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MOLECULAR ANALYSIS OF NEW DROUGHT TOLERANT SEGREGANTS SELECTED FROM F_2 POPULATIONS OF BREAD WHEAT CROSSES

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Abstract- New genetic recombinations in bread wheat hybrid populations are considered the main source of variation for practicing successful selection to develop drought tolerant genotypes. Diallel crosses (except reciprocals) among six wheat cultivars and lines were made in 2008/2009 season. F_2 seeds were produced on F_1 plants in 2009/2010 season. Selection for high grain yield was practiced in the F_2 populations in 2010/2011 season under water stress (WS) and well-watering (WW) conditions. The selected 115 F_3 families and their 6 parents were evaluated in the field in 2011/2012 season under both conditions. Among them, five F_3 families showed superiority under WS reaching 61.8% for SF9 over the best Egyptian cultivar in this study (Sakha-93). Molecular characterization based on simple sequence repeats (SSR) analysis of the 5 best recombinants (drought tolerant) and their five parents was performed. The 15 SSR primers produced 42 amplicons, 34 of them were polymorphic, with an average polymorphism of 78.59%. Ninety two amplicons were useful genotype–specific markers for the studied 10 wheat genotypes, in which 56 of them were positive, while 36 were negative markers. The 5 selected recombinants (SF8 through SF12) exhibited 3,1,2,6 and 11 positive markers and 8,5,4,2 and 6 negative markers, respectively. The SSR assay permitted the identification of two out of ten wheat genotypes by six unique markers. Maryout-5 and Aseel-5 were characterized by four positive unique markers, while maryout-5 was characterized by one negative unique marker. The Syrian drought tolerant cultivar Aseel-5 was characterized by two unique positive markers amplified by the primers WMS 198 (100bp) and WMC 322 (400bp). The genetic similarity ranged from 57 to 84%, suggesting that the new recombinants are different from their parents on the DNA level.

Keywords- *Triticum aestivum*, Selected recombinants, SSR, unique markers, genetic similarity, drought tolerance

Introduction

Wheat is one of the most important cereal crops of the world and provides over 20 % of calories and protein for human nutrition for over 35% of the world's population in more than 40 countries including Egypt. Across the last five years, the average annual consumption of wheat grains is about 14 million tons, while the average annual local production is about 8 million tons with an average grain yield of 18.0 ardab / feddan (6.43 t/ha) (Agricultural statistics, Arab republic of Egypt, 2012). Therefore, the gap between annual local production and consumption is about 6 million tons. This gap could be narrowed by increasing local production of wheat *via* two ways. The first way is through vertical expansion, *i.e.* increasing wheat production per unit area through the development of new cultivars of high yielding ability, early maturity, resistance to biotic and abiotic stresses, and the adoption of recommended cultural practices for growing these cultivars. The second way is through the horizontal expansion, *i.e.,* by increasing the area cultivated with wheat. But the limiting factor for this approach in Egypt is the availability of irrigation water. Potential expansion of wheat area is only possible in the North coast and Egyptian deserts. But the soil in these areas is sandy with low water holding capacity and thus exposes wheat plants to drought stress. Such drought stress causes great losses in wheat yield and its components [1-3]. Using drought tolerant wheat cultivars that consume less water, and can tolerate soil water deficit could solve this problem.

To start a proper wheat breeding program for improving drought tolerance, the source populations should possess genetic variability amenable for selection. Unfortunately, with present distribution of improved high yielding, pure line cultivars, selection from established cultivars would rarely isolate a new genotype [4]. Hybridization is the principal breeding procedure for the development of new recombinations, *i.e.,* inducing new genetic variability in wheat. The chief role of hybridization is to cross diverse genotypes and create hybrid populations with wide genetic variation from which new recombinations of genes may be selected [5]. Selection from segregating generations of wheat hybrid combinations succeeded to develop new genotypes that possess drought tolerance adaptive traits, such as early maturity [6-8], glaucosness [9-10], high water use efficiency [11] and high grain yield/plant under water deficit conditions [12-13].

Molecular markers have been proven to be powerful tools in the assessment of genetic variation and elucidation of genetic relationships within and among species on the contemporary of the morphological and biochemical markers which may be affected by environmental factors and growth practices [14-15]. A wide variety of DNA-based markers has been developed in the past few years and restriction fragment length polymorphism (RFLP) was the first molecular marker used for genome analysis and genome mapping [16]. This was followed by advances in polymerase chain reaction (PCR) technology which led to a number of useful markers, *e.g.*

random amplification of polymorphic DNA (RAPD) [17], simple sequence repeats (SSRs) [18] and amplified fragment length polymorphism (AFLP) [19], which vary in their specifity and resolution. SSRs are present in the genome of all eukaryotes and consist of several repeats to over hundreds of nucleotide motif and planked by sequence that can be used as primers so it is more specific than RAPD [20].

SSRs offer a potentially attractive combination of features that are useful as molecular markers. First, SSRs have been reported to be highly - polymorphic and this highly informative in plants, providing many different dosely related individuals [21-22]. Second SSRs can be analyzed by a rapid, technically simple, and inexpensive PCR-based away that requires only small quantities of DNA. Third, SSRs are co-dominant and simple Mendelian segregation has been observed. Finally, SSRs are both abundant and uniformly dispersed in plant genomes [21,23,24]. The primary advantage of SSRs as molecular markers is the cost and research effort required to clone and sequence SSR-containing DNA fragments from the plant species of interest [25]. Many investigators concluded that SSR molecular markers are significantly associated with wheat traits related to salinity tolerance [26] and drought tolerance [27-32].

The main objective of the present investigation was to develop new wheat recombinants of high grain yield under water stress conditions. The detailed objectives were to: 1. evaluate the agronomic and yield attributes of 115 selections (F_3 families) for drought tolerance, and 2. characterize the 5 best selections (drought tolerant recombinants) and their parents at the DNA level *via* SSR analysis.

Materials and Methods

The field work of this investigation was carried out during the three successive growing seasons from 2009 / 2010 to 2011 / 2012 at the Experimental Farm and Laboratories of the Plant Research Department, Nuclear Research Center, Inshas, El-Sharkyia Governorate (The latitude and longitude of the experimental site are 30° 24` N and 31° 35` E, respectively, while the altitude is 20 m above the sea level). The soil at the experimental site was loamy sand to sandy.

Materials

Six genotypes of bread wheat (*Triticum aestivum* L.) were used in the present study. Name, pedigree, origin and important traits of these genotypes are presented in [Table-1].

Table 1- Name, pedigree and the most important trait(s) of the studied bread wheat genotypes.

Name	Designation	Pedigree	Origin	Important Trait
Sids-4 cv.	Sd-4	Maya"S"Mon"S"/CMH74.A592/3/Sakha8 X2SD10002-140sd-3sd-1sd-0sd	ARC-Egypt	Earliness
Sakha-61 cv.	Sk-61	Lina/RL4220//7c/Yr"S"CM 15430-25-55-0S-0S	ARC-Egypt	Earliness
Maryout-5 Line	Mr-5	Giza 162 // Bch's /4/ PI-ICW 79Su511Mr-38Mr-1Mr-0Mr	DRC-Egypt	High yielding and Salt tolerant
Aseel-5 cv.	As-5	BIG INC 08 104	ICARDA-Syria	Drought tolerant
Sakha-93 cv.	Sk-93	Sakha 92/ TR 810328 S8871-1S-2S-1S-0S	ARC-Egypt	High yielding
Giza-168 cv.	Gz-168	Mrl / Buc // Seri CM 930468M-0Y-0M-2Y-0B	ARC-Egypt	High yielding

ARC = Agricultural Research Center, DRC = Desert Research Center, ICARDA = International Center for Agricultural Research in the Dry Areas, cv. = cultivar.

Experimental Procedures

Making the Diallel Crosses (Among 6 Parents): The six genotypes, presented in [Table-1] were grown in 2008/2009 season. All possible diallel crosses (excluding reciprocals) were made among the six genotypes, so seeds of 15 direct F_1 crosses were obtained.

Producing F_2 Seeds: F_1 seeds from each of the 15 crosses were sown in 2009/2010 season in the field under well water conditions in separate plots. Each plot consists of 6 rows; each row was 3 m long and 30 cm wide; spaces between hills were 10 cm. The plants were left for natural self pollination. At maturity F_2 seeds of each cross were separately harvested. The recommended cultural practices for wheat production at Inshas were followed in F_1 generation.

Growing F_2 Populations in Selection Fields: In 2010/2011 seasons, two selection fields were used to grow F_2 populations of 15 diallel crosses in separate plots under irrigation regimes; one irrigation regime for each selection field. Two irrigation treatments (starting from 21 days after sowing) were used, *i.e.*, irrigation every 5 days (well-watering; WW) and irrigation every 15 days (water-stress; WS), where total quantity of irrigation water for WS was 70 % of that for WW. Each selection plot consisted of 18 rows, each row was 3 m long and 30 cm wide; spaces between hills were 10 cm (plot size = 16.2 m^2).

Practicing Selection: Individual plant selection, using *ca* 1 % selection intensity was practiced in the same season (2010/2011), in the 15 $F_{2's}$ for high grain/yield plant and some other favorable traits, such as spike length, spike weight, spikes/plant, earliness, glaucousness…etc., under water stress and non-stress conditions. One hundred and fifteen individual plant selections were separately harvested (55 under WS and 60 under WW).

Field Evaluation of Selections and their Parents: A field experiment was conducted in 2011/2012 season to evaluate selected F_3 families (115) as compared to their parents (6). The experimental design used was a split-plot with balanced lattice (11x11) arrangement in three replications. Main plots were assigned to the two irrigation regimes (same as for F_2's) and sub-plots were devoted to 121 genotypes (115 selections + 6 parents). Each plot consisted of 4 rows, each row was 2.25 m long and 30 cm wide; spaces between hills were 10 cm (plot size = 2.7 m^2). Rainfall in both seasons was very light and intermittent with a total precipitation of 10.3 and 13.9 mm for the two seasons, respectively, suggesting that rainfall during the stress period was of negligible influence on moisture content of the experimental soil.

Data Recorded in the Field Experiment

Data were recorded on days to 50% heading (DTH), days to 50% anthesis (DTA), days to 50% physiological maturity (DTM), plant height (PH in cm), spike length (SL in cm), spikes/plant (SPP), grains/spike (GPS), spike weight (SW in g), 100-grain weight (100GW) in g and grain yield/plant (GYPP) in g. Data on the latter seven traits were measured on 27 individual plants/plot for M_2's and parents. Data on the 1st three traits were measured on a per plot basis.

Biometrical Analysis

The collected field data were subjected to the normal analysis of variance of the split-plot design and least significant differences (LSD) were calculated according to [33].

Molecular Characterization Using SSR Analysis

SSR analysis was used in the present study to investigate the genetic diversity among the best 5 selections (transgressive segregants) and their 5 parents on DNA level.

DNA Extraction

Extraction of DNA was carried out according to [34-35]. Young green leaves from each genotype were collected from ten days seedlings germinated from seeds of each genotype and quickly frozen in liquid nitrogen and then ground using mortar and pestle. The extraction buffer was preheated to 65°C in a water bath. The frozen powder (100-120 mg) was transferred to 2 ml eppendorf tubes using a self-made spatula from filter paper dipped into liquid nitrogen. The preheated extraction buffer of 500 µl was added to each tube with 10 µl of RNase (100mg/ml), mixed well by vortex and incubated at 65°C for 30 min in water bath. Samples were mixed well by vortex and returned to water bath twice in the course of these 30 minutes. The solution was left to cool down at room temperature, then 300 µl of 6M ammonium acetate, stored at 4°C, was added. The samples were mixed well by vortex and then kept for 15 minutes (at 4°C). The tubes were centrifuged at 13,000 rpm for 5 minutes at room temperature. The supernatants (the upper aqueous solution of approximately 700µl) were transferred to new microfuge tubes and 50 µl CTAB were added to each tube and mixed gently. Seven hundred µl chloroform- isoamylalcohol (24:1) were added and the tubes were swirled or inverted gently to avoid mechanical damage of to the DNA. Samples were centrifuged at 13,000 rpm for 5 minutes. Upper aqueous supernatant was transferred to new eppendorf tube. This upper phase contains the DNA. Two thirds volume of ice-cold isopropanol (~500 µl) was added to the eppendorf tube which contained the DNA. Tubes were inverted gently to avoid mechanical damage to the DNA and the DNA was allowed to precipitate for 15 min at -20°C or left standing on ice for 30 minutes. The samples were centrifuged for 20 minutes at maximum speed (13,000 rpm) in order to pellet the DNA. The DNA pellets should now be visible. The liquid was drained carefully, 1000 µl 70% ethanol was added and left for 3 minutes. Centrifuge was applied for 10 minutes at 10,000 rpm. The alcohol was drained and 1000 µl of 90% ethanol was added, centrifuged at 10,000 rpm for 10 min and the alcohol was drained and the pellet remaining at the bottom of the centrifuge tube was dried. Pellet in 100 µl TE was resuspended and left to dissolve at 4°C in the refrigerator for at least 30 minutes. The un-dissolved cellular debris was spun down by centrifuging the tube for 10 min at 13,000 rpm. The supernatant was transferred into a new tube and stored at 4°C for immediate use or -20°C for long term storage.

Detection of Polymorphism

The polymorphism among the 12 selections (7 putative mutants and 5 transgressive segregants) and their 7 parents was performed based on SSR analysis. These selections represent drought tolerant and high yielding M_3 and F_3 families. A set of fifteen random primers [Table-2] chosen according to Bousba, et al. [36] among the publicly available sets catalogued in the Grain Genes database (http://wheat.pw.usda.gov) as WMC (Xwmc) and as described by Roider, et al. [37] for WMS (Xgwm), specialized for *Triticum aestivum* and used for screening drought tolerance was used in the detection of polymorphism among the nineteen wheat genotypes. These primers were synthesized by BioShop® Canada Inc.

Table 2- Description of the SSR loci used in this study.

Sr. No.	Primer	Sequence
1	WMS 06	F : 5 - CGT ATC ACC TCC TAG CTA AAC TAG - 3
		R : 5 - AGC CTT ATC ATG ACC CTA CCT T - 3
2	WMS 30	F : 5 - ATC TTA GCA TAG AAG GGA GTG GG - 3
		R : 5 - TTC TGC ACC CTG GGT GAT TGC - 3
3	WMS 108	F : 5 - ATT AAT ACC TGA GGG AGG TGC - 3
		R : 5 - GGT CTC AGG AGC AAG AAC AC - 3
4	WMS 118	F : 5 - GAT GGT GCC ACT TGA GCA TG - 3
		R : 5 - GAT TG TCA AAT GGA ACA CCC - 3
5	WMS 149	F : 5 - CAT TGT TTT CTG CCT CTA GCC - 3
		R : 5 - CTA GCA TCG AAC CTG AAC AAG - 3
6	WMS 169	F : 5 - ACC ACT GCA GAG AAC ACA TAC G - 3
		R : 5 - GTG CTC TGC TCT AAG TGT GGG - 3
7	WMC 177	F : 5 - AGGGCTCTCTTTAATTCTTGCT - 3
		R : 5 - GGTCTATCGTAATCCACCTGTA - 3
8	WMC 179	F : 5 - CATGGTGGCCATGAGTGGAGGT - 3
		R : 5 - CATGATCTTGCGTGTGCGTAGG - 3
9	WMS 198	F : 5 - TTG AAC CGG AAG GAG TAC AG - 3
		R : 5 - TCA GTT TAT TTT GGG CAT GTG - 3
10	WMC 235	F : 5 - ACTGTTCCTATCCGTGCACTGG - 3
		R : 5 - GAGGCAAAGTTCTGGAGGTCTG - 3
11	WMS 304	F : 5 - AGGAAACAGAAATATCGCGG - 3
		R : 5 - AGG ACT GTG GGG AAT GAA TG - 3
12	WMC 307	F : 5 - GTTTGAAGACCAAGCTCCTCCT - 3
		R : 5 - ACCATAACCTCTCAAGAACCCA - 3
13	WMC 322	F : 5 - CGCCCCACTATGCTTTG - 3
		R : 5 - CCCAGTCCAGCTAGCCTCC - 3
14	WMS 375	F : 5 - ATTGGCGACTCTAGCATATACG - 3
		R : 5 - GGGATGTCTGTTCCATCTTAGC - 3
15	WMC 445	F : 5 - AGAATAGGTTCTTGGGCCAGTC - 3
		R : 5 – GAGATGATCTCCTCCATCAGCA - 3

F = Forward, R = Reverse

Polymerase Chain Reaction (PCR)

The PCR master mix for simple sequence repeat (SSR) primers consisted of 2 µL of 20 ng/µL genomic DNA template, 0.40 µL of 10µM a forward and reverse primer mixture, 0.18µL (0.9 U) of Taq polymerase, 1.20 µL of 10X buffer (10 mM Tris-HCL, 50 mM KCl, 1.5 53 mM MgCl2, pH 8.3), 0.96 µL of a 100 µM mixture of dNTPs and 7.26 µL of water bringing the total reaction volume to 12 µL. Reaction conditions for SSR markers were as follows: 8.33 µL ddH20, 2.4 µL 10X reaction buffer, 0.9 µL 50mM MgCl2, 1.92 µL 2.5mM dNTPs, 1.9 µL 1pM of 19bp M-13. The PCR master mix for sequence-tagged site (STS) was carried out in a volume of 20 µl and contained 200 ng of genomic DNA, 0.2 mM of dNTPs, 10 pmol of each primer, 2.0 mM of MgCl2, 50 mM of KCl, 10 mM of Tris-HCl (pH 9.0 at 25 °C), 0.1% TritonX-100 and 0.5 U of Taq DNA Polymerase The amplification products were resolved by electrophoresis in a 1.5% agarose gel containing ethidium bromide (0.5ug/ml) in 1X TBE buffer at 95 volts. PCR products were visualized on UV light and photographed using a Polaroid camera. Amplified products were visually examined and the presence or absence of each size class was scored as 1 or 0, respectively.

Genetic Similarities based on SSR Analysis

The banding patterns generated by SSR-PCR marker analysis were compared to determine the genetic relatedness of the genotypes. Clear and distinct amplification products were scored as '1' for pres-

ence and '0' for absence of bands. Bands of the same mobility were scored as identical. The genetic similarity coefficient (GS) between two genotypes was estimated according to Dice coefficient Sneath and Sokal [38] as follows: Dice formula: $GS_{ij} = 2a/(2a+b+c)$.Where GS_{ij} is the measure of genetic similarity between individuals i and j, a is the number of bands shared by i and j, b is the number of bands present in i and absent in j, and c is the number of bands present in j and absent in i.

Results and Discussion

Selection Experiment

In the present experiment, 115 individual plants with desirable traits related to drought tolerance were selected from F_2 populations of diallel crosses between five wheat genotypes; 55 of them selected under water stress and 60 under well-irrigation in 2010/2011 season. Progenies of these selections (115 F_3 families) were evaluated in 2011/2012 season along with their five parents for studied attributes under water stress and non-stress conditions.

Comparing Performance of Two Selection Groups with Parents

The evaluated 121 genotypes were partitioned into three groups, namely parents (6), F_3 families selected under WS (55) and F_3 families selected under WW (60). Summary of group mean and best genotype mean for each of these groups under WW and WS conditions is presented in [Table-3]. Results of this table indicated that means of all the two selection groups were higher than the mean of parents group when evaluating them either under water stress or non-stress conditions. Moreover, means of the best selection in each of the two selection groups were markedly higher in magnitude for grain yield attributes and were earlier in maturity (lower) than the best parent under both WW and WS conditions. Superiority of the selections over parents in grain yield and earliness traits under WS is advantageous for drought tolerance.

In general, selection in this experiment under WS is more efficient than selection under WW when the target environment is WS and the opposite is true, *i.e.*, selection under WW is more efficient than under WS when the target environment is WW conditions. In this context, literature includes two contrasting strategies for identifying genotypes that will be high yielding under stress environments: (i) Genotypes may be evaluated under the conditions in which they will be ultimately produced, namely a certain type of stress environment, to minimize genotype X environment interaction. Ceccarlli [39] has argued for this approach, but it may result in lower heritability, particularly across years. (ii) Genotypes may be evaluated under optimum conditions maximizing heritability; but perhaps encountering problems with genotype x environments. Braun, et al. [40] has argued for this approach, citing results from 17 years of CIMMYT winter performance.

Table 3- Summary of group mean ($\bar{x}$) and best (B) genotype mean of 6 Ps and 115 F_3 families selected from F_2's (55 under WS and 60 under WW) for studied traits under water stress (WS) and well watering (WW) conditions (2011/ 2012 season).

Group	Stress	Parameter	DTM	PH (cm)	SPP	GPS	100GW (g)	GYPP (g)
6 Parents	WW	$\bar{x}$	135	96	8.3	75	5.1	32
	WS	$\bar{x}$	134	90	7.6	70	4.6	26.6
	WW	B	133	107	10.1	90	5.5	37.3
	WS	B	132	100	9.1	84	5	33.3
55 F_3 Families	WW	$\bar{x}$	135	95	9.4	72	5.3	36.4
	WS	$\bar{x}$	133	88	8.2	66	4.6	28.2
Selected Under WS	WW	B	122	121	13.3	113	6.3	54.2
	WS	B	119	105	11.4	108	6	45.6
60 F_3 families	WW	$\bar{x}$	135	98	9.1	71	5.3	35.2
	WS	$\bar{x}$	133	92	7.9	64	4.6	27.2
Selected Under WW	WW	B	123	127	12.3	81	6.3	56.1
	WS	B	121	112	11	75	6.1	39.4
$LSD_{0.05}$			0.65	0.90	0.12	1.13	0.08	0.80

Our results are in favor of the first strategy. The direct selection under water deficit stress environment would ensure the preservation of alleles for drought tolerance [41], while direct selection under optimal environment would take advantage of the high heritability [40,42,43]. A third alternative, currently used at CIMMYT, this is simultaneously evaluation under near optimum and stress conditions, with selection of those genotypes that perform well in both environments [44]. However, ultimate evaluation must be performed in the target environment prior to recommendation for a cultivar for commercial production. Further selection and evaluation under drought stress conditions should be continued in the selected superior F_3 families derived from the present investigation in order to assure their superiority in drought tolerance and select the most stable and high yielding ones under drought stress conditions.

Selection in the F_2 populations under water stress (the group of 55 F_3 families selected under WS) gave the highest mean of grain yield attributes and the earliest and highest yielding selections under both WW and WS conditions [Table-3]. The best selection from this group (F_3 families selected under WS) was earlier than the earliest parent by 11 days under WW and 13 days under WS. In general, selection from segregating generations (F_2 in this study) under WS is more efficient when the evaluation of selections is under WS. On the contrary, selection in segregating generations under WW is the least efficient when the target environment is WS.

Agronomic Characterization of the 5 best F_3 Selected Families

The five highest yielding F_3 selected families under water-stress conditions were identified on the base that they showed significant superiority in grain yield/plant over the best parent of the corresponding cross under water stress conditions by about 15 % and more in [Table-4]. Out of the two selection groups (55 F_3 families selected under WS and 60 F_3 families selected under WW), 3 and 2 F_3 families, respectively significantly outyielded, by at least 15 %, the best parent under WS conditions. The five best selected families (SF) included three (SF9, SF10 and SF11) selected under WS, *i.e.*, Sd4 X Mr5-WS-PS2, Sk61 X As5-WS-PS3 and Sk61 X Sk93-

WS-PS2, respectively and two (SF8 and SF12) selected under WW, *i.e.,* Sd4 X Sk61-WW-PS8 and Mr5 X Sk93-WW-PS8, respectively.

Means of studied traits of the 5 best families and the 6 parental genotypes are presented under WS and WW in [Table-4]. On average, under WS conditions the group of 5 best F_3 families showed the highest mean grain yield (41.2 g), while the group of 6 parents exhibited the lowest grain yield (26.6 g). In the same manner, under WW conditions, group of the five best F_3 families showed higher average grain yield/plant (47.7 g) than that of parents group (32.0 g). Moreover, reduction in GYPP due to water stress in the best F_3 groups (13.3% on average,) was less than that of parental group (17.1%). This means that in this experiment, F_2 populations were effective in releasing higher yielding families under water stress of higher drought tolerance than the original parents and the success of the procedure, *i.e.,* hybridization followed by transgressive segregation, in isolating new variants of higher drought tolerance. This conclusion was previously confirmed in wheat by [8,9].

Table 4- Mean performance of the 5 best selected F_3 families and their parents for studied wheat traits under water stress and well watering conditions (2011/ 2012 season).

Genotype	DTH	DTM	PH (cm)	SL (cm)	SW (g)	SPP	GPS	100GW (g)	GYPP (g)	Red.
Water Stress										
SF8	89	131	103	13.5	3.6	10.9	67	5	38.2	11.6
SF9	82	131	97	14.3	4.1	11.2	71	5	45.6	6.9
SF10	92	132	90	12	4	9.7	72	5.5	38.5	29
SF11	88	133	85	13.9	3.9	11.4	64	5.6	44.2	6.2
SF12	87	131	85	16.3	5	8	64	5.6	39.4	12.6
Av. (F_3)	87.6	131.6	92	14	4.1	10.2	67.6	5.3	41.2	13.3
Sd-4	87	132	96	16.2	4.3	5.3	84	5	23.1	24.6
Sk-61	92	132	79	10.3	3.1	8.1	63	4.4	24.8	17.7
Mr-5	95	138	94	14.2	3.8	6.9	76	4.9	26.1	13.4
As-5	96	132	92	13.1	3.4	9.1	69	4.6	33.3	10.6
Sk-93	94	132	81	12.2	3.2	8.7	66	4.4	28.2	17
Gz-168	95	136	86	12.6	3.6	7.3	65	4.2	26	15.5
Av. (P)	92.9	133.5	89.9	13.1	3.5	7.6	70	4.6	26.6	17.1
$LSD_{0.05}$	0.67	0.56	1.08	0.13	0.08	0.13	0.9	0.07	0.8	
Well Watering										
SF8	92	133	106	13.6	3.8	11.5	73	5.2	43.2	
SF9	85	132	100	14.7	4.1	12.1	77	5.6	49	
SF10	93	134	104	12.8	4.6	11.9	77	6.3	54.2	
SF11	89	134	93	14.2	4.1	11.7	72	6.3	47.1	
SF12	89	133	93	17.1	4.8	9.5	73	5.7	45.1	
Av. (F_3)	89.6	133.2	99.2	14.48	4.3	11.3	74.4	5.8	47.7	
Sd-4	89	134	102	16.4	5.1	5.5	90	5.5	30.6	
Sk-61	94	134	83	11.7	3.4	9.1	68	4.8	30.1	
Mr-5	97	140	100	15.3	4.2	7.2	80	5.5	30.1	
As-5	97	133	100	13.5	3.8	10.1	73	5.2	37.3	
Sk-93	95	134	86	12.5	3.5	9.7	70	4.9	34	
Gz-168	96	138	94	13.7	3.7	8.3	71	4.7	30.8	
Av. (P)	95	135	96	13.8	3.9	8.3	75	5.1	32	
$LSD_{0.05}$	0.84	0.65	0.9	0.12	0.08	0.12	1.13	0.08	0.8	

Red. (Reduction %) = 100 (GYPP under WW - GYPP under WS) / GYPP under WW, P = Parent, F_3 = best F_3 families, Av. = average.

It is worth noting that the group of best F_3 families was, on average, earlier than the group of parents for DTH (by 5.3 and 5.4 days), DTA (by 3.9 and 2.8 days) and DTM (by 1.9 and 1.8 days) under WS and WW, respectively. Comparing all the 5 best families, it is interesting to mention that the best families in grain yield/plant under water stress were SF9 (selected from the F_2 cross Sd-4 X Mr-5 under WS) (45.6 g), followed by SF11 (selected from the F_2 cross Sk-61 X Sk-93 under WS) (44.2 g) with a very low reduction due to water stress (6.9 and 6.2 %, respectively). The latter family was the second highest yielding under well watering (49.0 g). In the 1st rank under WW was SF10 (selected from the F_2 cross Sk-61 X As-5 under WS) (54.2 g). It is interesting to note that the three best families under WS and under WW were a result of selection for high yield under water stress conditions. The earliest F_3 family was SF9 (DTM was 131 and 132 days) as compared with the earliest parent Aseel-5 (DTM was 132 and 133 days) under water stress and non-stress, respectively. The best F_3 families for grain yield/plant were characterized by high value of one or more yield attributes under both WS and WW conditions. Superiority of the best F_3 families in grain yield over parents was associated with superiority in number of spikes/plant (10.2 and 11.3 for best F_3 versus 7.6 and 8.3 for parents under WS and WW, respectively).

Actual gain (%) from selection relative to the better parent of the respective cross (for the 5 best F_3 families) was estimated and presented in [Table-5]. Practicing selection in the F_2 generation of the studied crosses of wheat resulted in an actual significant superiority (actual selection gain) over the better parent in grain yield/plant ranging from 15.48 % for SF10 to 74.71 % for SF9 under water stress and from 32.76% for SF12 (selected from the F_2 cross Mr-5 X Sk-93 under WW) to 60.24 % for SF9 under non-stress conditions. The selected F_3 family SF9 showed the highest actual selection gain under both water stress and non-stress conditions. The five selected F_3 families showed significant superiority in grain yield over their better parents under both stress and non-stress conditions. These superior families in grain yield are the result of transgressive segregation and could be considered promising lines of tolerance to drought conditions.

Table 5- Actual selection gain and superiority (%) in grain yield of the 5 best F_3 families over the better parent and over each of studied wheat parents under water stress and well watering conditions (2011/ 2012 season).

Designation	F_3 Family	Actual gain (%) over				Superiority (%) over		
		Better Parent	Sd-4	Sk-61	Gz-168	Mr-5	Sk-93	As-5
Water Stress								
SF8	Sd4 X Sk.61-WW-PS8	54.2	65.7	54.2	46.7	46.4	35.6	14.6
SF9	Sd4 X Mr5-WS-PS2	74.7	97.8	84.1	75.1	74.7	61.8	36.8
SF10	Sk61X As5-WS-PS3	15.5	67	55.4	47.8	47.5	36.6	15.5
SF11	Sk61 X Sk93-WS-PS2	56.9	91.8	78.4	69.7	69.3	56.8	32.6
SF12	Mr5 X Sk93-WW-PS8	39.8	70.9	59.1	51.3	51	39.8	18.2
			Sk-61	Mr-5	Sd-4	Gz-168	Sk-93	As-5
Well Watering								
SF8	Sd4 X Sk.61-WW-PS8	41.3	43.5	43.5	41.2	40.1	27.2	15.8
SF9	Sd4 X Mr5-WS-PS2	60.2	62.8	62.8	60.1	58.9	44.2	31.3
SF10	Sk61 X As5-WS-PS3	45.3	80.1	80.1	77.1	75.8	59.6	45.3
SF11	Sk61 X Sk93-WS-PS2	38.7	56.5	56.5	53.9	52.8	38.7	26.2
SF12	Mr5 X Sk93-WW-PS8	32.8	49.8	49.8	47.4	46.3	32.8	20.9

Superiority = 100 (Selection - Parent)/Parent

Transgressive segregation is a phenomenon specific to segregating hybrid generations and refers to the individuals that exceed parental phenotypic values for one or more characters [45]. Such plants are produced by an accumulation of favorable genes from both parents as a consequence of combination. Observations on transgressive segregations in segregating hybrid generations were previously explained by many research workers [46-48]. The selection of new recombinants and transgressive segregants was previously reported in wheat [47-49].

The expected selection gain for GYPP in the 5 F_2 crosses Sd-4 X Sk-61, Sd-4 X Mr-5, Sk-61 X As-5, Sk-61 X Sk.93 and Mr-5 X Sk-93 (the corresponding F_2 populations of the 5 best families SF8 through SF12 under the corresponding irrigation regime) was 4.5, 10.7, 33.2, 4.1 and 52.5 %, respectively. The best actual gain for GYPP (74.71% under WS and 60.24% under WW) shown by SF9 was much higher than the expected one (10.7 %). The low estimates of expected GA might be due to the underestimation of heritability percentages in the broad sense (h^2_b), which were 29.6, 17.5, 49.3, 8.7 and 70.1%, respectively.

Superiority of the best four selected F_3 families (SF8 through SF11) in grain yield over their better parent could mainly due to their high superiority in number of spikes/plant under both WW and WS conditions [Table-5]. The success of this study in obtaining new wheat genotypes of higher yield and earlier maturity than their parents under water stress conditions could be attributed to the presence of sufficient amount of additive and additive X additive types of genetic variance, amenable for high selection efficiency, in the F_2 generation of the studied crosses, besides to accumulation of favorable genes of yield traits from both parents as a result of new recombinations and transgressive segregations. Superiority in grain yield of the 5 best families over each of the 6 parents (as checks) used in this study [Table-5] reached to 97.8 % for SF9 over the Egyptian cultivar Sids-4 followed by SF11 (91.8 % superiority over the same cultivar) under water stress and reached 80.1 % for SF10 over Sakha-61 and Maryout-5 followed by 62.8 % superiority (by SF9) over Sakha-61 under well watering.

All the five best selected families outyielded, significantly, all the parents in this study under both water stress and non-stress conditions. The lowest superiority of these families was recorded over Aseel-5 (ranging from 15.5 to 36.8 % under water stress and from 15.8 to 45.3 % under well watering) because Aseel-5 cultivar was the highest yielding cultivar in this study. These five selected families should further be selfed for more homozygosity and producing pure lines and then tested for their stability under a variety of water stress conditions.

Detailed characterization of the 5 best F_3 families on the morphological and phenological levels is presented hereafter as follows:

SF8: It is a putative transgressive segregant in the F_3 generation resulted from selection for high yield in the F_2 population of the cross Sd-4 X Sk-61 under WW conditions. It showed high GYPP under both WW and WS; with low yield reduction due to water stress. It recorded the tallest plant [Fig-1] and was earlier than the earliest parent under both environments. Superiority in GYPP ranged from 14.6 and 15.8 % (over Aseel-5) to 65.7 and 43.5% (over Sids-4) under WS and WW, respectively.

SF9: It is a putative transgressive segregant in the F_3 generation, resulted from selection for high yield in the F_2 population of the cross Sd-4 X Mr-5 under WS conditions. It showed the highest GYPP under WS and the second highest under WW; with the second lowest yield reduction (6.9%) due to WS, *i.e.* the 2nd most drought tolerant F_3 family. It recorded the earliest F_3 for DTH and DTA [Fig-2] under both environments. Superiority in GYPP ranged from 36.8 and 31.3% (over Aseel-5) to 97.8 and 62.8% (over Sids-4) under WS and WW respectively.

SF10: It is a putative transgressive segregant in the F_3 generation, resulted from selection for high yield in the F_2 population of the cross Sk-61 X As-5 under WS conditions. It recorded the highest GYPP under WW (54.29 g), but showed the highest reduction in grain yield due to WS (29.0 %), 29.0 %), thought it recorded significantly higher yield than the best parent (Mr-5) under drought stress conditions. This family (SF10) recorded also the heaviest grain (100GW) [Fig-3] under both environments. Superiority in GYPP ranged from 15.5 and 45.3 % (over Aseel-5) to 67.0 and 80.1 % (over Sids-4) under WS and WW, respectively.

SF11: It is a putative transgressive segregant in the F_3 generation, resulted from selection for high GYPP in the F_2 population of the cross Sk-61 X Sk-93 under WS conditions. It proved the most drought tolerant selected family; since reduction in yield due to water stress was the lowest (6.2 %). Its yield under WS ranked the second highest and that under WW ranked 3rd amongst the 5 best F_3 families. This selected family showed the heaviest 100GW [Fig-3] under both WW and WS conditions. Superiority in GYPP ranged from 32.6 and 26.2% (over Aseel-5) to 91.8 and 56.5% (over Sids-4) under WS and WW, respectively.

Fig. 1- The earliest maturity and tallest plant shown by SF8 as compared with the better parent Sids-4.

Fig. 2- The earliest heading shown by SF9 as compared with the better parent Sids-4.

Fig. 3- The heaviest grains shown by SF10 and SF11 as compared with the better parent Sakha-61.

SF12: It is a putative transgressive segregant in the F_3 generation, resulted from selection for high GYPP in the F_2 population of the cross Mr-5 X Sk-93 under WW conditions. It is a high yielding family under both WW and WS; with low yield reduction (12.6 %) due to water stress. It is characterized by the longest and heaviest spike [Fig-4].

Superiority in GYPP over all parental cultivars (as checks) ranged from 18.2 and 20.9 % (over the highest yielding check Aseel-5) to 70.9 and 49.8 % (over the lowest yielding check Sids-4) under WS and WW, respectively.

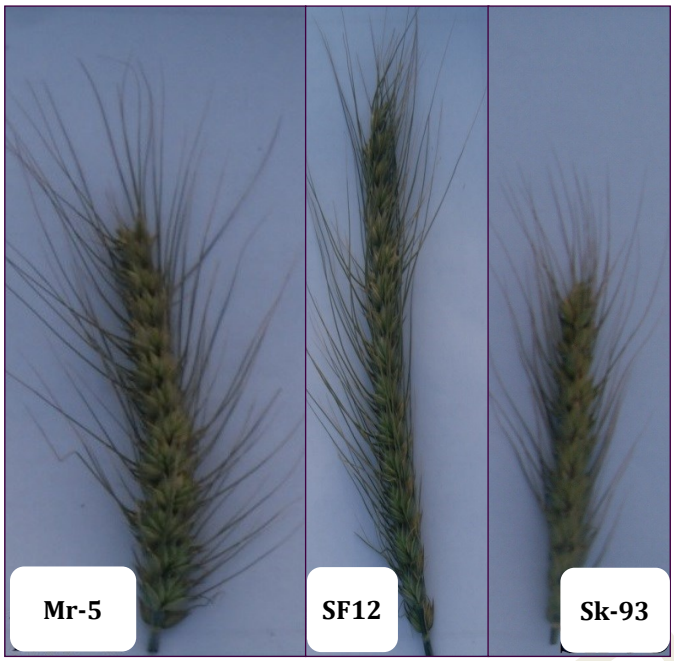

Fig. 4- The longest and heaviest spike of SF12 as compared with the better parent Maryout-5 and Sakha-93.

Molecular Characterization of the 5 Best Segregants and Their 5 Parents

Genetic Diversity Among 10 Wheat Genotypes

Fifteen SSR primers revealed discernible amplification profiles, therefore were employed to investigate the genetic polymorphism among the 10 wheat genotypes [Table-6] and [Fig-5], [Fig-6]. The 15 SSR primers produced a total of 42 amplicons, 34 of them were polymorphic, with an average percentage of 78.59% polymorphism. The number of polymorphic amplicons per primer ranged from 1 (WMS30 and WMC177) to 6 (WMS108) with an average of 2.27 polymorphic amplicons / primers. While two primers (WMC235 and WMS 304) showed no polymorphism. The size of amplified fragments varied with the different primers, ranging from 75 to 1500 bp [Fig-5], [Fig-6].

In this regard, Naghavi, et al. [50] used PAPD and SSR analyses to estimate genetic diversity among bread wheat genotypes including nineteen Iranian cultivars and two lines (Shain and Line 518). The level of polymorphism was 88% with RAPDs compared to 100% with SSRs. Wjhani, et al. [51] used three types of molecular markers (RAPD, AFLP and SSR) to study the genetic diversity among 14 wheat accessions, including 8 Syrian local cultivated varieties and 6 wild wheat genotypes. The three molecular systems differed in the ratio of polymorphism detected; the lowest was the AFLP technique (90.4%), while it was 92.3% in RAPD and 100% in SSR. Moreover, Abd El-Hadi [31] investigated the genetic diversity among three durum wheat cultivars and their six selected drought tolerant lines wing ISSR analysis. He reported that out of 99 amplified DNA fragments, 70 were polymorphic, representing a level of 71.42% polymorphism. Moreover, Bousba, et al. [36] reported that a total of 136 fragments were obtained from the 26 SSR primers and all the bands were polymorphic across all the genotypes screened. They added that polymorphism information content (PIC) values ranged from 38% to 94%, with an average of 74%. The results of the present study are in good agreement with those reported in the literature and confirm that polymorphism is a general phenomenon in wheat populations resulting from hybridization followed by segregating generations, as in the case of this study.

Table 6- Total no of amplicons, number of monomorphic and polymorphic amplicons and percentage of polymorphism, as revealed by SSR primers for 5 selected families and their 5 parents.

Sr. No.	Primers	Total No. of Amplicon	No of Mono-Morphic Amplicon	No of Poly-Morphic Amplicon	Polymorphism (%)
1	WMS 06	2	0	2	100
2	WMS 30	1	0	1	100
3	WMS 108	6	0	6	100
4	WMS 118	3	0	3	100
5	WMS 149	3	1	2	66.67
6	WMS 169	2	0	2	100
7	WMC 177	1	0	1	100
8	WMC 179	7	3	4	57.14
9	WMS 198	5	1	4	80
10	WMC 235	1	1	0	0
11	WMS 304	1	1	0	0
12	WMC 307	2	0	2	100
13	WMC 322	2	0	2	100
14	WMS 375	2	0	2	100
15	WMC 445	4	1	3	75
	Total	42	8	34	
	Average	2.8	0.53	2.27	78.59

Genotype Identification by Unique DNA Markers

As shown in [Table-7], the SSR assay permitted the identification of two out of ten wheat genotypes by unique positive and/ or negative markers.

The two genotypes, *i.e.,* maryout-5 and Aseel-5 were characterized by four positive unique markers, while one genotype (Maryout-5) was characterized by one negative unique marker.

The salinity tolerant line maryout-5 developed by Desert Research centre (DRC), Egypt was characterized two unique positive markers amplified by the primes WMC 179 (250bp) and WMC 307 (150bp) and one unique negative marker amplified by the primer WMC 179 (200bp).

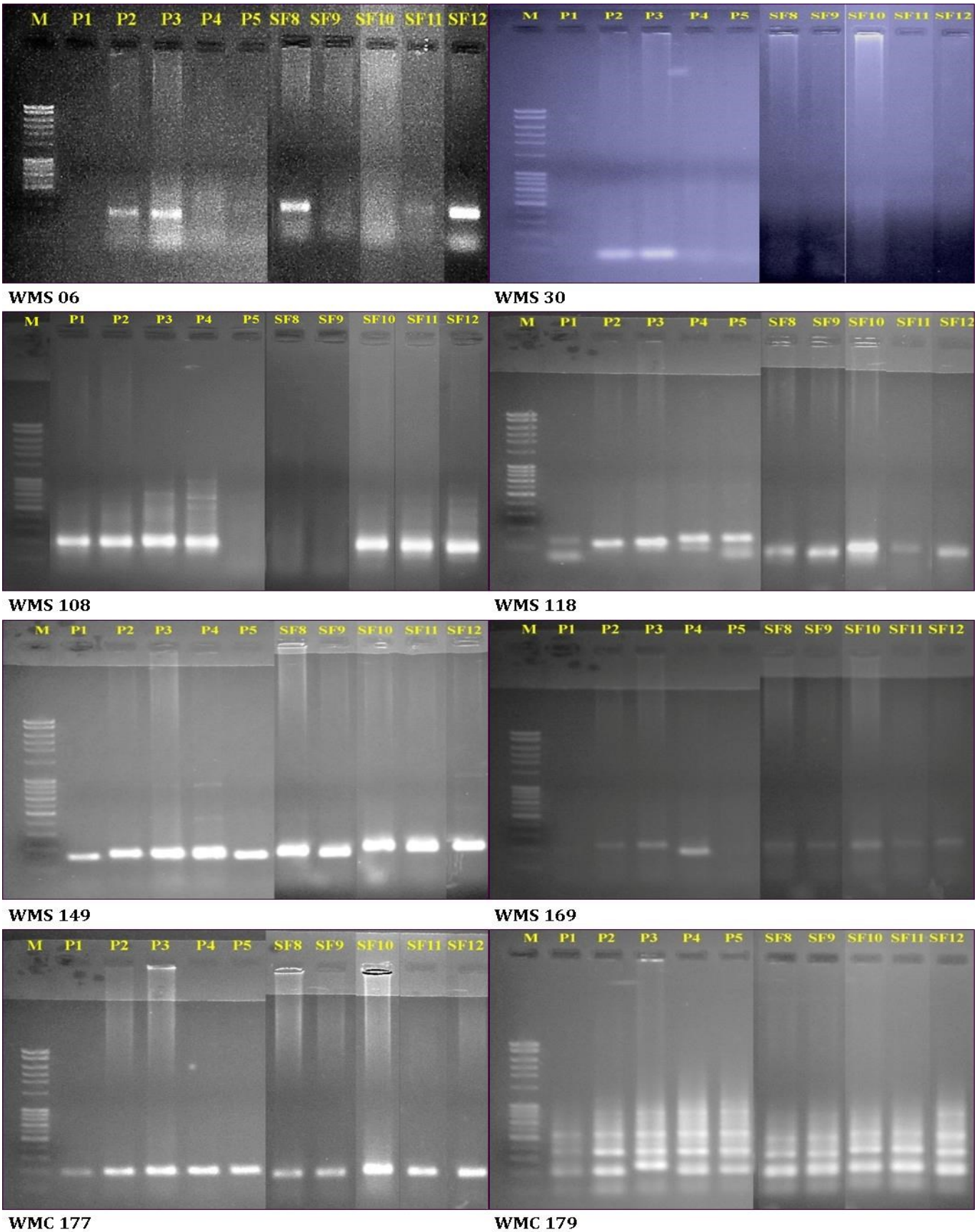

Fig. 5- Banding patterns of ten bread wheat genotypes amplified with the SSR primers WMS 06, WMS 30, WMS 108, WMS 118, WMS 149, WMS 169 and WMC 177 and WMC 179. M: 100bp DNA ladder, Lane 1: Sd-4, Lane 2: Sk-61, Lane 3: Mr-5, Lane 4: As-5, Lane 5: Sk-93, Lanes 6 to 10: selected families from SF8 to SF12.

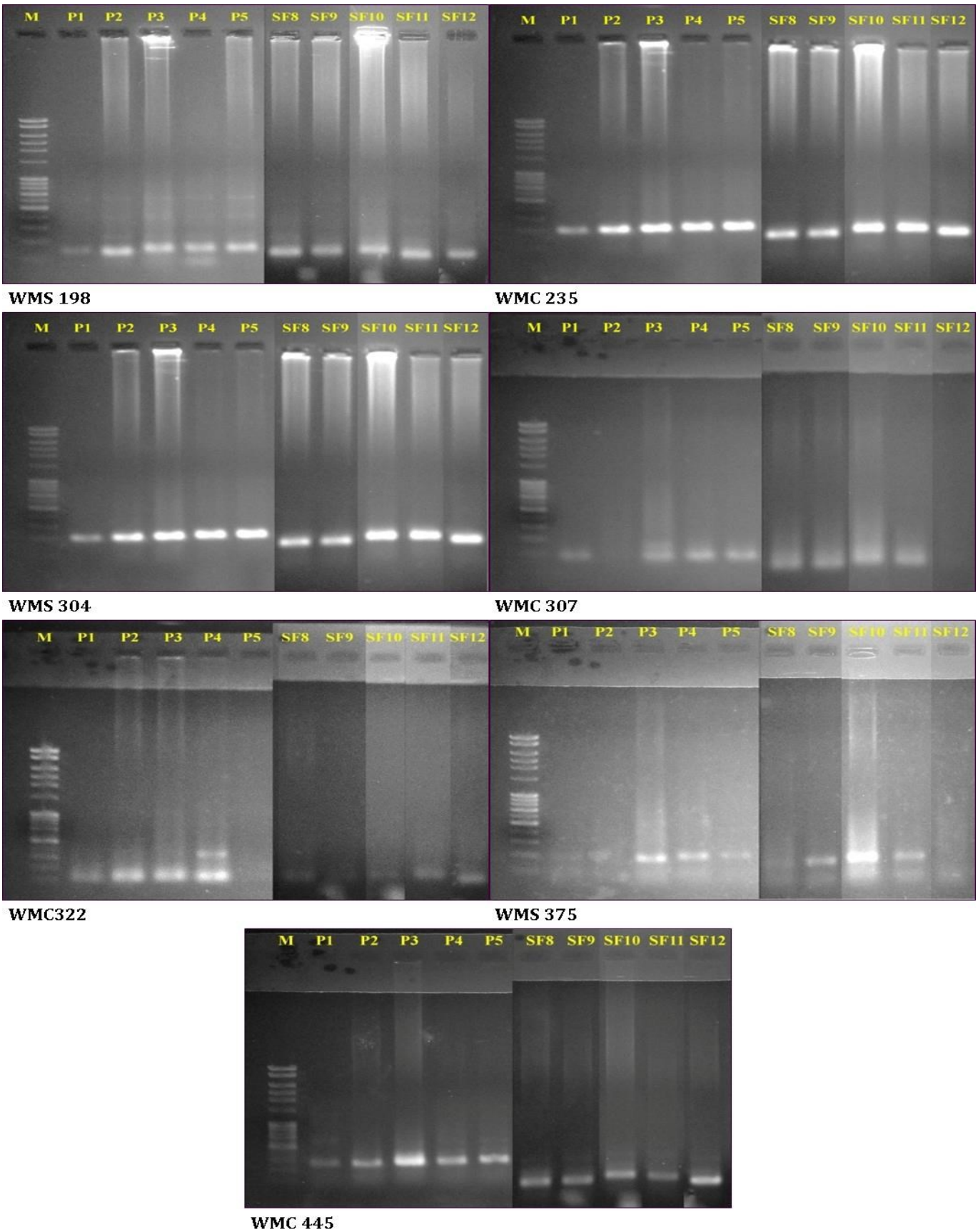

Fig. 6- Banding patterns of ten bread wheat genotypes amplified with the SSR primers WMS 198, WMC 235, WMS 304, WMC 307, WMC 322, WMS 375 and WMC 445. M: 100bp DNA ladder, Lane 1: Sd-4, Lane 2: Sk-61, Lane 3: Mr-5, Lane 4: As-5, Lane 5: Sk-93, Lanes 6 to 10: selected families from SF8 to SF12.

Table 7- Unique positive and negative SSR markers generated for 10 wheat genotypes (5 selected families and their 5 parents), marker size (bp) and total number of markers identifying each genotype.

Genotype	Positive Unique Markers Primer (Size/bp)	Total No.	Negative Unique Markers Primer (Size/bp)	Total No.	Grand Total
Sids-4	-		-		
Sakha-61	-		-		
Maryout-5	WMC179 (250), WMC 307 (150)	2	WMC 179 (200)	1	3
Asseel-5	WMS198 (100), WMC 322 (400)	2	-		2
Sakha-93	-		-		
SF8	-		-		
SF9	-		-		
SF10	-		-		
SF11	-		-		
SF12	-		-		
Total		4		1	5

The Syrian drought tolerant cultivar Aseel-5 was characterized by two unique positive markers amplified by the primers WMS 198 (100bp) and WMC 322 (400bp). While the remaining eight genotypes did not exhibit any unique marker [Table-7]. The size of these unique markers ranged from 100 to 400 bp.

In this regard, Moghaieb, et al. [25] determined the genotype specific SSR markers in nine bread and pasta wheat genotypes. They reported that 13 markers can be considered as a useful marker for screening for salt tolerance in these wheat genotypes. Abd El-Hadi [31] reported that in durum wheat, ISSR analysis showed four genotype-specific markers for the drought tolerant putative line S_3 that has a high significant increase in grain yield/plant than their parents under drought stress conditions and thus, these bands can be verified as markers associated with drought tolerance in durum wheat breeding programs.

Subsequent experiments need to be achieved to determine the linkage between the genotype-specific SSR markers in the present study and gene(s) for drought tolerance in the studied bread wheat genotypes. Using SSR analysis, we were able to identify eleven markers associated with drought tolerance in wheat. The present results support the idea that SSR analysis can provide fast detection of species-specific markers linked to drought stress tolerance in dread wheat these markers could help in breeding programs aiming at improving wheat productivity under drought stress conditions.

Genetic Relationships Among the 10 Wheat Genotypes

The recorded data from the SSR analysis in this study were used to compute the similarity matrices according to Dice coefficient [38]. As shown in [Table-8], the genetic similarity coefficient (GS) ranged from 57% (between SF8 and Aseel-5) to 84% (between Aseel-5 and Maryout-5 and between SF9 and SF10). The results of this investigation indicated that all the five selected drought tolerant families differ on the DNA level from their parents, where the average of genetic similarity (GS) between selections and their parents was about 70 %.

In this context, Abd El-Hadi [31] reported that the genetic similarity between six selected putative durum wheat mutants (derived *via* gamma rays) and their three parents depending on ISSR analysis ranged from 12.7 to 87.4 %. Moreover, Munir [26] reported that genetic similarity coefficients for SSR markers between 18 salt tolerant wheat accessions ranged from 0.45 to 0.95.

Table 8- Genetic similarity (GS) matrices among the ten wheat genotypes (5 selected families and 5 parents).

	Sids-4	Sakha-61	Maryout-5	Aseel-5	Sakha-93	SF8	SF9	SF10	SF11
Sids-4	1								
Sakha-61	0.68	1							
Maryout-5	0.63	0.82	1						
Aseel-5	0.69	0.8	0.84	1					
Sakha-93	0.73	0.72	0.67	0.72	1				
SF8	0.7	0.68	0.62	0.57	0.61	1			
SF9	0.79	0.72	0.65	0.68	0.64	0.8	1		
SF10	0.8	0.73	0.67	0.69	0.73	0.81	0.84	1	
SF11	0.7	0.77	0.7	0.73	0.8	0.7	0.77	0.78	1
SF12	0.6	0.67	0.65	0.64	0.71	0.73	0.71	0.68	0.79

In conclusion, the use of molecular markers can increase the efficiency of conventional plant breeding by identifying markers associated with the quantitatively inherited traits controlled by several genetic loci and their genetic components are difficult to measure. Consequently, wheat breeder can use molecular methods such as SSR to select specific genotypes for drought tolerance using specific unique markers. The SSR analysis used in the present investigation proved that it was possible to create new genes or gene combinations of high grain yield/plant under drought stress conditions *via* mutation and hybridization breeding procedures, respectively.

Conflicts of Interest: None declared.

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