MOLECULAR DIVERSITY OF nrDNA ITS REGION AFFECTED HETEROSIS IN SUNFLOWER (*Helianthus annuus*)

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ABSTRACT

A study was conducted to specify mutations of the internal transcribed spacer (ITS) in the nucleotides of nuclear ribosomal DNA (nrDNA) and their molecular relationship with heterosis exhibited by the six A- lines and three R-testers of sunflower. The trail was conducted at the fields of the College of Agriculture, University of Anbar, Abu Ghraib during two seasons (spring and fall 2016). Parental lines were crossed according to lineXtester crossing scheme to produce 18 single hybrids, genotypes were sown to assess their phenotypic performance in the fall season. Results of the molecular analysis of ITS sequencing showed that the rearrangement of single nucleotides had accumulated in a higher rate in the F_1 hybrids compared with their ancestor inbreds. The total number of mutations was 268, and deletion mutations accounted for the largest proportion with 209 mutation (71 in lines and 138 in hybrids). In contrast, the transition mutations were 25 all occurred in the hybrids, however the number of transversions recorded 17only. Based on DNA sequence of the nrDNA ITS region the total genotypes were separated into two main groups in cluster analysis following the nearest neighbor method. There was a significant convergence between R2 and both A4XR2 and A6XR3 hybrids scoring higher values of similarity in context ITS sequence. To be specific, the hybrid A2XR3 exhibited the best desirable heterosis for days to 75% flowering and leaf area, while hybrid A5XR3 had the highest heterosis for head traits, height and area.

Keywords: nrDNA; ITS; sunflower; *Helianthus annuus*; CMS; heterosis
***Part of Ph.D. dissertation of the 1st author**

التنوع الجزيئي لمنطقة nrDNA ITS أثّر في قوة الهجين في زهرة الشمس (*Helianthus annuus*)

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المستخلص

أجريت الدراسة لتحديد الطفرات ضمن منطقة ITS في نكليوتايدات الحامض النووي الريبوزي (nrDNA) وعلاقتها الجزيئية مع قوة الهجين التي تظهرها ست سلالات عقيمةً (A–lines) وثلاثة خصبة (R–testers) من زهرة الشمس. نفذت التجربة في حقول كلية الزراعة ، جامعة الأنبار ، أبو غريب خلال الموسمين الربيعي والخريفي 2016. ضربت السلالات الأبوية وفقًا لتضريب السلالةx الفاحص لإنتاج 18 هجينًا فرديًا. تم زراعة التراكيب الوراثية لتقييم أدائها المظهري في الموسم الخريفي. أظهرت نتائج التحليل الجزيئي أن إعادة ترتيب النكليوتايدات الفردية قد تراكم بمعدل أعلى في الهجن مقارنة مع السلالات الأبوية. وكان العدد الإجمالي للطفرات 268 طفرة، شكلت طفرات الحذف النسبة الأكبر مع 209 طفرة (71 في السلالات و 138 في الهجن). بالمقابل، كانت جميع الطفرات الانتقالية (25 طفرة) في الهجين، في حين بلغت عدد طفرات التحول 17 طفرة فقط. استناداً إلى تتابعات الدنا لمنطقة nrDNA ITS ، انفصلت التراكيب الوراثية إلى مجموعتين رئيسيتين في التحليل العنقودي وفقا لطريقة الجار الأقرب. كان هنالك تقارب كبير بين الفاحص R2 وكل من الهجينين A4XR2 و A6XR3، عندما سجلت أعلى قيم للتشابه فيما يتعلق بتسلسل ITS. على وجه التحديد، كان للهجين A2XR3 أفضل قوة هجين لعدد الأيام إلى 75٪ تزهير ومساحة الأوراق، بينما أعطى الهجين A5XR3 أعلى قوة هجين لمساحة القرص وارتفاع النبات.

الكلمات المفتاحية: nrDNA، ITS ، زهرة الشمس، *Helianthus annuus*، CMS ، قوة الهجين

***جزء من اطروحة دكتوراه للباحث الاول**

***Received:22/8/2018, Accepted:24/2/2019**

INTRODUCTION

Sunflower (*Helianthus annuus*) is an important crop belongs to the largest plant family (Asteraceae), and mainly growing for premium quality oil production. The developing sunflower crop is highly restricted by the narrow genetic background due to limited gene pool. Thus, much attention has been directed to widen the genetic base of sunflower via either conventional or modern biotechnologies (14). Sunflower crop has undergone substantial genetic and phenotypic changes during domestication process. Therefore, the discovering of cytoplasmic male sterility (CMS) and exploring the restorer fertility (RF) considered a main pillars for the previously mentioned changes that lead to considerable alteration in sunflower production to hybrid breeding (5),(15). The revolutionary effect of heterosis in different plant species encourages specialists to pay considerable attention to this unique phenomenon. Consequently, there is always a strong demand to know the way of genes action and interaction that serve in tuning such actions via customizing breeding methods and developing heterotic groups (12). The genetic mechanism laying behind heterosis still not consistent, hence researchers continuing to push forward but not for sure new assumptions to solve the puzzle of heterosis (20). However, in the last two decades, significant progress was made in developing new molecular tools and/or updating previous strategies that facilitating the researchers mission. High priority has been given to study Internal Transcribed Spacer (ITS) as a prominence sequence data for phylogenic studies in plants (7),(18). Apparently, the detection of single nucleotide polymorphisms (SNPs) is an important addition to the molecular diagnosing of genetic diversity for many reasons. For instance, such domains are associated with key phenotypic and physiological variants in many plant species cv(8),(3),(1). Furthermore, the nucleotides diversity in the nuclear ribosomal DNA (nrDNA) of ITS region can provide an efficient tool for characterizing genotypes heterogeneity as well as the classification of different species (2).The study was proposed to evaluate the possible polymorphism in the nucleotides of nuclear ribosomal DNA (nrDNA) and their relationship with heterosis in different cms sources of sunflower

MATERIALS AND METHODS

Genomic DNA extraction

A field study was conducted during two growing seasons (spring and fall, 2016) using six male sterile A-lines (A1 to A6) and three fertility restorer R-lines (R1, R2 and R3) obtained from Seed Test and Certification Board (STCB), Ministry of Agriculture. The genotypes were crossed according to line X tester matting system in the first season to be evaluated in the second. DNA was extracted from seven leaf-stage seedlings in the STCB labs. using Genomic DNA Mini Kit-Plant (Geneaid Biotech Ltd., South Korea).

DNA Quantity and Quality

Nanodrop was used to check the DNA quality according to the following formula:

$$Purity\ of\ DNA = O.D_{260} / O.D_{280} = \geq 1.8$$

Reads ranged between 1.8-2, and D oNA concentration was adjusted to 50 ng/µl final concentration(18).

Amplification of ITS region

Polymerase chain reaction (PCR) was performed and the specific primer (F-5'-TCCGTAGGTGAACCTGCGG-3) was used to amplify the ITS region in the targeted genomes (2). The mixture of PCR reaction was 12.5 µl of Green Master Mix, 1µl of primer, 3 µl of DNA template, finally volume was completed to 25 µl using nuclease-free water. The samples were submitted to the PCR machine for amplification step with the following thermal profile; Initial denaturation and denaturation at 95^{o}C for 4 min, Denaturation step was at 55^{o}C for 30 sec., extension and final extension was at 72^{o}C for 1 and 7 min., respectively. The ITS region sequence of the pooled plant samples were identified by applying Sanger sequencing method in (Macrogen Inc., Seoul, Korea) with the aid of ABI 3730 Genetic Analyzer (Applied Biosystems, USA).

Electrophoresis

Agaros gel was prepared by adding 2 g of agarose to 100 ml of 1X TAE buffer (16). Ten microliters from each amplification product were loaded into the wells. Electrophoresis was performed at a voltage of 5 volt cm^{-1} till DNA reached the edge of the gel. Agarose gel

was exposed to a UV transilluminator, pictured and documented at 340 nm cannon.

Statistical analysis

DNA sequencing of ITS region was analyzed using MEGA6 software (Molecular Evolutionary Genetics Analysis version 6.0) to identify SNP cases (single nucleotide polymorphism) within the targeted genomes. Cluster analysis of SNP results were estimated using Euclidean distance according to the nearest neighbor method (2).

Heterosis

Heterosis was calculated as stated by Laosuwan and Atkins (1997) using the following formula: Heterosis (H) % = {(F1-BP)/BP} ×100.

RESULTS AND DISCUSSION

Single nucleotide Polymorphism (SNP) of the nrDNA ITS sequence

The results of nrDNA ITS sequences (Figure 1) showed that alterations in the single nucleotides have occurred at a higher rate in hybrids compared to their parental inbred lines and testers . The counted total of mutations was about 268 (Table 1), deletion mutations took the lead as it scored a total of 209 detected within most studied genotypes (71 in the inbreds and 138 in hybrids). These kinds of mutations were residents in a specific loci 450, 549, 550, 570, 579, 580, 661 and 667, except for R2, A4×R2 and A6×R3 genotypes that showed addition mutation in loci 540, 549, 570, 579, 661 or adding guanine (G) in loci 550, 580 and 667. Mutation of transition occurred in A2×R3, A3×R1, A3×R2, A4×R1, A4×R2, A5×R2 and A6×R3 hybrids, most of which were G>A (guanine to adenin), while there were three mutations of A>G (adenine to guanine) in A3×R1, A5×R1 and A6×R3 hybrids and three mutations of T>C (thymine to cytosine) exhibited by the three hybrids (A5×R1, A5×R3, A6×R3). Only one mutation was found to be C>T (cytosine to thymine) in A6×R3 hybrid at the nucleotide loci of 576. Transversion mutations recorded a total of 17 mutations, substitution of adenine (A) with thymine (T) occurred in five hybrids (A2×R3, A3×R1, A3×R2, A5×R3 and A6×R3). The other type of substitution was T>A occurred at single locus in A4×R2 hybrid and at two loci (502 and 613) in A6×R3 hybrid. The A nucleotide substituted C (C>A) in A3×R1, A5×R1 and A6×R3 hybrids, however T>G was a single transversion mutation displayed by A5×R1 hybrid, meanwhile G>T transversion revealed by A6×R3 hybrid and A>C transversion by A2×R3 hybrid.

Table 1 (A). Single nucleotides polymorphism of the nrDNA ITS sequence in sunflower

No.	Genotype	Type of substitution	Location	Nucleotide
1	A1	Deletion	540, 549, 550, 570, 579,580, 661, 667, 692	------
2	A2	Deletion	540, 549, 550, 570, 579,580, 661, 667	------
3	A3	Deletion	540, 549, 550, 570, 579,580, 661, 667	------
4	A4	Deletion	540, 549, 550, 570, 579,580, 661, 667,687	------
5	A5	Deletion	540, 549, 550, 570, 579,580, 661, 667,692	------
6	A6	Deletion	540, 549, 550, 570, 579,580, 661, 667,692	------
7	R1	Deletion	540, 549, 550, 570, 579,580, 661, 667	------
8	R2	Deletion	10	------
9	R3	Deletion	540, 549, 550, 570, 579,580, 661, 667	------
10	A1×R1	Deletion Transition Addition	540, 549, 550, 570, 579,580, 661, 667 697 692	------ G>A G
11	A1×R2	Deletion	540, 549, 550, 570, 579,580, 661, 667,694,695	-------
12	A1×R3	Deletion Addition	540, 549, 550, 570, 579,580, 661, 667,682 692	------- A
13	A2×R1	Deletion	540, 549, 550, 570, 579,580, 661, 667,692	--------
14	A2×R2	Deletion	540, 549, 550, 570, 579,580, 661, 667	
15	A2×R3	Deletion Transition Transversion Transversion	540, 549, 550, 570, 579,580, 661, 667 693, 696, 697 685 689	------- G>A A>T A>C
16	A3×R1	Deletion Transversion Transversion Transversion Transition	540, 549, 550, 570, 579,580, 661, 667, 688 1 690 693 694, 695	-------- A>T C>A G>C A>G
17	A3×R2	Deletion Transition Transversion	540, 549, 550, 570, 579,580, 661, 667, 692 78 686	------ G>A A>T
18	A3×R3	Deletion	540, 549, 550, 570, 579,580, 661, 667,692	------
19	A4×R1	Deletion Transition	540, 549, 550, 570, 579,580, 661, 667 694,698	------ G>A
20	A4×R2	Deletion Transition Transversion Addition Addition	10 456 502 540,549,570, 579, 661 550,667,580	------ G>A T>A T G

Table 1(B). Single nucleotides polymorphism of the nrDNA ITS sequence in sunflower

No.	Genotype	Type of substitution	Location	Nucleotide
21	A4× R3	Deletion	540, 549, 550, 570, 579,580, 661, 667	------
22	A5× R1	Deletion	540, 549, 550, 661, 667, 570, 579,580	------
		Transition	683	T>C
		Transition	696	A>G
		Transversion	684	C>A
		Transversion	688	T>G
23	A5× R2	Deletion	540, 549, 550, 661, 667, 570, 579,580,686,687	------
		Transition	691	G>A
		Transversion	693	G>C
24	A5×R3	Deletion	540, 549, 550, 661, 667, 570, 579,580	------
		Transition	698	G>A
		Transition	683	T>C
		Transversion	682	A>T
25	A6×R1	Deletion	540, 549, 550, 661, 667, 570, 579,580	------
		Transition	78	G>A
		Transition	194	A>G
26	A6×R2	Deletion	540, 549, 550, 661, 667, 570, 579,580,692	------
27	A6×R3	Deletion	10	------
		Transition	551, 557,588,581,650, 670	G>A
		Transition	675	A>G
		Transition	576	C>T
		Transition	577	T>C
		Transversion	502,613	T>A
		Transversion	697	G>T
		Transversion	687, 688	A>T
		Transversion	678	G>C
		Transversion	623	C>A
		Addition	540,549,570, 579, 661	T
		Addition	550,667,580	G

Cluster analysis of nrDNA ITS sequence

Cluster analysis for single nucleotide polymorphism (SNP) of the ITS region in inbred lines, testers and their F1 hybrids was accomplished according to the nearest neighbor method (Figure 2). Results indicated that the studied genotypes distributed into two main groups, the first consisted of three genotypes (R2, A4×R2 and A6×R3), meanwhile the second group divided into many sub-groups. It can be noticed that most of the studied genotypes tended to organize in pairs according to its ITS sequence showing high level of genetic similarity. Some other genotypes revealed unique pattern of ITS sequence, hence genetic distinctness by occupying single sub-clusters (A2, A6, R2, A1×R3 and A5×R3). In the same context, hybridization process had an effective role in shaping unique DNA sequence in hybrids, like A5×R2 that was the most divergent genotype in term of nrDNA sequence. The estimated Euclidean distance among the studied cms genotypes of sunflower (Table 2) approved the existence of common relationship between the R2 tester and its descended hybrid A4×R2 and A6×R3 hybrids resulting in too high values with all other genotypes not less than 0.243. This is in part of it genotypes expressed almost the mutation types (Table 1). Meanwhile, the highest value (0.305) was against the inbred line A3. In addition, A4×R2 and A6×R3 hybrids showed the highest value of the Euclidean distance with all other hybrids. The highest value was 0.0336 for A4×R2 hybrid against A1×R2 hybrid. The A6×R3 single hybrid gave the highest value (0.0352) with A6×R1 hybrid. where they showed the lowest value of the Euclidean distance (0.0015) in A4×R3, A3×R3, A6×R2 and A4×R2 hybrids

Figure 1. Single nucleotide Polymorphism (SNP) of the nrDNA ITS sequence in inbreds (1-6), testers (7-9) and lineXtester hybrids (10-27) of sunflower

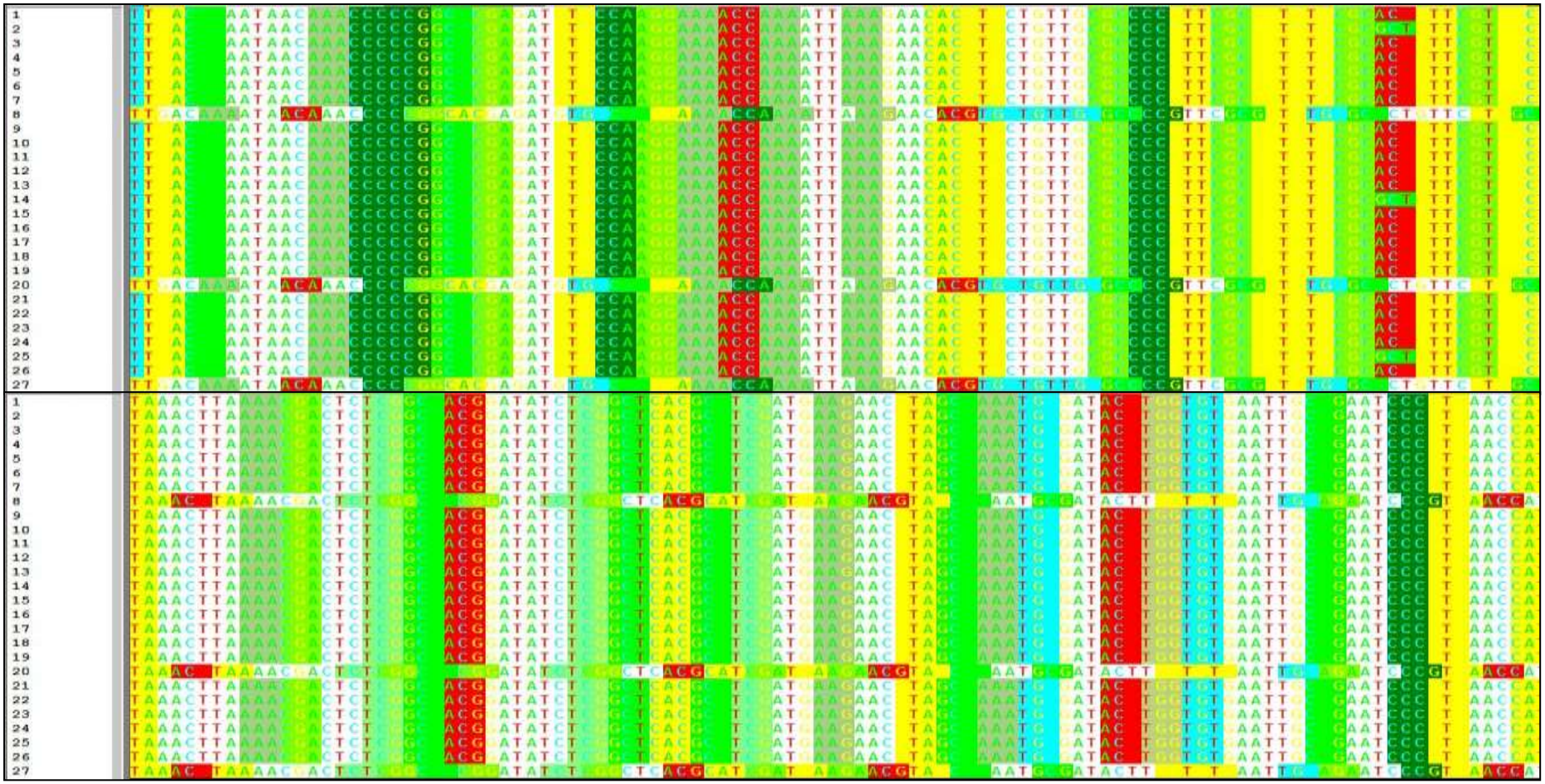

Figure 2. Dendrogram diagram of SNPs for the nrDNA ITS sequence of A-Lines (1-6) R-Lines (7-9) and hybrid (10-27) of sunflower

Heterosis

Number of days to 75% flowering

Heterosis for flowering time was calculated according to the deviation of the first generation over the earliest parent (Table 3). The observed heterosis was in significant negative values for some hybrids and ranging from -6.01% in A2×R3 hybrid to -1.27% in A1×R2 hybrid, which approved the overdominance effect of the early parents genes implying that these hybrids could mature earlier and avoid the undesirable environmental conditions. The other hybrids acted in different way as it exhibited a significant positive effect of partial dominance genes of the early parent when it had positive values ranging from 4.62% for A3×R3 hybrid to 1.24% for A2×R1 hybrid. These findings may due to the high frequency of different mutations (deletion, transition and transversion) within ITS region of hybrids genomes (9),(19), (10).

Plant height (cm)

Although negative heterosis for plant height is desirable, most of the tested hybrids tended to be taller than their inbred parents, hence they had a positive heterosis degrees (Table 3). Among the 18 experienced F_1 hybrids, ten exhibited significant positive values of heterosis ranging from 13.29% in A5×R2 hybrid to 7.47% in A3×R1 hybrid. Such estimates indicated the effect of overdominance gene action of the taller parent for plant height trait. The different polymorphism rate of ITS region may play a key role in magnifying the total diversity, which in turn improved the chances of getting heterosis in the desired direction (13), (6),(17).

Leaf area (cm^2)

Heterosis expressed by the single hybrids for leaf area show in Table 3. The magnitude of heterosis ranged from significant positive to negative values for the this trait. However, the majority of the studied hybrids (17 hybrid) expressed significant heterosis values. The highest positive estimate was 63.51% in A2×R3 hybrid against the lowest (3.38%) showed by A6×R3 hybrid. Whereas, others hybrids gave significant negative values of heterosis ranged between -67.79% in A6×R2 hybrid to -3.59% in A3×R2 hybrid. The positive values of heterosis indicated the presence of over-dominance genes action, meanwhile the negative values pointed to partial-dominance gene of action of the best parent (17).

Table 2. Values of Euclidean distance of SNPs for nrDNA ITS sequence in A-Lines (1-6) R-Lines (7-9) and hybrid (10-27) of sunflow

1																											
2	0.0030																										
3	0.0105	0.0105																									
4	0.0060	0.0090	0.0105																								
5	0.0030	0.0030	0.0075	0.0060																							
6	0.0030	0.0030	0.0075	0.0060	0.0030																						
7	0.0045	0.0045	0.0060	0.0075	0.0045	0.0015																					
8	0.0274	0.0305	0.0305	0.0243	0.0274	0.0274	0.0290																				
9	0.0045	0.0045	0.0060	0.0075	0.0015	0.0045	0.0060	0.0290																			
10	0.0120	0.0120	0.0120	0.0135	0.0090	0.0120	0.0135	0.0336	0.0090																		
11	0.0030	0.0030	0.0105	0.0075	0.0030	0.0030	0.0045	0.0289	0.0045	0.0090																	
12	0.0075	0.0075	0.0030	0.0105	0.0045	0.0075	0.0060	0.0305	0.0030	0.0090	0.0075																
13	0.0000	0.0030	0.0105	0.0060	0.0030	0.0030	0.0045	0.0274	0.0045	0.0120	0.0030	0.0075															
14	0.0030	0.0000	0.0105	0.0090	0.0030	0.0030	0.0045	0.0305	0.0045	0.0120	0.0030	0.0075	0.0030														
15	0.0105	0.0105	0.0000	0.0105	0.0075	0.0075	0.0060	0.0305	0.0060	0.0120	0.0105	0.0030	0.0105	0.0105													
16	0.0060	0.0090	0.0105	0.0000	0.0060	0.0060	0.0075	0.0243	0.0075	0.0135	0.0075	0.0105	0.0060	0.0090	0.0105												
17	0.0030	0.0030	0.0075	0.0060	0.0000	0.0030	0.0045	0.0274	0.0015	0.0090	0.0030	0.0045	0.0030	0.0030	0.0075	0.0060											
18	0.0030	0.0030	0.0075	0.0060	0.0030	0.0000	0.0015	0.0274	0.0045	0.0120	0.0030	0.0075	0.0030	0.0030	0.0075	0.0060	0.0030										
19	0.0045	0.0045	0.0060	0.0075	0.0045	0.0015	0.0000	0.0290	0.0060	0.0135	0.0045	0.0060	0.0045	0.0045	0.0060	0.0075	0.0045	0.0015									
20	0.0274	0.0305	0.0305	0.0243	0.0274	0.0274	0.0290	0.0000	0.0290	0.0336	0.0289	0.0305	0.0274	0.0305	0.0305	0.0243	0.0274	0.0274	0.0290								
21	0.0045	0.0045	0.0060	0.0075	0.0015	0.0045	0.0060	0.0290	0.0000	0.0090	0.0045	0.0030	0.0045	0.0045	0.0060	0.0075	0.0015	0.0045	0.0060	0.0290							
22	0.0120	0.0120	0.0120	0.0135	0.0090	0.0120	0.0135	0.0336	0.0090	0.0000	0.0090	0.0090	0.0120	0.0120	0.0120	0.0135	0.0090	0.0120	0.0135	0.0336	0.0090						
23	0.0045	0.0075	0.0120	0.0060	0.0060	0.0075	0.0090	0.0274	0.0060	0.0120	0.0060	0.0090	0.0045	0.0075	0.0120	0.0060	0.0060	0.0075	0.0090	0.0274	0.0060	0.0120					
24	0.0120	0.0120	0.0090	0.0150	0.0105	0.0120	0.0105	0.0352	0.0090	0.0060	0.0090	0.0060	0.0120	0.0120	0.0090	0.0150	0.0105	0.0120	0.0105	0.0352	0.0090	0.0060	0.0105				
25	0.0030	0.0000	0.0105	0.0090	0.0030	0.0030	0.0045	0.0305	0.0045	0.0120	0.0030	0.0075	0.0030	0.0000	0.0105	0.0090	0.0030	0.0030	0.0045	0.0305	0.0045	0.0120	0.0075	0.0120			
26	0.0030	0.0030	0.0075	0.0060	0.0030	0.0000	0.0015	0.0274	0.0045	0.0120	0.0030	0.0075	0.0030	0.0030	0.0075	0.0060	0.0030	0.0000	0.0015	0.0274	0.0045	0.0120	0.0075	0.0120	0.0030		
27	0.0274	0.0305	0.0305	0.0243	0.0274	0.0274	0.0290	0.0000	0.0290	0.0336	0.0289	0.0305	0.0274	0.0305	0.0305	0.0243	0.0274	0.0274	0.0290	0.0000	0.0290	0.0336	0.0274	0.0352	0.0305	0.0274	

Head area (cm^2)

Head area represents a key secondary component of yield trait simply because it accommodates many and/or large seeds. Heterosis percentage for head area varied in response to the genetic and epigenetic differences between the crossed parental lines. Heterosis percentage was positive and significant for this trait in two hybrids only, nevertheless A5×R3 was in the lead scoring 44.06%. These results were further supported by ITS sequencing, especially as this hybrid has two genetically distinct parents (A5 and R3) raising a great single nucleotide polymorphism in ITS domain. The negative values ranged between the minimum(-66.45%) in hybrid A6×R2 and the highest (-5.94%) in A6×R1. These results indicate a clear effect of the over-dominance gene action in the hybrids that gave positive heterosis, while the effect of partial-dominance marked the hybrids with negative heterosis (4), (10).

Table 3. Percentage of heterosis of line×teser matting sachem in sunflower

Hybrids	Days to 75% flowering	Plant height (cm)	Leaf area (cm^2)	Head area (cm^2)
$A_1 \times R_1$	1.88	14.98	-29.54	-53.81
$A_1 \times R_2$	-1.27	0.16	-25	-57.11
$A_1 \times R_3$	-3.03	12.06	45.45	23.65
$A_2 \times R_1$	1.24	1.87	-18.42	-50.51
$A_2 \times R_2$	-0.63	8.66	44.73	16.31
$A_2 \times R_3$	-6.01	8.10	63.15	11.39
$A_3 \times R_1$	-1.98	7.47	00.00	-9.88
$A_3 \times R_2$	-1.33	-2.83	-56.89	-61.97
$A_3 \times R_3$	4.62	9.08	-8.62	-10.53
$A_4 \times R_1$	-1.92	5.45	15.38	12.45
$A4 \times R_2$	-1.93	4.85	-53.84	-52.14
$A_4 \times R_3$	1.26	9.14	3.84	2.87
$A_5 \times R_1$	-4.45	4.29	-9.80	1.52
$A_5 \times R_2$	-4.51	13.29	-50.98	-52.10
$A_5 \times R_3$	-0.63	16.18	41.17	44.06
$A_6 \times R_1$	-4.41	-5.98	3.38	-5.94
$A_6 \times R_2$	-3.21	-2.22	-67.79	-66.45
$A_6 \times R_3$	-1.79	-4.63	3.38	7.48
S.E (sij)	1.19	6.10	0.016	23.33

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