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Single marker analysis to associate SSR marker to yield component traits in interspecific hybridization between *Helianthus annuus* L. and *Helianthus argophyllus* Torr. & A. Gray

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Abstract
In the present investigation sunflower marker-trait association were studied for eight yield component traits using sixteen polymorphic SSR markers with a set of 123 BC_1F_1 population of the cross CMS 38B × HEL 153/83. Association of mean performance of BC_1F_2 families of corresponding BC_1F_1 individuals and with corresponding marker score were assessed for single marker analysis by using student's t test in SPSS version 16. A total of fourteen markers gave significant association with at least one of the eight traits studied. Most of the markers were found to be related to more than one trait. The markers ORS 1146 was found to be associated with three traits *viz.*, days to 50% flowering, head diameter and oil content. This indicates that the same gene is controlling the expression of these characters. Some of the markers were associated to only one trait. Moreover, phenotypically these characters have more association with each other. Hence these markers may be useful for marker assisted breeding programme on further validation in different genetic backgrounds.

Keywords: Sunflower, Single marker analysis, SSR markers, marker trait association

Introduction
Sunflower is an important oilseed crop of the world. Consideration of seed yield and oil content are important to breeding for high oil yield in sunflower. However, yield being a complex trait depends on many characters, especially yield attributing traits which are controlled by several genes. These complex traits are referred to as quantitative traits (also called as polygenic or multifactorial traits) and the regions within genomes that contain genes associated with a particular quantitative trait are known as quantitative trait loci (QTLs). These QTLs can be identified with the use of molecular markers at molecular level by quantitative trait locus (QTL) analysis using statistical approaches. Till today several efforts were made in plant breeding and the major effort in breeding has changed from traditional phenotypic-pedigree based selection systems to molecular genetics with emphasis on quantitative trait loci (QTL) identification and marker assisted selection (MAS) in crop plants. In sunflower several studies on marker trait association has been done by several researchers *viz.*, Burke *et al.* (2002) [4] identified 14 QTL for seed weight, seed length and seed width, two QTL sin for grain oil content (Mokrani *et al.*, 2002) and Tang *et al.* (2006) [7, 9] detected 34 QTLs for seven seed traits etc.. In the present study, attempts were made to associate the SSR markers to various yield and yield component traits using populations BC_1F_1 and BC_1F_2 derived from interspecific cross *H. annuus* CMS 38B and *H. argophyllus* HEL 153/83.

Material and method
In this present investigation 123 BC_1F_1 individuals and corresponding BC_1F_2 families derived from interspecific cross between *Helianthus annuus* CMS 38B and *Helianthus argophyllus* HEL 153/83 were evaluated for yield component traits. The interspecific hybrid (F_1) obtained from cross CMS 38B × HEL 153/83 was backcrossed with cultivated *H. annuus* L. CMS 38B parent. This was carried out during summer 2015 at MARS Raichur. Staggered sowing of cultivated *H. annuus* L. CMS 38B was taken to coincide with flowering period of F_1 (CMS 38B × HEL 153/83). Method of hand emasculation and pollination was carried out. Here emasculation was done in F_1 (CMS 38B × HEL 153/83) which was taken as female parent and pollens were collected from CMS 38B and pollination was done. At maturity of the crop, the seeds were harvested separately, cleaned and taken for next season which constituted BC_1F_1 material.

BC_1F_1 seeds were sown during *kharif* 2015. The genomic DNA from each BC_1F_1 individual plants was isolated by Saghai-Maroof *et al.* (1984) [8] method with some modification. Isolated genomic DNA was quantified by electrophoresis method using 0.8 % agarose gel. The images of gels were documented under UV illumination using Uvi Tech gel documentation system. The concentrated DNA was diluted to optimum level for further PCR amplification process. A total of 123 BC_1F_1 individuals were genotyped using 16 polymorphic markers (table 1) which have shown polymorphism between parents. Polymerase Chain Reaction (PCR) was performed by using a Touch - Down PCR using the programme given in Table 2 with varying annealing temperature different for each polymorphic primers. DNA amplification was performed in 10 µl reaction mixture. The 10 µl reaction mixture contained 2 µl of 10 ng/µl template DNA, 1 µl of 10 pM / µl SSR primers pair (Forward: 0.5µl and Reverse: 0.5µl), 0.8 µl of 2 mM $MgCl_2$, 0.125 µl of 10mM dNTP mix, 1 µl of 10X PCR buffer and 0.1 µl of 5U/µl Taq DNA polymerase and 5.775 µl HPLC water. After the completion of PCR, the products were stored at 4 ºC until the gel electrophoresis setup was ready. The amplified products were separated using 4% agarose gel (3g high resolution agarose and 1g low EEO agarose) and viewed under gel doc. Based on the band size each individuals were scored as 1 or 2. Score 1 indicates allele of parent CMS 38B and score 2 indicated heterozygous type having both the alleles of parents.

Table 1: List of sunflower SSR primers used in the study

Sl. No	Primer	Forward (5′-3′)	Reverse (3′ -5′)	Ta	Approximate allele size (bp)	
					CMS 38B	HEL 153/83
1	ORS 691	GCATCTGAGCAACTGCGTTA	ACCGTCCTTAGCTCTTGTGAG	54	500	480
2	ORS 1146	GGCTCATCACTTGCATCTATTGT	TGAAGACACCATCTCCAATGC	50	410	380
3	HA 3638	GACATAATCACTAGTTGTTGGTGC	CTCCTCCCACCTCAACAATTTC	55	300	270
4	ORS 349	CCCTAACCAATATGCTCCCATT	TGGATAAACGAGTGAATGGTGT	52	260	290
5	ORS 716	CCCCACAACCCATAGCCTAA	GAACTAACCGCCATCCAAGA	53	340	300
6	ORS 510	CATCGCGTCCCTCTCTCTAA	CCAACCATCACAGCAATCAG	53	280	300
7	ORS 229	TCCGACCCGAATCTTATGAACC	GACCCGAATGAGACCCAAACTG	56	160	130
8	ORS 1065	ACCGCTGTCAACACCTTAAACTC	GGCTGGGAATCAACTGCTACTAC	56	330	300
9	ORS 176	CCCTAACTGGTTTTCTGACCC	AACTTTTGTTTGTTTGTCCAGG	52	570	530
10	ORS 677	TCCTTCCTTCATTCTCTGCATT	CTCCATACCGTTGCCATCAT	52	600	560
11	ORS 606	CACATAACCCATCATCATTCG	GACTCCAGATTCAAAATTCAAACC	52	250	300
12	ORS 613	GTAAACCCTAGGTCAATTTGCAG	ATCTCCGGAAAACATTCTCG	52	350	400
13	ORS 307	CAGTTCCCTGAAACCAATTCA	GCAGTAGAAGATGACGGGATG	53	110	140
14	ORS 833	GTATGAGCGTGGAATGGCTAGAT	ACATAGAACCGTTGGACGATAGC	56	170	190
15	ORS 561	CTTTGCACGTTGGTCATCAT	ACCAGCACCTTCCTCAACTG	52	600	630
16	ORS 1147	TCTGAGTAGCCGCCGTATCAC	AAACATCACTTTCCACTTATACCCTTC	56	300	260

Table 2: Programme of thermal cycler for the SSR primers used in the study

Sl. No.	Steps	Temperature (º C)	Duration (Minutes)	Number of cycles
1	Initial Denaturation	94	5 min	1
2	Final Denaturation	94	50 sec	
3	Annealing	50 º C - 56 º C + 5 º C temperature decreased by one in each cycle	50 sec	10
4	Primer Extension	72	1 min	
5	Final Denaturation	94	50 sec	
6	Annealing	50 º C - 56 º C	50 sec	25
	Primer Extension	72	1 min	
	Final Extension	72	10 min	1
	Hold	4	Until removed	

The heads of each individual BC_1F_1 plants were covered with cloth bags before opening of flower and later gently rubbed with hands to ensure self-pollination and seed setting. Seed setting was observed on each BC_1F_1 plant at varying level and at maturity seeds were harvested separately from each plant, each representing BC_1F_2 family. A total of 132 BC_1F_2 families along with four checks CMS 38B, RSFH 1887, KBSH 44 and GK 202 were taken during *Kharif* 2016 in the fields of UAS Raichur. The purpose of use of checks is to have comparition of test entries with the checks. The experiment was carried out in alpha lattice design with three replications each with 10 blocks and each block accommodating 14 families (plots). All the recommended agronomic practices were carried out. Phenotypic observations were taken on yield component traits *viz.,* days to 50 *percent* flowering, plant height, head diameter, stem diameter and days to maturity. The plants were left for open pollination and seeds from each plant of each family harvested separately and cleaned and packed in different packets and post-harvest observations were recorded on test weight (100 seeds), seed yield per plant (g) and oil content (%). Oil content was estimated using NIR machine at UAS Dharwad.

Data scoring and data analysis

The amplified PCR products were observed for clear and unambiguous bands represented as alleles and these bands were scored for their presence or absence with the score 1 indicating the presence of CMS 38B parental allele *i.e.*, homozygous and score 2 indicating the presence of alleles of both the parents *i.e.,* heterozygote. The data matrix of binary codes thus obtained was subjected to further analysis.

Phenotypic values of BC_1F_2 individuals were subjected to associate with corresponding marker score for its significance by using Student's t test in SPSS software (version. 16). Thus, genotypic data of 123 genotypes of BC_1F_1 population and phenotypic data of the same 123 genotypes in BC_1F_2 generation was compiled and analyzed using Student's t test. A significant difference indicates that the marker is associated to the trait under study. Difference between the phenotypic means provides an estimate phenotypic variance contributed by the marker for the particular trait under study. This approach associated the marker with the quantitative trait studied.

Results

Phenotype analysis

The phenotypic variation observed among 136 BC_1F_2 families is summarized in the Table 3. All the families were significantly different for eight yield component traits. The traits 50% flowering, days to maturity and plant height were showing low coefficient of variation. All other traits had medium coefficient of variation. The non-significant values of skewness and kurtosis also indicated that the traits have normal distribution. Hence these data were subjected into single marker analysis.

Single marker analysis

Individuals of BC_1F_1 population were genotyped using polymorphic markers and the bands representing the alleles of each individuals were scored as 1 and 2 and the genotypic data and corresponding phenotypic data of BC_1F_2 families were analysed with single marker analysis to obtain marker trait association. The BC_1F_1 population genotyped with 16 polymorphic markers. Out of 16 polymorphic markers the molecular data of ORS 1146 and ORS 307 is shown in Fig1 and Fig 2 respectively.

Table 3: Analysis of Variance in BC_1F_2

Source	DF	Oil content (%)	Plant height (cm)	Stem diameter (cm)	Head diameter (cm)	Days to 50% flowering	Days to maturity	Test weight. 100 seeds (g)	Seed yield / plant (g)
		Mean Square							
Replication	2	8.663	2162.974	125.078	149.043	25.759	21.760	1.659	22.454
Genotype	139	38.10 **	559.45 **	11.026**	11.355**	42.243**	43.025**	0.688**	75.078**
Block(Replication)	27	26.462	818.759	28.089	24.286	3.719	3.534	0.462	7.672
Residual	251	20.974	138.980	5.665	4.106	5.235	5.255	0.302	5.634
CV %		13.53	10.35	10.35	16.49	3.47	2.39	18.41	19.55

Student's t test was performed for each of the phenotypic traits with all the marker classes. The potential relationship between the marker and trait was established considering the significance of the t test. It was found that a single marker was related with many traits and a single trait related to many markers. The marker which is having a strongest relationship can be judged from its adjusted R^2 value which will give the overall percentage of variability of that particular trait that the marker can explain. Single marker analysis for mean value of BC_1F_2 families for various traits is presented in Table 4.

Days to 50 *percent* flowering

Three markers were found to be associated with days to 50 *percent* flowering. Among them ORS 176 described the highest phenotypic variance of 4.45 with a p-value of 0.038. The marker ORS 1146 explained the phenotypic variance of 3.58 % with a p-value of 0.036 and the marker ORS 510 explained the phenotypic variance of 3.78 % with a p-value of 0.030.

Days to maturity

Four markers were found to be associated with days to maturity. Among these, ORS 1065 described the highest phenotypic variance of 9.94 % with a p-value of 0.005. The other markers viz., ORS 307 explained the phenotypic variance of 9.30 % with a p-value of 0.001, ORS 229 with phenotypic variance of 3.56% having p-value 0.044. The marker ORS 1147 explained phenotypic variance of 3.14% with p value 0.031.

Head diameter (cm)

The marker ORS 1146 was found to be associated with the head diameter with phenotypic variance of 2.46% having p-value 0.083

Oil content (%)

Four markers were found to be associated with oil content. Among these, the marker ORS 1065 described the highest phenotypic variance of 8.14 with a p-value of 0.01. The markers viz., ORS 307 explained the phenotypic variance of 5.32 % with a p-value of 0.041, ORS 613 with phenotypic variance of 3.80% having p-value 0.031 and the marker ORS 1146 exhibited phenotypic variance of 3.79% with p-value 0.03

Plant height (cm)

Three markers *viz.,* ORS 176, ORS 677, ORS 716 and ORS 833 were found to be associated with plant height exhibiting phenotypic variance of 3.68 %, 4.61 %, 3.59 % and 5.12 % with p-value 0.034, 0.017, 0.036 and 0.042 respectively.

Stem diameter (cm)

The marker ORS 606 was found to be associated with stem diameter exhibiting phenotypic variance of 3.19 % with p-value 0.011.

Table 4: Marker trait association: SSR markers associated with yield and yield component traits identified through single marker analysis

Trait	Markers	Probability value	r^2	r^2 (%)
Days to 50 *percent* flowering	ORS 176	0.038	0.04455	4.45
	ORS 1146	0.036	0.03581	3.58
	ORS 510	0.030	0.03702	3.78
Days to maturity	ORS 229	0.044	0.03557	3.56
	ORS 307	0.001	0.09297	9.30
	ORS 1065	0.005	0.09937	9.94
	ORS 1147	0.05	0.03138	3.14
Head diameter (cm)	ORS 1146	0.083	0.02460	2.46
Oil content (%)	ORS 307	0.041	0.05322	5.32
	ORS 613	0.031	0.03796	3.80
	ORS 1065	0.01	0.08142	8.14
	ORS 1146	0.03	0.03791	3.79
Plant height (cm)	ORS 176	0.034	0.03680	3.68
	ORS 677	0.017	0.04606	4.61
	ORS 716	0.036	0.03588	3.59
	ORS 833	0.042	0.05123	5.12
Stem diameter (cm)	ORS 606	0.011	0.03192	3.19
Test weight. 100 seeds (g)	ORS 677	0.05	0.03143	3.14
	ORS 3638	0.05	0.02906	2.91
Seed yield / plant (g)	ORS 613	0.06	0.06079	6.08
	ORS 691	0.03	0.03814	3.81
	ORS 1065	0.05	0.13008	13.01

Test weight 100 seeds (g): The two markers *viz.*, ORS 677 and ORS 3638 were found to be associated with test weight exhibiting phenotypic variance of 3.14 % and 2.91 % having p - value 0.05 and 0.05 respectively.

Seed yield / plant (g): Among three markers associated with yield / plant, ORS 1065 explained the highest phenotypic variance of 13.01 with a p-value of 0.05. The markers viz., ORS 613 explained the phenotypic variance of 6.08 % with a p-value of 0.06 and ORS 691 with phenotypic variance of 3.81% having p-value 0.03.

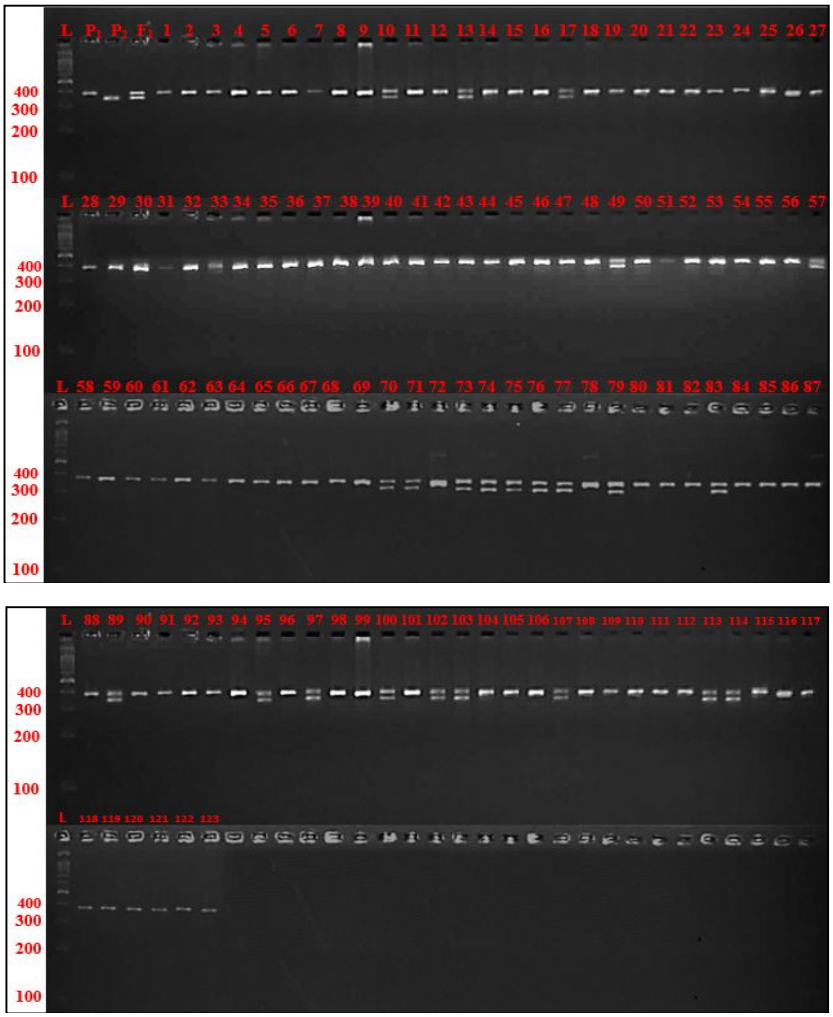

Fig 1: Genotyping of BC_1F_1 population (well: 1 to 123) with the SSR marker ORS 1146 along with the parents P_1: CMS 38B, P_2: HEL 153/83 and F_1: (CMS 38B × HEL 153/83).

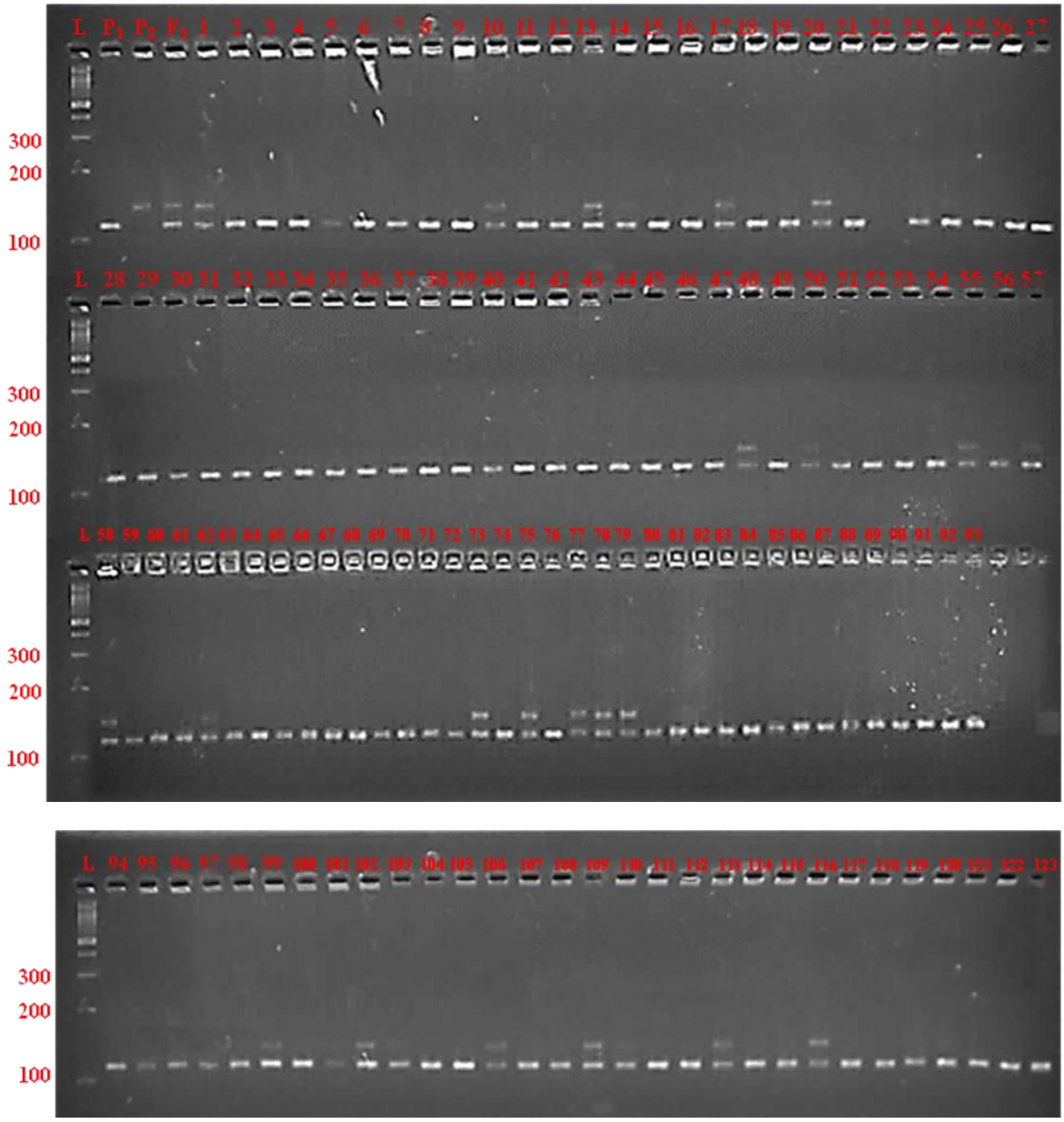

Fig 2: Genotyping of BC_1F_1 (well: 1 to 123) population with the SSR marker ORS 307 along with the parents P_1: CMS 38B, P_2: HEL 153/83 and F_1: (CMS 38B × HEL 153/83) using L: 100kb ladder

Discussion

A total of 14 markers gave significant association with at least one of the eight traits studied or more than one trait. Most of the markers were found to be related to more than one trait. In the present study, the marker ORS 1065 was found to be associated with days to maturity, oil content and seed yield per plant. The marker ORS 1146 was associated with days to 50 *percent* flowering, head diameter and oil content. Likewise, ORS 176 with days to 50 *percent* flowering and plant height, ORS 677 with plant height and test weight, ORS 613 with oil content and seed yield per plant and marker ORS 307 with days to maturity and oil content, ORS 229 with days to maturity, ORS 510 with days to 50 *percent* flowering, ORS 1147 with days to maturity, ORS 716 with plant height, ORS 833 with plant height, ORS 606 with stem diameter and ORS 691 with seed yield per plant.

ORS 1065 explained the highest phenotypic variance of 13.01 % for seed yield per plant followed by ORS 613 explaining the phenotypic variance of 6.08 %. Three markers were found to be associated with days to 50 *percent* flowering. Among them ORS 176 described the highest phenotypic variance of 4.45. Four markers were found to be associated with days to maturity. Among these, ORS 1065 described the highest phenotypic variance of 9.94 %. Four markers were found to be associated with oil content. Among these, the marker ORS 1065 described the highest phenotypic variance of 8.14 %. Three markers *viz.*, ORS 176, ORS 677, ORS 716 and ORS 833 were found to be associated with plant height exhibiting phenotypic variance of 3.68 %, 4.61 %, 3.59 % and 5.12 % respectively. Thus, a single marker was found to be associated with several traits in this study.

The same markers were also associated with different traits as reported by Bert *et al.* (2003) [2] ORS 229 was associated with stem diameter. In the studies of Vanitha *et al.* (2014) [10] the markers ORS 613 was associated with plant height, ORS 677 with head diameter, ORS 606 with volume weight, ORS 833 with test weight and oil content and ORS 1065 with kernel weight. ORS 510 and ORS 176 were associated with days to 50 *percent* flowering in the studies of Benjamin *et al.* (2011) [1]. ORS 716 was associated with plant height, ORS 307 for

head diameter in the studies of David and Jhon (2007) [5]. In the studies of Birkin *et al.* (2014) [3] the marker ORS 561 found to be associated with plant height using SNP based linkage map. In the studies of Junfang Chen *et al.* (2006) [6], the primers ORS 613, ORS 691 were linked to *ms9* gene.

Conclusion

In the present study, out of sixteen polymorphic SSR loci, fourteen of them have shown association with specific traits. This result is surprising and this needs thorough validation in large population size in different genetic backgrounds upon which these markers can be used in marker assisted selection.

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