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GENETIC DIVERSITY FOR SUNFLOWER A, B AND RF LINES USING MOLECULAR APPROACHES

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ABSTRACT

Behavior of ten cytoplasmic male sterile (CMS) lines and combination with 10 restorer lines have been studied. The aim was to determine the stability of male sterility in the field and the similarity among the CMS lines per seed and between CMS lines and restorer lines via molecoular markers. Fertility restoration was determined for all cytoplasmic male sterility sources. The results showed that CMS lines A 9-18 and 32 were stable lines. Also, lines 9, 21 and 32, showed good matching in flowering date with the maintainer. Molecular markers showed that genetic distances, cytoplasmic male sterility (CMS) lines were divided into 2 sub -clusters, line 34 was in one sub cluster, the second sub cluster included 2 groups. The first group included lines 32; 33; 21; 17; 27 and 24, group 2 included lines 18; 9 and 19. The second cluster fertility restorer (Rf) was divided into 2 sub- clusters only, Rf15 was in the first sub- cluster. But the second sub-cluster , included 2 groups : Group 1 contains Rf 12,11 and 10 , and group 2 contains Rf 8; 7;4; 3; 2 and 1 . The combined data showed that line 34 was the most diverse line in relation to its genetic back ground. Besides, heterotic effects are more expected when using this line female with Rf parent .In contrast line A 19 showed low similarity index with Rf 1; 2 and 3 indicating low expected heterotic effects.

The best in five hybrids gave hybrids A33xRf15 was the earliest in flowering ,while sakha 53 was the latest , A33x Rf 15 gave the highest value of plant height , while the hybrid A34x Rf15 was the shortest .Also stem diameter sakha 53 gave the thick in stem diameter ,while A21xRf15 gave the thin in stem diameter .Also head diameter the hybrid A34xRf 15 give the best size in head diameter, while hybrid A32 x Rf 15 gave the small size in head diameter .Also hybrid A33xRf15 gave the highest value of 100 seed weight , while the hybrid A19XRf10 had the lowest one .also hybrid A33xRf 15 gave the highest seed yield /plant, while hybrid A32xRf15 gave the lowest yield . Also seed yield /faden, the hybrid A33x Rf15 gave the highest seed yield /fed an, while A32 xRf 15 gave the lowest .Also hybrid A32XRf 15 gave the highest in %of oil ,while sakha 53 gave the lowest.

Key words: ***Sunflower, (CMS), (B), (Rf) lines, Breeding, Molecular markers.***

INTRODUCTION

The first induction of cytoplasmic male sterility (CMS) in sunflower was reported by Leclercq (1969). He found that cytoplasmic male sterility was inherited as maternal trait. The first CMS type was discovered in a progeny of inter-specific crosses between *Helianthus petiolris* Nutt and *Helilanthus. annuss* L. (Leclercq 1969).

The identification and development of male sterile and fertility restorer lines was a major step for the success of sunflower hybrid breeding programs. Modern sunflower breeding with the development of F_1 hybrids began after the discovery of cytoplasmic male sterility (Leclercq 1970) and fertility restorer genes (Kinman1970). More than forty sources of cytoplasmic male sterility are known (Miller 1992). The discovery of a restorer gene encouraged the development of commercial sunflower hybrid production in a large scale since 1972.The restoration of pollen fertility in *Petiolaris* (PETI) cytoplasm has been reported to be controlled by a single dominant gene (Enns 1972, Leclercq 1 972, Jan *et al* 2000 and Seiler 2000).

However, many sunflower hybrids that are commercially grown have a single source of CMS. This lead to homogeneity and potential risk. Diversification of CMS sources is inevitable in any hybrid breeding program, more than 62 new CMS sources of different origins have been reported in sunflower (Serieys 1999). Diversity of the new sources and importance of CMS was assessed based on cytoplasmic male sterility and fertility restoration system (Serieys and Vincourt 1987 and Serieys 1994). Only few investigations described the interaction between cytoplasm and nuclear genes in the expression of several quantitative and qualitative characters in sunflower, whereas, beneficial cytoplasmic nuclear interaction have been reported in various crops (Jan1992).In sunflower, a unique cytoplasmic nuclear interaction caused reduction in chlorophyll content. New CMS sources and their respective fertility restorer genes have been found (Serieys 1969 and 1999). Different cytoplasmic backgrounds to be utilization hybrids production will permit the variability increase of cultivated sunflower and help solving parent problems that occur with cytoplasmic male sterility from the source of (CMS)-widely used i.e, *petiolris* sources (PETI). In some cases, restoration complementary dominant genes are also reported (Jan *et al* 2000). The development of new fertility restorers by traditional back -crossing is costly and time consuming because it requires extensive crossing programs .Identification of molecular markers closely linked to fertility restoring genes facilitates the breeding of new restorer lines. Random amplified polymorphic DNA (RAPD)markers have been developed (Wiliams *et al* 1990, Welsh 1990 and McCleland, 1990).RAPDs is generated by amplification of genomic DNA using a single primer of arbitrary nucleotide sequence to drive the amplification reaction.

Many researchers are now using RAPD to construct genetic maps. The most useful application of RAPD markers is to quickly generate markers within a genomic region of interest using near isogenic lines (Martin *et al* 1991).The advantage of this technology is that markers are targeted to a smaller region within the genome (Michelmore *et al* 1991). The main goal of sunflower breeding program as an oil crop is to maximize seed production /head and seed oil content, which could be reached by hybrid

production. Many investigations have been carried out to establish conventional breeding programs for hybrid production but the main obstacle was the production of cytoplasmic male sterile (CMS) lines. Conventional methods could be used to maintain CMS and restore its fertility but they are costly, time and labor consuming. In Egypt some investigations for this purpose were previously conducted but, still, the main problem is how to identify CMS in early stages (seedling stage) and how can we select parents to be used in sunflower hybrid production.

The aims of the study are, 1) Search for CMS sources that possess restoration systems other than CMS-PETI and to investigate alternative CMS –Rf systems and their utilization in breeding programs.
2) Estimate the genetic relationships among these CMS and Rf, and compare the ability of these different markers in detecting the genetic diversity, of ten CMS, and fertility restorer (Rf lines).

MATERIALS AND METHODS

Plant Material The material of the present work included ten cytoplasmic male sterility sources to be used as female parents in any hybrid(A lines).They were lines number 19, 9, 18, 24, 27, 17, 21, 33, 32 and 34 and their ten isogenic maintainer B lines in addition to ten pollen parents that are included. They fertile and can restorer they fertility of the female (A) parent (Rf) lines male were also were lines maintainers 1, 2, 3, 4, 7, 8, 10, 11, 12and 15. All lines (A,Band Rf) were obtained from the Oil Crops Research Section, Field Crops Research Institute (FCRI),Agriculture Research Center (ARC). Evaluation was performed during the summer seasons 2006-2008, at Giza Research Station. ARC, Giza, Egypt. The description of material is presented in Table (2). Selection of different inbred lines was based on matching in flowering date, with the restorer lines their, stability in cytoplasmic male sterility, and their morphology.

Similarity and /or diversity via molecular markers: Extracted DNA from different inbred lines were tested against twenty arbitrary chosen 10 mer RAPD primers, the universal names and sequences of tested primers are presented in Table (1).

Methods Seeds from all studied lines including (A and B) were planted in 2006,2007 and 2008 seasons, mean while Rf lines were sown 11-13 days earlier. Phenological observation and measurements were made during the vegetation period. Number of fertile and sterile plants was counted during the stage of flowering date.

Extraction and purification of genomic DNA was performed using a modified CTAB (hexadecyl trimethyl ammonium bromide).The procedure is based on the protocol suggested by Sue- Porebsk *et al* (1997). Random

Table 1. Sequence of the random ten mer RAPD primers

No	Primer	Sequences 5------3	GC%
1	OPA-9	5GGGTAACGCC3	70%
2	OPA-12	5TCGGCGATAG3	60%
3	OPA-17	5GACCGCTTGT3	60%
4	OPA-19	5CAAACGTCGG3	60%
5	OPB-07	5AGATGCAGCC3	60%
6	OPC-15	5GACGGATCAG3	60%
7	OPC-19	5GTTGCCAGCC3	70%
8	OPD-3	5GTCGCCGTCA3	70%
9	OPD-4	5TCTGGTGAGG3	60%
10	OPD-12	5CACCGTATCC 3	60%
11	OPF-1	5ACGGATCCTG3	60%
12	OPF-7	5CCGATATCCC3	60%
13	OPF-13	5GGCTGCAGAA3	60%
14	OPF-16	5AGCGTCCTCC3	70%
15	OPL-10	5TGGGAGATGG3	60%
16	OPN-4	5GACCGACCCA3	70%
17	OPN-12	5CACAGACACC3	60%
18	OPN-20	5GGTGCTCCGT3	70%
19	OPZ-07	5CCAGGAGGAC3	70%
20	OPZ-09	5CACCCCAGTC3	70%

amplified polymorphic DNA (RAPD) analysis was carried out using ten CMS inbred lines and their fertility restorer (Rf). Genomic DNA was used as template for polymerase chain reaction (PCR) amplification and performed as described by Williams *et al* (1990). A set of 20 arbiratar primers operon (Table 1) were synthesized in Germany, to produce distinct marker profiles for ten CMS lines and their restorer fertility PCR reaction were performed in 50 UL, using 15 ng as DNA template, 4Mm MgL_2 ,10 Mm Tris HCL_2, 1Mm EDTA, pH8.4, 200 UM each of dNTPs, 40 p moles of each primer, and 2.5 U Taq DNA polymerase reaction were overlaid by mineral oil. Programmed for 5 min at 94 C° for one cycle. One min 94C°, 1.5 min 36C°, 2min 72C°,during 40 cycle ,and 5 min end , extension at 72 C°. Then followed by soaking at 4C° Amplification products were separated by electrophoresis on 1.5 % agarose gel in 1XT BE buffer.

The five hybrids and a check cultivar sakha 53 were grown in the field following randomized complete block design with three replications. Each entry was grown in 3 rows length 4.5 m /replication with a spacing of 60x20.Thining was done leaving one plant/hill .Data recoded on a sample of Ten plants individual plants per plot, flowering date (days),plant height (cm), stem diameter (cm), head diameter (cm), 100 seed weight (g) seed

yield/plant(g) seed yield /faden and %of oil .Means were compared by using Duncans Multiple Rang Test as outlined by Steel and torrie(1980).

Each flowering was also carried out to ensure complete seed set in the hybrids. Ten plants per plot were randomly chosen to record observations on eight quantitive characters. Then mean values of different hybrids were compared to assess the influence of cytoplasm on selected characters.

RESULTS AND DISCUSSION

1- Stability of the sterility in the field: The stability of selected CMS lines was determined all over the investigated period of the three seasons (Table 2). The studied CMS lines showed different levels of reversion to normal fertile lines, stability varied from88.9 to 100%. The three lines. A9, A-18 and A-32 showed no reversion .The results in Table (2) indicated that the lines under study were stable in CMS performance and could be considered true CMS lines.

Table2. Stability of CMS lines determined according to number of reversed plants all over the tested season.

Inbred lines code	Number of tested plants*	Number of reversed plants*	CMS stability %
A-19	25	2	92
A-9	23	0	100
A-18	14	0	100
A-24	27	3	88.9
A-27	21	1	95.2
A-17	21	2	90.5
A-21	20	2	90
A-33	23	1	95.7
A-32	23	0	100
A-34	19	1	94.7

*Mean number of tested plants over all seasons.

2-Floweing in matching among related lines: Ten lines were consistence depending on their stability and matching in flowering with the studied (Rf) inbred lines in relations to flowering date and 50% flowering. Table (3) presents the mean number of days from sowing to flowering and to 50% flowering .It is clear that there are three pairs (A and B) of lines i.e. 9, 21 and 32, which had identical estimates of flowering initiation and 50% flowering but all lines were matching in 50% flowering. It is clear that all lines (A & B) showed good matching dates to each other.

Table 3. Mean number of days from sowing to first flower initiation and 50%flowering for CMS and Maintainer lines during 2006-2007and 2008

Inbred lines (A)	flowering Initiation		50% flowering (A lines)	50% flowering(B lines)
	CMS lines	(B lines)		
19	41	42	45	45
9	42	42	44	44
18	42	43	45	45
24	41	42	44	44
27	42	43	45	45
17	41	42	44	44
21	42	42	45	45
33	42	41	46	46
32	42	42	45	45
34	42	43	44	44

3- Molecular identification of similarity and /or diversity. DNA was extracted from the selected ten A-lines and ten Rf lines aiming to investigate the genetic relationships among these genotypes. Twenty arbitrary chosen RAPD primers were used for this purpose (Table 1).Out of them, ten successfully amplified DNA fragments were varying in their molecular size as shown in figures 1 and 2.Nine primers revealed polymorphic banding patterns while primer OPB-07 gave only two monomorphic fragments. Number of amplified fragments varied among tested primers and maximum number of fragments (15) was generated with primer OP-C 15, while the minimum number (2) was revealed by primer OPB-07.

A dendrogram (Fig. 3) reflecting the genetic relationships between ten CMS inbred lines and genetic diversity data for 20 primer systems indicated that, the lines were divided into 2 main clusters. The first cluster, which included CMS lines, was divided into 2 sub cluster. Line 34 was a unique line and was separated solely in a sub cluster. The second sub cluster included 2 groups of lines; group 1 included lines 32, 33, 21, 17, 27 and 24, while group 2 included lines 18, 9 and 19. The second cluster for fertility restorer lines (Rf) were divided into 2 sub clusters, Rf 15 was also a unique Rf lines separated in sub cluster. The second sub cluster included 2 groups; group 1 contained Rf 12, 11, and 10 and group 2 contains 8, 7, 4, 3, 2 and 1.

The genotypes distributed on the consensus tree according to the banding patterns in RAPD. The combined data of similarity matrix, as shown in the consensus tree Fig. (3) clearly showed that inbred line 34 was the most diverse genotype in relation to its genetic background and that heterotic effects is more expected when using this parent as an R f with different inbred lines (Table 4) .In contrast, line 19 showed low similarity index with Rf 1, 2 and 3 indicating low expected heterotic effects.

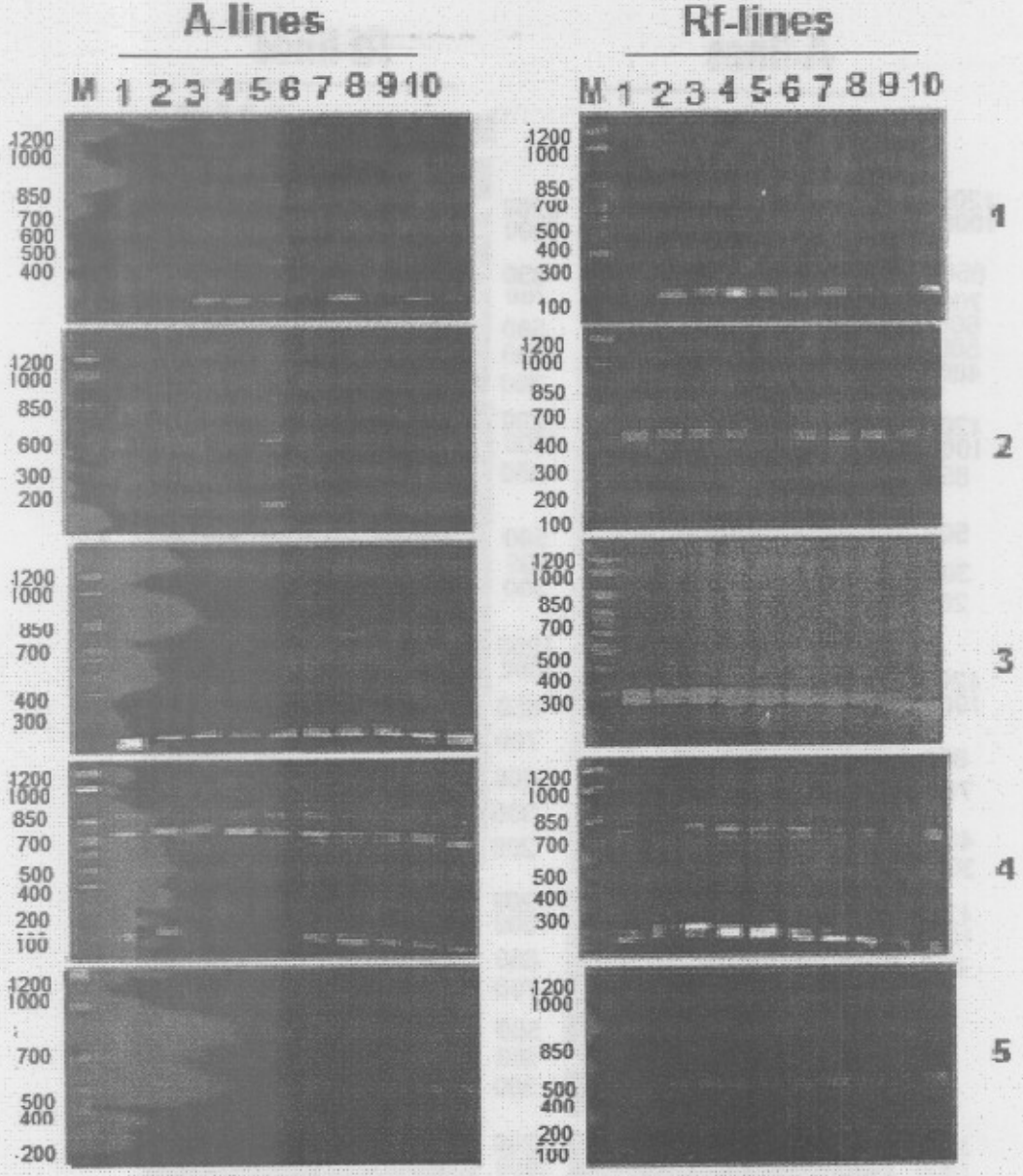

Fig. 1. DNA banding patterns as revealed by RAPD-PCR Primers (1-5).

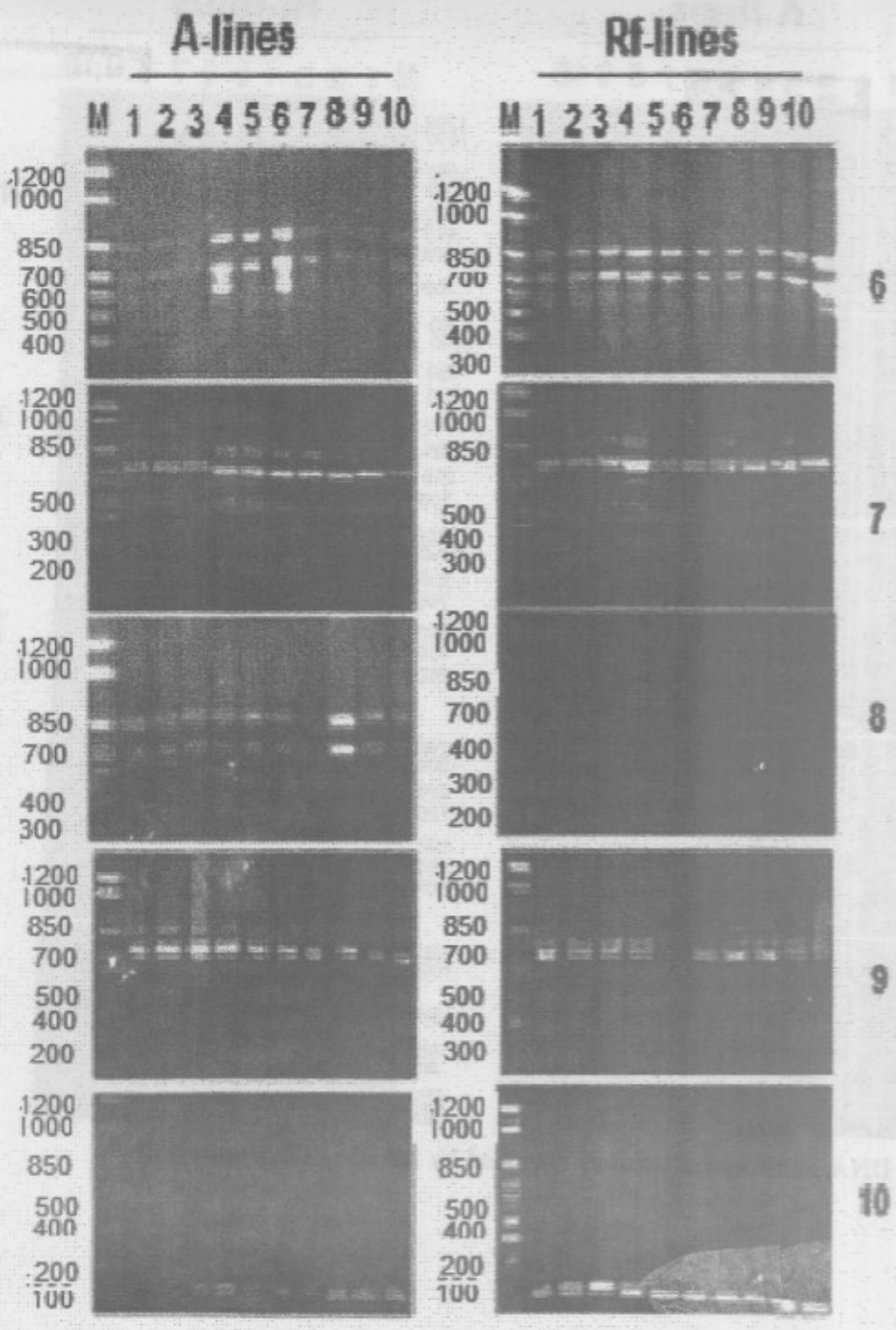

Fig. 2. DNA banding patterns as revealed by RAPD-PCR Primers (6-10).

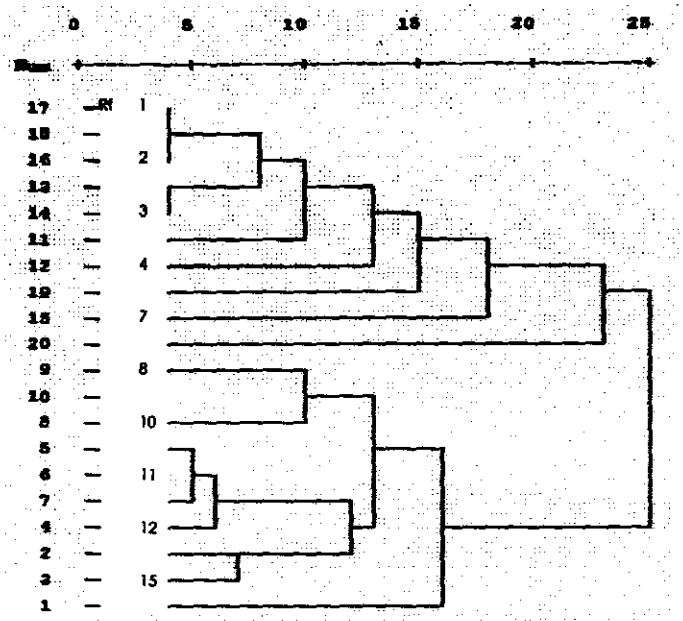

Fig. 3. A dendrogram showing the genetic relationship among the studied lines

The analysis of Morphological attributes; yield component, yield and oil content of lines hybrids on the based of their similarity and for diversity behavior. The analysis of five hybrids obtained by crossing diverse cytoplasmic male sterility (CMS) and fertility restorer (Rf) lines showed the presence of fertility restoration in all combinations. The hybrids showed significant variations for flowering date. Hybrid A33xRf15 was the earliest in flowering, while the check cultivar sakha 53 was the latest. Regarding plant height the hybrid A34 xRf 15 was the shortest plants while Sakha 53 had the tallest plants. Concerning stem diameter the hybrid A21x Rf 15 gave the most thin stems while Sakha 53 gave showed the thick diameter.

With respect to attributes, hybrid A34x Rf 15 showed wide head diameter, while hybrid A32XRf15 gave the smallest head. Also 100 seed weight hybrid A19xRf 10 gave the lowest in 100 seed weight, while the hybrid A33xRf 15 gave the highest in 100 seed weight. Regarding seed yield per plant the hybrid A32x Rf 15 gave the lowest in seed yield /plant, while hybrid A33xRf15 gave the highest seed yield /plant. Also seed yield/fed was shown by the hybrid A32xRf 15 gave the lowest in seed

Table 4. Similarity matrix generated by using ten RAPD-PCR primers

Var.	Var.																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1																				
2	.79																			
3	.76	.88																		
4	.80	.83	.84																	
5	.76	.81	.82	.89																
6	.75	.86	.83	.90	.92															
7	.69	.79	.80	.89	.90	.91														
8	.73	.77	.70	.80	.82	.83	.76													
9	.80	.79	.74	.84	.82	.85	.78	.83												
10	.84	.83	.80	.85	.83	.86	.81	.84	.93											
11	.73	.71	.66	.69	.63	.68	.61	.61	.65	.71										
12	.57	.61	.67	.64	.64	.63	.64	.53	.56	.62	.82									
13	.62	.68	.69	.71	.71	.71	.66	.63	.65	.69	.83	.89								
14	.63	.68	.69	.72	.71	.70	.68	.61	.63	.68	.80	.82	.92							
15	.57	.57	.63	.64	.62	.60	.56	.62	.54	.62	.73	.73	.80	.84						
16	.70	.72	.69	.68	.68	.71	.61	.65	.69	.74	.84	.77	.87	.89	.79					
17	.71	.75	.69	.74	.69	.74	.66	.66	.72	.76	.89	.76	.84	.88	.75	.90				
18	.67	.75	.69	.71	.69	.73	.64	.66	.70	.73	.85	.78	.86	.87	.74	.92	.96			
19	.54	.65	.58	.58	.58	.61	.58	.55	.60	.63	.72	.76	.76	.77	.61	.82	.79	.81		
20	.61	.62	.59	.64	.60	.60	.59	.56	.67	.65	.70	.64	.65	.72	.56	.69	.76	.72	.77	

yield/fed an, while the A33xRf15 and A34 X Rf 15 gave the highest value yield/fed an. %of oil the hybrid A32 x Rf15 give the highest oil content and the lowest Sakha 53, was obtained for compared to (38.8- 41.93%) oil content in the two season.

Table5. Mean values of flowering date ,plant height, stem diameter ,head diameter ,100 seed weight ,seed yield /plant, seed yield/fedan and oil % .

Hybrid and check	Flowering date day (cm)			Plant height (cm)			Stem diameter (cm)			Head diameter (cm)			100 seed weight (g)			Seed yield / plant (g)			Seed yield/ fad.(K.g)			oil %		
	2007	2008	Mean	2007	2008	Mean	2007	2008	Mean	2007	2008	Mean	2007	2008	Mean	2007	2008	Mean	2007	2008	Mean	2007	2008	Mean
A 34 XRF15	ab 48.00	b 46.67	Bc 47.33	a 137.00	A 133.33	a 135.17	b 2.08	b 2.00	b c 2.04	B 20.36	b 18.25	b 19.30	a-c 5.61	Ab 4.67	bc 5.14	C 52.18	c d 45.13	d 48.66	C 1827	cd 1580	d 1703	b 41.38	bc 40.52	b 40.95
A 19 XRF10	ab 47.33	b 45.33	B 46.33	C 174.33	C 170.33	cd 172.33	ab 1.99	b 1.98	b 1.99	Ab 17.83	b 16.71	b 17.27	a 4.90	a 4.10	a 4.50	bc 46.90	bc 40.38	C 43.64	bc 1462	bc 1414	C 1528	a 39.93	a 38.91	a 39.42
A33 XRF15	a 44.67	a 42.67	A 43.67	d 185.67	D 181.00	e 183.33	b 2.17	b 2.08	C 2.13	B 20.27	b 18.24	b 19.26	C 6.44	b 5.28	d 5.86	C 50.88	d 46.73	d 48.81	C 17.81	d 1636	d 1708	b 41.65	b.c 40.54	b.c 41.09
A21 XRF15	a 45.00	b 45.67	Ab 45.33	C 170.67	C 166.00	C 168.33	a 1.81	a 1.78	a 1.79	B 20.11	b 17.95	b 19.03	bc 5.88	ab 5.02	cd 5.45	ab 41.10	Ab 37.39	ab 39.25	ab 1438	a.b 1389	a.b 1374	a 39.30	a 38.31	a 38.80
A32 XRF15	bc 49.67	C 48.67	C 49.17	b 160.33	B 155.67	b 158.00	b 2.06	b 2.01	bc 2.03	A 15.43	a 13.44	a 14.44	bc 5.99	ab 4.86	cd 5.42	a 37.21	A 34.11	a 35.66	a 1303	a 1194	a 1249	b 42.14	C 41.72	C 41.93
Sakha53	C 52.33	d 51.33	D 51.83	cd 179.00	Cd 175.00	d 177.00	C 2.62	C 2.53	D 2.58	Ab 17.94	ab 15.90	b 16.92	ab 5.26	A 4.15	ab 4.71	b 44.20	Bd 41.71	bc 42.96	b 1547	b-d 1468	bc 1504	a 39.61	ab 39.14	a 39.37

CONCLUSION

The CMS sources are important for sunflower breeding programs and hybrid seed production. In this study molecular approaches were used to distinguish the relationships among the available CMS –Rf system. The calculated similarity matrix showed considerable genetic variability in the studied line . This variability seems to be adequate to select among them and to start a breeding program for hybrid seed production. Studying flowering initiation and 50%flowering that matches during reproduction of A lines via crossing with B lines allows the hybridization to maintain CMS trait in the parents we recommended the parent male Rf15 and female A34 and 32 used in best hybrid production in sunflower . On the other hand, estimating the genetic distances among CMS and Rf permit the selection of good combiners for hybrid seed production.

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التباعد الوراثي فى دوار الشمس بين السلالات العقيمة الذكر سيتوبلازميا والسلالات الملقحة والسلالات المعيدة للخصوبة بإستخدام المعلمات الجزيئية

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يهدف هذا البحث إلي استخدام الوراثة الجزيئية في تحديد نسبة القرابه والتباعد الوراثى بين السلالات العقيمة الذكر سيتوبلازميا والسلالات المعيدة للخصوبة في إنتاج هجن دوار الشمس

تم إنتخاب عشرون سلالة من السلالات العقيمة الذكر سيتوبلازميا من بين ثلاثة واربعون سلالة والانتخاب تم على التوافق فى مواعيد الازهار والثبات الوراثى ومن العشرون سلالة تم انتخاب عشرة سلالات من احسن السلالات فى التوافق فى مواعيد الازهار والثبات الوراثى والتجانس فى الشكل المورفولوجى .

تم استخدام عشرين بادئ لاثبات اكثر السلالات المتباعدة وراثيا لاستخدامها فى انتاج هجن دوار الشمس .

ومن النتائج اتضح ان السلالات العقيمة الذكر سيتوبلازميا وهى ١٨ و ٢٤ و ٣٢ هى اكثر السلالات العقيمة ثباتة وراثيا بنسبة ١٠٠% وايضا السلالات ٩ و ٢١ و ٣٢ هى اكثر السلالات المتوافقة فى مواعيد التزهير مع السلالات الملقحة .

واظهرت النتائج ان السلالتين A34 ، A32 كانتا اكثر السلالات المتباعدة وراثيا حيث انفصلت السلالة A34 في مجموعة واحدة وكانت السلالة Rf15 المعيدة للخصوبة حيث انفصلت فى مجموعه منفردة

وأظهرت النتائج ان السلالات A21 ، A17 ، A27 ، A24 ، اقل السلالات فى التباعد الوراثي يليها السلالات A18 ، A9 ، A19 في التباعد الوراثي .

- اتضح من النتائج ان الهجين Rf15 × A34 أعطي حجم قرص اكبر ولكن السلالة الملقحة لم يكن لها القدرة علي إعادة الخصوبة كاملة

أما الهجين Rf15× A33 أعطي اعلي وزن في صفات وزن ١٠٠ بذرة ، محصول النبات ومحصول الفدان من البذرة إما بالنسبة المئوية الزيت فكان الهجين Rf15 × A32 أعطي نسبة زيت اعلي. واظهرت النتائج ان استخدام الاباء Rf15 مع الامهات ٣٣ و ٣٤ اعطى اعلى محصول بذور للفدان . كما اظهر استخدام الاباء Rf15 مع الام ٣٢ أعطى أعلى نسبة مئوية للزيت.

- أظهرت النتائج أن استخدام الهجن في برامج التربية حقق نسبة اعلي في محصول الفدان من البذرة من ٢٠ – ٢٥ % والنسبة المئوية للزيت بنسبة تتراوح بين ٣٨,٨ – ٤١,٩٣ %

مقارنة بصنف المقارنة مفتوح التلقيح سخا ٥٣ حيث كانت النسبة المئوية للزيت ٣٩,٣٧ %.