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Research Article

Allelic diversity of simple sequence repeats among elite inbred lines and mutants of cultivated Sunflower (*Helianthus annuus* L.)

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Abstract
Ten simple sequence repeat (SSR) markers were used to evaluate genetic relationships in a set of twelve parental lines of sunflower representing the genetic stock, including restorers, maintainer and mutant lines of the classical cytoplasmic male sterility. A total of 26 loci were detected among the twelve genotypes. Of this, 23 loci were polymorphic for the inbred lines investigated. Average number of bands and polymorphic bands per primer were 2.6 and 2.3 respectively. NTSYS-pc (ver 2.02) was used to calculate the jaccard's similarity coefficients. Genetic dissimilarity estimates based on simple matching coefficient revealed more genetic diversity among the genotypes tested. The greatest genetic diversity was observed between the RHA-851A and RHA-265 (0.87) followed by RHA-851A and R-298 (0.857) and more similarity was between RHA-265 and R-298 (0.08). Unweighted pair group method with arithmetic mean (UPGMA) cluster analysis grouped the 12 genotypes into four groups at 0.74 similarity coefficient.

Key words
Sunflower**,** Simple sequence repeats (SSRs), Sunflower, Genetic dissimilarity, Micro satellites

Introduction:

The sunflower (*Helianthus annuus* L.), an annual diploid (X=17) species, is an important source of oil, feed and a universally known and widely grown ornamental. *Helianthus annuus* is one of the 48 *Helianthus* species belonging to the family Compositae. Sunflower breeding programs are focused basically on yield, oil content and disease resistance. Use of germplasm of *Helianthus* species for hybrids development requires detailed genotype control. Molecular genetic polymorphism investigations that analyze the variability of certain DNA sites are becoming one of the most efficient approaches in plant genetics.

Microsatellites or simple sequence repeats (SSRs) are short, tandemly repeated DNA sequences flanked by unique, conserved DNA sequences. SSRs were originally discovered in the human genome and other mammalian genomes and have since been isolated from the genomes of several plant species (Akkaya, *et al.*, 1992). SSRs have become one of the principle classes of DNA markers used for DNA fingerprinting, genetic mapping and molecular breeding in crop plants. Their utility and popularity can be attributed to several factors. First, SSR markers tend to be monoallelic and highly polymorphic.SSR repeat length variants (alleles) are produced by DNA replication slippage and unequal crossing over between sister chromatids (Levison and Gutman,1987; Jeffreys *et al.*, 1994). Multiple alleles proliferate through the cyclical recurrence of these phenomena across generations. Second, SSR markers can be multiplexed by length using virtually any genotyping system. When assayed using semi-automated, multicolor, genotyping systems, SSR markers can be multiplexed by length and color and the genotyping throughput can be doubled or tripled depending on the number of fluorophores handled by the system. Third, a significant percentage of SSR markers, depending on the complexity of the host genome, amplify a single orthologous locus across genotypes. Such markers are ideal for DNA fingerprinting and genetic mapping and, more specifically, for cross referring maps.

In sunflower SSR markers are significantly more polymorphic than AFLPs (Hongtrakul *et al.,* 1997) or allozymes (Croon *et al.,* 1997). PCR amplification of sunflower micro satellites was used to uncover a genetic diversity among elite inbred lines (Yu *et al.,* 2000)(Paniego at al., 2002).

This work describes the use of SSR marker system for investigation of allelic diversity in *Helianthus*. The objective of this study was to use ten SSR primers to measure the genetic diversity of ten elite inbred lines and two mutant lines which were selected for their performance with respect to seed yield.

Material and methods

The SSRs were screened for length polymorphisms among six sterility maintainer (B) lines (CMS-234A,DSF-15A,VRF X DSF-2,VRF X NDOL-2,RHA-851A and 4546A X NDOL-3) , four fertility restorer (R) lines (6-D-1, RHA-857, RHA-265 and R-298) and mutant lines (6-D-1P#1 and 6-D-1P#2). These lines were selected based on their performance with respect to seed yield and other component characters. Among these lines, 6-D-1P#1 and 6-D-1P#2 were mutant lines which were developed from the line 6-D-1 through induced mutation.

DNA extraction:

The DNA was extracted following the protocol (CTAB method) described by Gentzbittel (Gentzbittel *et al.,* 1992) with modification, from frozen leaves stored at -80°C. The leaves cut into pieces were completely homogenized in liquid nitrogen. Extraction buffer (100 mM Tris HCl (pH 8.0), 20 mM EDTA, and 1.4 mM NaCl, 2% CTAB per liter) was added in 50 ml tubes filled with leaf powder to a volume of 15 ml and mixed well. The tubes were incubated at 65°C for 30 minutes with repeated shaking. Equal volume of chloroform: isoamylalcohol mix (24:1) was added and mixed thoroughly for 15 minutes, followed by centrifugation at 4000 rpm for 30 minutes. Equal volume of isopropanol was added to the supernatant. DNA was hooked out after half an hour and washed in 70% ethanol and suspended in 500 µl of TE buffer (pH 8.0). The DNA was incubated with 10-15 µl (10 µg/µl concentration) of RNAse for 30 minutes. To this, equal volume of chloroform: isoamyl alcohol was added and centrifuged at 12,500 rpm for 10 minutes. Twice the volume of absolute ethanol and 1/10th volume of 3 M sodium acetate were added to the aqueous layer and incubated overnight. The content was centrifuged at 12,500 rpm for 15 minutes and supernatant was discarded. The pellet was washed with 70% ethanol and air dried. Then, the pellet was dissolved in 500 µl of TE and stored at -20°C. The quality and quantity were checked through 0.8% agarose gel by electrophoresis. DNA concentration for PCR reaction was estimated, by comparing the band intensity produced by the known dilution that gave good amplification.

SSR analysis:

Genomic DNA was used as template for PCR amplification as described by Williams (Williamsl *et al.,* 1990). A set of ten primers (Paniego *et al.,* 2002) (Table 1) were used to produce distinct marker profiles for twelve parental lines. Amplification reactions were in the volumes of 20 µl containing dATP, dCTP, dGTP and dTTPs each at 2.5 mM, 5 pM/µl primer, 15 ng of genomic DNA and 0.5 unit of Taq DNA polymerase (Bangalore Genei Pvt. Ltd., Bangalore). Amplification was performed with Thermal Controller programmed for 25 cycles. After initial denaturation for five minutes at 94°C, each cycle consisted of one minute at 94°C, one minute at 48±5°C and one minute at 72°C. The 25 cycles were followed by five minutes of final extension at 72°C. PCR amplified products were subjected to electrophoresis on 2 per cent agarose gel in 1X TAE buffer. The electronic images of ethidium bromide stained gels were captured and documented using Electrophoresis Documentation and Analysis System (EDAS 120).

Data analysis:

Data generated from the polymorphic fragments was scored as present (1) or absent (0) for each of the 12 genotypes. The diversity among the lines was worked out by subjecting the SSR scores to cluster analysis. Sequential Agglomerative Hierarchic Non-overlapping (SAHN) clustering was performed on simple matching similarity matrix by Unweighted Pair Group Method with Arithmetic Averages (UPGMA). The data analysis was done using the software NTSYSPC version 2.02.

Results and Discussion

In this work we applied primers to sunflower micro satellite loci that have been developed by Paniego *et al*. (2002) to reveal the diversity in mutant and elite inbred lines. A set of ten primers (Table 1) was used in PCR reaction to amplify DNA fragments from 12 genotypes (six maintainer and six restorers). Among, these ten primers, seven produced polymorphic across all genotypes. The remaining was monomorphic among the genotypes tested.

Allelic diversity estimation is an important step in knowing diversity between parental lines. Such systems have to comply with two rules: 1) optimum quantity of loci with maximum alleles and 2)

amplification products of such loci should be simple and unambiguous.

The primers Hal 209-ar, Hal 287-ar, Hal 354-ar and Hal 442-ar gave highest (100%) polymorphism. The number of loci ranged from three (primers Hal 494-ar, Hal 354-ar and Hal 442-ar) to five (primers Hal 354-ar) with an average of 3.71 loci per primer. The primers Hal 209-ar and Hal 494-ar (66.7%) and Hal 327-ar (66.7%) showed the least polymorphism.

Some primer combinations evidenced the amplification of multiple band patterns. This kind of amplification profile can be explained by at least two main reasons: (I) Heterozygosity of the inbred lines, indicating that they are derived from heterozygous cultivar selections; and (ii) presence of two (or more) duplicated micro satellites loci (including their border sequences) in the sunflower genome.

Similarity analysis were conducted using a complete BDM comprising data from the analysis of 26 micro satellites and estimated with the NTSYS 1.8 program (Exeter software, Setauket, N. Y.; Rohlf) using the jaccard association coefficient of similarity (Sneath and Sokal 1973). The resulting pair wise similarity values were expressed as dissimilarity values in triangular dissimilarity matrices. Cluster analysis were conducted by applying the SAHN option on similarity estimates using the unweighted pair group method arithmetic average (UPGMA) and the resulting clusters, were expressed as dendrograms using the option TREE PLOT. Genetic distances among the 12 inbred lines ranged from 0.08 for R-298 X RHA-265 to 0.87 for RHA-265 X RHA-851A (Table 2). All the parental lines showed diversity among themselves indicating that there is considerable amount of variation and these can be used in breeding programme.

The dendrogram constructed from the pooled data, primarily separated sunflower inbred lines along fertility restorer (R) – maintainer (B) boundaries. Similarly, lines from different sources like mutant (6-D-1) and non-mutant (6-D-1P#1) types of maintainer lines tended to cluster together. Sunflower B and R lines, apart from one cultivar (6-D-1P#2), formed four diverse, but distinct clusters separated by a genetic distance of 0.74. Among all the restorer lines, mutant line 6-D-1 P#2 clustered together with B lines indicating, the similar genetic make up as B lines.

Molecular markers analysis of the twelve genotypes using ten primers produced polymorphism for most of the studied loci. As per the diversity index, the genotypes were grouped into eight clusters. The highest distance between the genotypes RHA-851A and RHA-265 (0.87) indicated high divergence between them. Low diversity indices were observed between RHA-265 and R-298 (0.08) and between 6-D-1 and 6-D-1P#1 (0.13) which indicated low divergence. Crossing between the genotypes with high diversity coefficient will manifest high heterosis.

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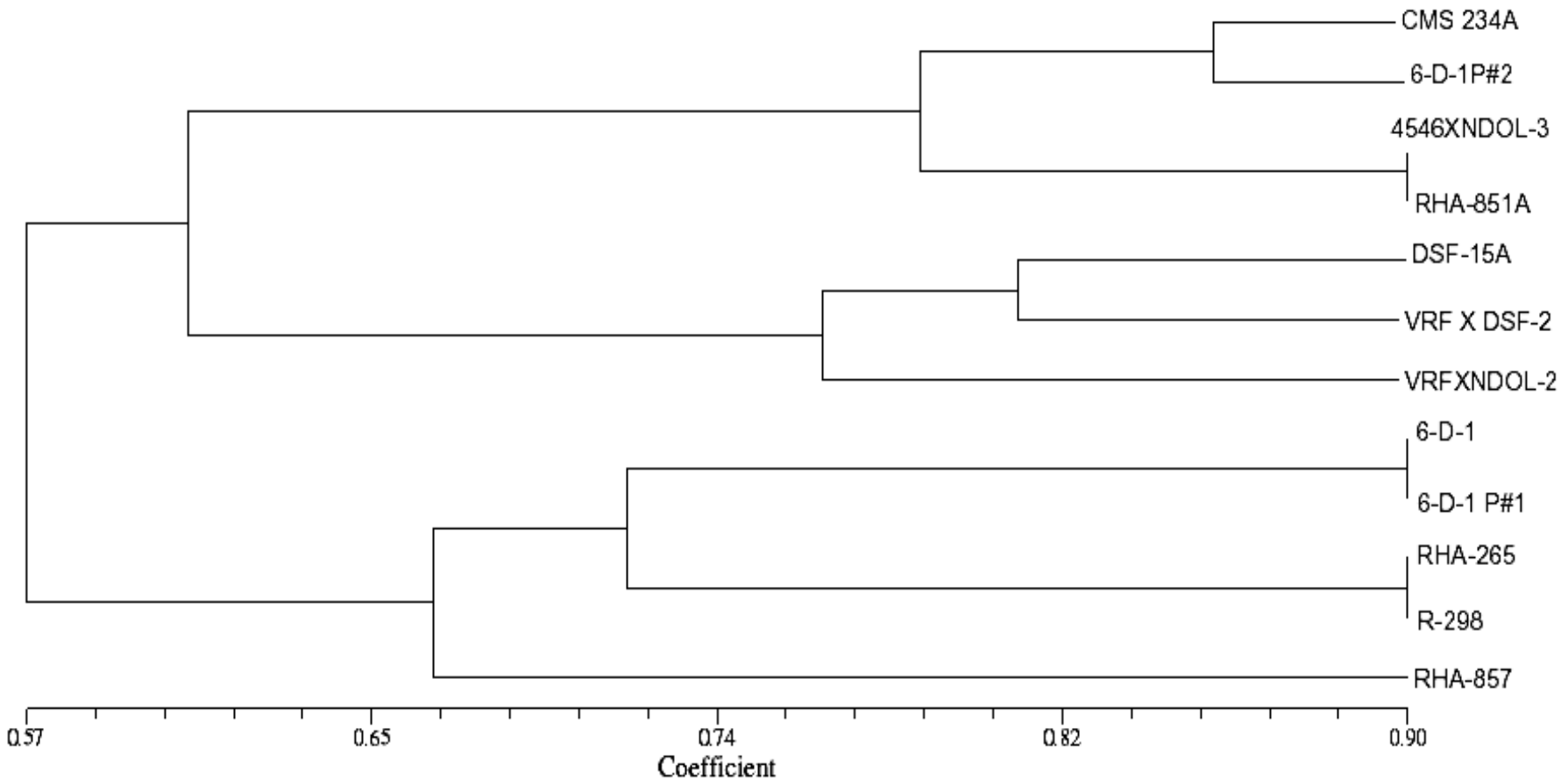
Table 1. PCR primers amplifying micro satellites from cultivated sunflower

Primer	Motif	Primer sequence		Expected size range (bp)	Allele No.
		Forward (5' – 3')	Reverse (5' – 3')		
Ha95-ar	(GA)24	GAACATGGCCATAACTCATAGACG	CCTTCGACCCAACATC	166-162	5
Ha494-ar	(GA)17A(GA)2N12T15	CGTTGGTTAAGGCCTGAGGTC	GAGCAGTAAACAGAGGGTACACC	209-193	4
Hal209-ar	(ATT)26	CATTGGGCCTCACAAACACTTG	GATGTGAAACAGCTCCATACTC	160-142	5
Hal1287-ar	(GA)26	GATATGAGCCCATCACTCATC	GAAGATATGTCAGGTCACACCC	171-151	8
Hal1327-ar	(ATT)30	CCGTTAGGTATGTTACTTGCGACC	GGTGGGGGGAATATTCTGACGTG	225-201	6
Hal1354-ar	(AT)16AATN7(GA)4T2(GA)11	CATATGTGGCACATTCATGG	GGTTGATTGGGGTTAAGGAG	212-182	8
Hal1442-ar	(ATT)31	GCTTATGTGCTTACGTGTTCCTG	CTAAACAGTTCGGCGAGTGTAGG	244-202	8
Hal1608-ar	(ATT)25	GATCTTAGGTCCGCCAC	GATGGCATTTGGCTAGAC	331-169	7
Hal1626-ar	(ATT)35	GATGTTACACGTTAGCAACG	GAACTCAGCCTAAAAGTC	170-146	6
Hal1796-ar	(ATT)33	CGAAGGAAGGAACCTGCCTG	CCATACGCGTTTACTTCTCAGG	230-152	7

Table 2: Genetic distance between all possible pairs of sunflower parental lines

Genotypes	Dissimilarity (1-SM) x 100											
	CMS 234A	DSF-15A	VRF x DSF-2	VRF x NDOL-2	4546A x NDOL-3	RHA-851A	6-D-1	6-D-1#1	6-D-1# 2	RHA-857	RHA-265	R-298
CMS 234A	0											
DSF-15A	43	0										
VRF x DSF-2	50	20	0									
VRF x NDOL-2	54	30	26	0								
4546A x NDOL-3	49	40	44	81	0							
RHA-851A	71	82	85	80	65	0						
6-D-1	57	33	40	53	75	82	0					
6-D-1#1	50	37	44	60	57	78	13	0				
6-D-1#2	33	54	47	67	75	67	54	45	0			
RHA-857	37	40	45	58	74	85	40	33	47	0		
RHA-265	56	45	42	62	65	87	28	40	53	33	0	
R-298	56	36	33	53	52	85.7	18	30	53	25	8	0

Fig. 1: Dendrogram constructed by UPGMA cluster analysis of the genetic distance matrix for twelve inbred lines of sunflower estimated from the genotypes of 23 polymorphic SSR marker loci



Diversity (1 – SM) × 100