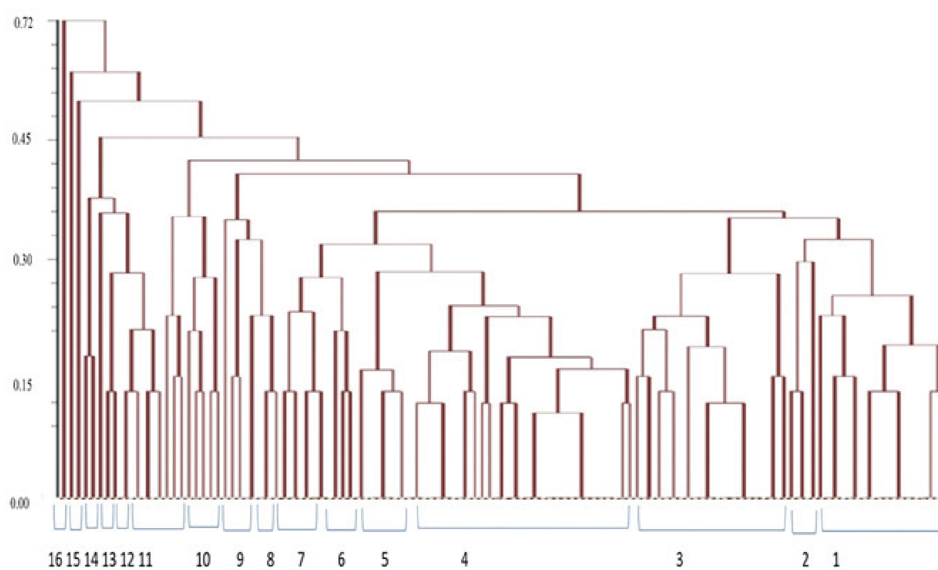

Fig. 2: Dendrogram of 121 sunflower inbred lines using Dice coefficient and UPGMA method (Number indicates the cluster)

contains 9 inbred lines followed by cluster X (8 inbred lines), cluster VI (6 inbred lines), cluster IX (5 inbred lines), cluster X (3 inbred lines), cluster VII and cluster XIII (2 inbred lines). Remaining Clusters VIII, XII, XIV, XV and XVI had only one inbred line each indicating maximum dissimilarity and genetic distance with the inbred lines in the remaining clusters. Molecular profiling of marker ORS 1088 and ORS 938 amplifying 2 alleles is represented in Fig. 1a and 1b.

The inbred lines grouped in the same cluster share same characters or co-ancestry and are related by descent. The present study is supported by the findings of Duca *et al.* (2013), Zia *et al.* (2014) and Kirandeep *et al.* (2017) who carried out genetic diversity studies in sunflower using microsatellites.

In conclusion, it would therefore require careful selection of the advanced inbred lines to exploit heterosis and develop heterotic groups from the present study. The inbred line resistant for powdery mildew can be chosen as one of the parents during hybridization programs to develop powdery mildew resistant hybrids and as a donor parent for transferring the resistant alleles to the elite lines through back cross breeding. In that case, the polymorphic markers will be helpful in recovering the genetic background of the recurrent parent through marker assisted back-cross (MABC) breeding programme.

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