World Research Journal of Agronomy
ISSN: 2320-3404 & E-ISSN: 2320-5644, Volume 2, Issue 1, 2013, pp.-015-025.
Available online at http://www.bioinfopublication.org/jouarchive.php?opt=&jouid=BPJ0000009

UNIQUE SSR MARKERS FOR DROUGHT TOLERANCE IN NEWLY-DEVELOPED BREAD WHEAT MUTANTS

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Received: November 07, 2013; Accepted: December 09, 2013

Abstract- Drought tolerance is the ability of a variety to remain relatively more productive than others under limited water conditions. Irradiation of seeds of 7 bread wheat genotypes with gamma rays was used in this study to create genetic variability amenable for efficient selection of new putative drought tolerant mutants. The objectives were to evaluate morphological traits of the mutants under water stress (WS) and well watering (WW) conditions and to characterize the drought tolerant mutants and their parents on the DNA level. A total of 57 putative induced mutants were selected from M_2 populations, 23 of them were selected under WS and 34 under WW. Out of them, seven M_3 families (SF1 through SF7) outyielded significantly their parents by at least 15% under WS. The most superior M_3 family was SF3 (56.34 and 65.62% superiority over its parent Giza-168 under WW and WS, respectively). Fifteen SSR primers were used for RCR amplification of the genomic DNA of the best 7 M_3 families (mutants) and their 4 parents. The genetic similarity (Gs) ranged from 33 to 88%. The mutants SF3 and SF4 exhibited very low Gs (42 and 40%, respectively), with their common parent (Giza-168), indicating the efficiency of gamma rays in creating new genetic variability. The total number of amplicons detected by SSR was 45, while the number of polymorphic amplicons was 43, with an average polymorphism of 86.67%. The SSR analysis identified 11 genotype specific unique markers for the drought tolerance mutants and parents (5 positive and 6 negative unique markers). Thus, these bands can be verified as markers associated with drought tolerance in bread wheat and could help in breeding programs aiming at improving wheat productivity under drought conditions.

Keywords- *Triticum aestivum*, Drought tolerance, SSR, Unique markers, Mutants, Gamma rays

Introduction

The ideal genotype for moisture stress conditions must combine a reasonably high yield potential with specific plant characters, which could buffer yield against severe moisture stress [1]. To start a proper wheat bread breeding program for improving drought tolerance, the source populations should possess genetic variability amenable for selection. Gamma rays was effective in creating new genetic variability in the present pure line wheat cultivars, helping plant breeders to practice an efficient selection in the M_2 and next mutated generations [2-5]. Induced mutants *via* gamma rays have been obtained in bread wheat for resistance to drought leading to the release of 26 varieties worldwide [6].

Traditionally, the assessment of the new genetic variation in crop plants has been conducted on basis of phenotypic and cytogenetic characters, which frequently lack the resolving power needed to identify individual genotypes [7]. In the last decades, molecular markers such as RFLP, RAPD, ISSR and AFLP have been used to assess genetic variation at the DNA level, allowing an estimation of degree of relatedness between individuals without the influence of environmental variation [8]. Among different classes of molecular markers, simple sequence repeats (SSRs) markers also known as microsatellites, are useful in a variety of applications like plant genetics and breeding because of their reproducibility, multiallelic nature, codominant inheritance, relative abundance and good genome coverage. SSRs continue to be the main marker type for quantitative trait loci (QTL) studies in wheat, either alone or in combination with other types of markers, and they cover all 21 wheat chromosomes. Several investigators concluded that SSR molecular markers are significantly associated with wheat traits related to salinity tolerance [9] and drought tolerance [10-15].

The main aim of the present investigation was to develop drought tolerant wheat mutants *via* gamma radiation. The detailed objectives were to: (i) evaluate in the field 57 putative mutants selected from M_2 populations of seven gamma irradiated wheat genotypes, (ii) characterize the best putative drought tolerant mutants at the DNA level *via* SSR analysis and (iii) identify unique molecular markers for drought tolerance in bread wheat.

Materials and Methods

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

Seven genotypes of bread wheat (*Triticum aestivum* L.) were used in this study [Table-1].

Table 1- Name, pedigree and the most important traits of the studied 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
Sahel-1 cv.	Sh-1	NS 732 / PIMA // VEERY "S"	ARC-Egypt	Drought tolerant

Experimental Procedures

First season (2009/2010)

Producing M_2 Seeds: Irradiated seeds of the seven parents with the selected dose of gamma ray (350 GY) were immediately sown on 20 Nov., 2009 in separate plots to obtain M_1 plants of each bulk. Each plot consisted of 30 rows; each row was 4 m long and 30 cm wide. Spaces between each two plants were 10 cm in each row. The plants were left for natural self pollination. At harvest, ten seeds were taken randomly from each M_1 plant (M_2 seed). The 10 M_2 seeds from each plant of each bulk were blended to represent seed of the respective M_2 bulk. These seeds of M_2 bulks were kept for use in experiments of the second season (2010/2011). The recommended cultural practices for wheat production at Inshas were followed in M_1 generation.

Second Season (2010/2011)

Growing M_2 Populations in Selection Fields: In 2010/2011 seasons, two selection fields were grown by the seven M_2 bulks, the first under well-watering (WW) (irrigation every 5 days) and the second under water-stress (WS) (irrigation every 15 days starting from 21 days after sowing), where total quantity of irrigation water for WS was 70% of that for WW. Each 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), *i.e*., for high grain/yield plant and some other favorable traits, such as spike length, spike weight, spikes/plant, earliness, etc., under water stress and non-stress conditions. Fifty seven individual plant selections were separately harvested from M_2 (23 under WS and 34 under WW).

Third Season (2011/2012)

Field Evaluation of Selections and their Parents

A field experiment was conducted to evaluate selected individual genotypes as compared to their parents. The experimental design used was a split-plot with balanced lattice (8x8) arrangement in three replications. Plots were assigned to two irrigation regimes and sub-plots were devoted to 64 genotypes (57 selections + 7 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 of M_2's and M_3 selections were subjected to the normal analysis of variance of the split-plot design and least significant differences (LSD) were estimated according to [16].

Molecular Characterization (SSR Analysis)

SSR analysis was used in the present study to investigate the genetic diversity among the 7 best putative mutants (SF1 through SF7) and their 4 parents (Aseel-5, Sakha-93, Giza-168 and Sahel-1) and to identify markers associated to drought tolerance.

DNA Extraction

Extraction of DNA was carried out according to [17,18]. 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 re-suspended 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 selections (7 putative mutants) and their 4 parents was performed based on SSR analysis. These selections represent drought tolerant and high yielding M_3 families.

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

A set of fifteen random primers [Table-2] chosen according to [19] among the publicly available sets catalogued in the Grain Genes database (http://wheat.pw.usda.gov) as WMC (Xwmc) and as described by [20] for WMS (Xgwm), specialized for *Triticum aestivum* and used for screening drought tolerance was used in the detection of polymorphism among the eleven wheat genotypes. These primers were synthesized by BioShop® Canada Inc.

Polymerase Chain Reaction (PCR)

The PCR master mix for simple sequence repeats (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 presence 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 [21] 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.

Cluster Analysis Based on SSR

The similarity matrix was used in the cluster analysis. The cluster analysis was employed to organize the observed data into meaningful structures to develop taxonomies. At the first step, when each accession represents its own cluster, the distances between these accessions are defined by the chosen distance measure (Dice coefficient). However, once several accessions have been linked together, the distance between two clusters is calculated as the average distance between all pairs of accessions in the two different clusters. This method is called unweighted pair group method using arithmetic average (UPGMA) according to [21].

Results and Discussion

Selection Experiment

In the present experiment, 57 individual plants with derisible traits related to drought tolerance were selected from M_2 bulks (23 of them selected under water stress and 34 under well-watering) in 2010/2011 season. Progenies of these selections (57 M_3 families) were evaluated in 2011/2012 season along with their seven parental genotypes for grain yield and earliness attributes under water stress and non stress conditions.

Comparing Performance of Four Selection Groups

The evaluated 64 genotypes were partitioned into three groups, namely parents (7), M_3 families selected under WS (23) and M_3 families selected under WW (34). 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 the two selection groups were higher than the mean of parents group (non-irradiated genotypes) when evaluating them either under water stress or non-stress conditions. Moreover, means of the best selection (mutant) 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 (mutants) over parents in grain yield and earliness traits 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.

Further selection and evaluation under drought stress conditions should be continued in the selected superior M_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.

Table 3- Summary of group mean ($\bar{x}$) and best (B) genotype mean of 7Ps, 57 families selected from M'$_2$s (23 under WS and 34 under WW) for studied traits under water stress (WS) and well watering (WW) conditions (2011/ 2012 season).

Group	Stress	Parameter	DTH	DTA	DTM	PH (cm)	SL (cm)	SW (g)	SPP	GPS	100GW (g)	GYPP (g)
7 parents	WW	$\bar{x}$	95	102	135	96	13.8	3.9	8.3	75.0	5.1	32.0
	WS	$\bar{x}$	93	101	134	90	13.1	3.5	7.6	70.0	4.6	26.6
	WW	B	87	96	133	107	16.4	5.1	10.1	90.0	5.5	37.3
	WS	B	87	95	132	100	16.2	4.3	9.1	84.0	5	33.3
23 M3 families Selected Under WS	WW	$\bar{x}$	96	107	136	94	13.0	3.6	9.4	73.0	4.9	32.3
	WS	$\bar{x}$	95	104	134	89	12.8	3.2	8.5	68.0	4.4	27.6
	WW	B	92	100	132	104	15.7	4.2	13.6	85.0	5.6	48.2
	WS	B	91	92	130	102	14.9	3.7	13.3	80.0	4.9	42.8
34 M3 families Selected Under WW	WW	$\bar{x}$	97	108	137	93	13.0	3.6	9.4	73.0	5.1	33.6
	WS	$\bar{x}$	94	105	135	89	12.8	3.3	8.5	67.0	4.5	28.0
	WW	B	88	96	128	103	15.5	4.5	13.3	88.0	6.1	53.3
	WS	B	82	92	126	101	14.1	4.0	11.7	79.0	5.1	42.0
LSD 0.05			0.84	0.66	0.65	0.9	0.12	0.08	0.12	1.13	0.08	0.8

Field Characterization of the 7 Best M_3 Putative Mutants

Out of the two selection groups (23 M_3 families selected under WS, 34 M_3 families selected under WW) 2 and 5 families, respectively significantly out yielded, by at least 15%, the best parent under WS conditions. The seven best selected M_3 families included two (SF2 and SF3) selected under WS, *i.e.*, Sk-93-WS-PM2 and Gz-168-WS-PM2, respectively and five (SF1, SF4, SF5, SF6 and SF7) selected under WW, *i.e.*, As-5-WW-PM5, Gz-168-WW-PM5, Gz-168-WW-PM6, Sh-1-WW-PM6 and Sh-1-WW-PM7, respectively.

Means of studied traits of the seven best M_3 families and the 7 parental genotypes are presented in [Table-4]. Under WW conditions, the best M_3 families groups showed higher average

grain yield / plant (46.2 and 47.7 g, respectively) than that of parents group (32.0 g). Moreover, reduction due to water stress in the best M_3 group (12.0% on average) was less than that of parents group (17.1%). This means that in this experiment, selection practiced in M_2 populations was effective in producing higher yielding families under water stress of higher drought tolerance than the original parents and the success of the gamma-ray mutation induction in isolating new variants of higher drought tolerance. This conclusion was previously confirmed by [3-5].

Superiority of the best M_3 families in grain yield over parents was associated with superiority in number of spikes/plant (11.6 and 12.3 against 7.6 and 8.3 for parents under WS and WW, respectively).

Practicing selection for high grain yield in the M_2 populations derived from gamma radiation treatment of seven parents genotypes of wheat resulted in an actual progress over the corresponding original parent in GYPP ranging from 26.27 to 64.36% under WS and from 25.44 to 56.34% under WW for SF1 and SF3, respectively [Table-5].

The selected M_3 family (SF3) showed the highest actual selection gain followed by SF6 (62.62 and 48.03% under WS and WW, respectively). These two M_3 families showed also superiority in SPP and in DTM, *i.e.*, earliness of maturity, under both water stress and non-stress conditions.

Superiority of the seven M_3 families in grain yield is also mainly due to superiority over their original parents in number of spikes/plant.

The selected M_3 family (SF3) showed the highest actual selection gain followed by SF6 (62.62 and 48.03% under WS and WW, respectively). These two M_3 families showed also superiority in SPP and in DTM, *i.e.*, earliness of maturity, under both water stress and non-stress conditions.

Superiority in grain yield of the seven best families over each of the 7 parental cultivars (as checks) used in this study reached 85.7% for SF3 over the Egyptian cultivar Sids-4 under water stress and 60.1% over Sakha-61 under well watering. All the seven best M_3 selected families outyielded significantly all the parental cultivars in this study under both water stress and non-stress conditions. The lowest superiority of these families was recorded over Aseel-5 (ranging from 14.6 to 28.4% under water stress and from 19.0 to 29.2% under well watering) because Aseel-5 cultivar was the highest yielding cultivar in this study.

Detailed agronomic and yield characterization of the seven best M_3 families is presented hereafter as follows:

SF1: It is a putative mutant in the M_3 generation resulted from Aseel -5 variety *via* gamma irradiation under WW conditions. It is high yielding under both WS (2nd highest best M_3's) and WW (3rd highest best M_3's) with low reduction (10.0%) due to water stress, *i.e.*, drought tolerant. It recorded the highest number of grains/spike amongst the 7 best families [Fig-1]. Superiority in GYPP over all parents (checks) ranged from 26.3 and 25.4% (over Aseel-5) to 82.6% over Sids-4 and 55.5% over Sakha-61 and Maryout-5 under WS and WW, respectively.

SF2: It is a putative mutant in the M_3 generation resulted from selection for high yield in the M_2 population of irradiated seed of Sakha -93 under water stress conditions. It is a high yielding family under both drought stress and non-stress conditions, with low yield reduction due to water stress (drought tolerant). It recorded the highest number of spikes [Fig-2] under both water stress (13.3) and non-stress (13.6) conditions. Superiority in GYPP ranged from 26.0 and 28.1% (over Aseel-1) to 82.2% over Sids-4 and 58.8% over Sakha-61 and Maryout-5 under WS and WW, respectively.

Table 4- Mean performance of the seven best M_3 selected families and seven parents for studied wheat traits under water stress and well watering conditions (2011/ 2012 season).

Genotype	DTH	DTA	DTM (cm)	PH (cm)	SL	SW (g)	SPP	GPS	100GW (g)	GYPP (g)	Red. %
Best M_3						**Water stress**					
SF1	95	111	141	95	13.7	3.6	11.7	75	4.4	42.1	10
SF2	102	112	141	96	14.1	3.3	13.3	68	4.7	42	12.1
SF3	91	102	135	89	14.1	3.7	11.9	74	4.3	42.8	11.2
SF4	94	103	137	87	13.7	4	10.1	71	4.4	39.9	10.1
SF5	93	102	137	84	13.5	3.5	11.3	65	4.6	39.3	11.9
SF6	95	105	129	101	13.4	3.7	10.9	68	4.8	40.2	13
SF7	98	111	139	80	13.1	3.3	11.9	64	4.8	38.2	15.3
Aver. Parents	95	107	137	90	13.7	3.6	11.6	69.3	4.6	40.6	12
Sd-4	87	95	132	96	16.2	4.3	5.3	84	5	23.1	24.6
Sk-61	92	100	132	79	10.3	3.1	8.1	63	4.4	24.8	17.7
Mr-5	95	103	138	94	14.2	3.8	6.9	76	4.9	26.1	13.4
As-5	96	101	132	92	13.1	3.4	9.1	69	4.6	33.3	10.6
Sk-93	94	101	132	81	12.2	3.2	8.7	66	4.4	28.2	17
Gz-168	95	102	136	86	12.6	3.6	7.3	65	4.2	26	15.5
Sh-1	94	107	133	100	13.3	3.3	7.5	68	4.8	24.7	20.8
Aver.	93	101	134	90	13.1	3.5	7.6	70.1	4.6	26.6	17.1
$LSD_{0.05}$	0.67	0.58	0.56	1.08	0.13	0.08	0.13	0.9	0.07	0.8	
Best M_3						**Well watering**					
SF1	96	112	142	100	14	3.8	12.3	80	5	46.8	
SF2	103	113	142	103	14.6	3.5	13.6	72	5.2	47.8	
SF3	92	104	136	95	14.7	3.9	12.7	80	4.9	48.2	
SF4	96	104	139	91	14.1	4.1	10.9	78	5.3	44.4	
SF5	95	105	139	90	14.5	3.6	12.5	71	5.5	44.6	
SF6	98	109	131	103	13.6	4	11.5	74	6.1	46.2	
SF7	104	117	141	85	13.3	3.7	12.3	70	5.7	45.1	
Aver. Parents	98	109	139	95	14.1	3.8	12.3	75	5.4	46.2	
Sd-4	89	96	134	102	16.4	5.1	5.5	90	5.5	30.6	
Sk-61	94	102	134	83	11.7	3.4	9.1	68	4.8	30.1	
Mr-5	97	104	140	100	15.3	4.2	7.2	80	5.5	30.1	
As-5	97	102	133	100	13.5	3.8	10.1	73	5.2	37.3	
Sk-93	95	102	134	86	12.5	3.5	9.7	70	4.9	34	
Gz-168	96	104	138	94	13.7	3.7	8.3	71	4.7	30.8	
Sh-1	97	109	135	107	13.5	3.8	8.2	72	5.2	31.2	
Aver.	95	102	135	96	13.8	3.9	8.3	75 .0	5.1	32	
$LSD_{0.05}$	0.84	0.66	0.65	0.9	0.12	0.08	0.12	1.13	0.08	0.8	

Table 5- Actual selection gain and superiority (%) in grain yield of the 7 best M_3 families over each of studied wheat parents under water stress and well watering conditions (2011/ 2012 season).

Sr. No.	Best M3 family	Actual Gain (%) Over Better Parent	Superiority (%) Over						
			Sd-4	Sh-1	Sk-61	Gz-168	Mr-5	Sk-93	As-5
			Water Stress						
SF1	As-5-WW-PM5	26.27	82.6	70.3	70	61.7	61.3	49.4	26.3
SF2	Sk-93-WS-PM2	49.04	82.2	69.9	69.6	61.3	60.9	49	26
SF3	Gz-168-WS-PM2	64.36	85.7	73.1	72.8	64.4	64	51.9	28.4
SF4	Gz-168-WW-PM5	53.23	73.1	61.4	61.1	53.2	52.9	41.6	19.7
SF5	Gz-168-WW-PM6	50.92	70.5	59	58.7	50.9	50.6	39.5	17.9
SF6	Sh-1-WW-PM6	62.62	74.4	62.6	62.3	54.4	54	42.7	20.6
SF7	Sh-1-WW-PM7	54.53	65.7	54.5	54.2	46.7	46.4	35.6	14.6
			Sk-61	**Mr-5**	**Sd-4**	**Gz-168**	**Sh-1**	**Sk-93**	**As-5**
			Well Watering						
SF1	As-5-WW-PM5	25.44	55.5	55.5	52.9	51.8	50	37.8	25.4
SF2	Sk-93-WS-PM2	40.71	58.8	58.8	56.2	55	53.2	40.7	28.1
SF3	Gz-168-WS-PM2	56.34	60.1	60.1	57.5	56.3	54.4	41.9	29.2
SF4	Gz-168-WW-PM5	44.02	47.5	47.5	45.1	44	42.3	30.7	19
SF5	Gz-168-WW-PM6	44.66	48.2	48.2	45.8	44.7	42.9	31.3	19.5
SF6	Sh-1-WW-PM6	48.03	53.5	53.5	51	49.9	48	36	23.8
SF7	Sh-1-WW-PM7	44.5	49.8	49.8	47.4	46.3	44.5	32.8	20.9

SF3: It is a putative mutant in the M_3 generation resulting from selection for high grain yield in the M_2 population of irradiated Giza-168 cultivar under water-stress conditions. It ranked first in grain yield/plant amongst the 7 best M_3 families under both water-stress and non-stress conditions, with low yield reduction due to water stress, *i.e.*, a drought tolerant family. It recorded also the largest number of grains/spike [Fig-2] under WW and the 2nd largest under WS and the longest spike [Fig-1] and the earliest in DTH and DTM under WW and WS. Superiority in GYPP ranged from 28.4 and 29.2% (over Aseel-5) to 85.7% over Sids-4 and 60.1% over Sakha-61 and Maryout-5 under WS and WW conditions, respectively.

SF4: It is a putative mutant resulted from selection for high yield in the M_2 population of irradiated Giza-168 cultivar under WW conditions. It is a high yielding family under both WW and WS, with low yield reduction due to water stress, *i.e.,* drought tolerant. It recorded the heaviest spike and grain [Fig-3] under both environments. Superiority in GYPP over all parents (checks) ranged from 19.7 and 19.0% (over Aseel-5) to 73.1% over Sids-4 and 47.5% over Sakha-61 and Maryout-5 under WS and WW, respectively.

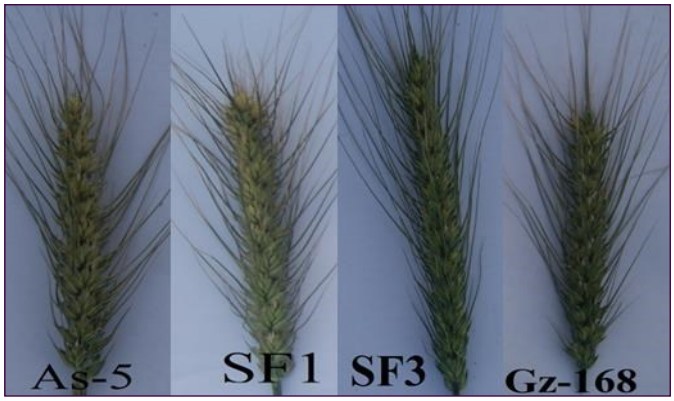

Fig. 1- The highest number of grains/spike for (SF1 and SF3) as compared with their parents As-5 and Gz-168, respectively. And the longest spike for SF3.

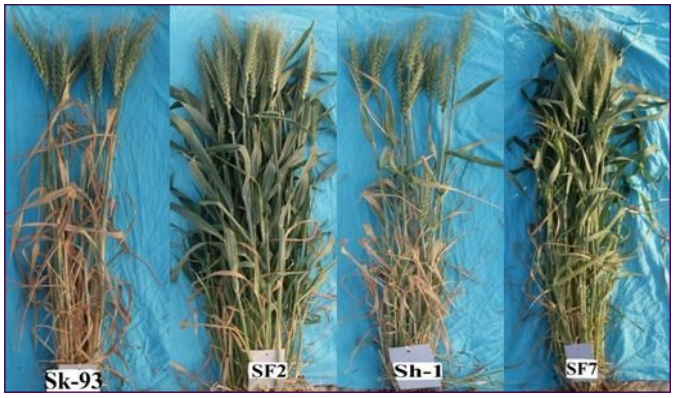

Fig. 2- The highest number of spikes for SF2 and SF7 as compared with their parents Sk-93 and Sh-1, respectively.

Fig. 3- The heaviest grain for SF4 and (SF6 and SF7) as compared with their parents Gz-168 and Sh-1, respectively.

SF5: It is a putative mutant in the M_3 generation resulted from selection for high yield in the M_2 bulk of irradiated Giza-168 cultivar under WW conditions. It is high yielder under both WW and WS conditions, with low reduction in GYPP due to water stress, *i.e.,* a drought-tolerant family. Superiority in GYPP ranged from 17.9 and 19.5% (over Aseel-5) to 75.5% over Sids-4 and 48.2% over Sakha-61 and Maryout-5 under WS and WW, respectively.

SF6: It is a putative mutant in the M_3 generation resulted from selection for high yield in the M_2 bulk of irradiated Sahel-1 cultivar under WW conditions. It is high yielder under both WW and WS conditions, with low reduction in GYPP due to water stress, *i.e.,* a drought-tolerant family. It recorded the earliest family in (DTM) amongst the best 7 families and the 7 parents, under both WW (131 days) and WS (129 days) conditions. It recorded the heaviest grain [Fig-3] under both environments. Superiority in GYPP ranged from 20.6 and 23.8% (over Aseel-5) to 74.4% over Sids-4 and 53.5% over Sakha-61 and Maryout-5 under WS and WW, respectively.

SF7: It is a putative mutant in the M_3 generation resulted from selection for high GYPP in the M_2 bulk of irradiated Sahel-1 cultivar under WW conditions. It is a high yielding M_3 family under both WW and WS conditions, with low yield reduction due to water stress. It is characterized by the shortest plant height, the heaviest grain [Fig-3] and recorded highest in SPP [Fig-2] amongst the 7 best selected families. Superiority in GYPP ranged from 14.6 and 20.9% (over Aseel-5) to 65.7% over Sids-4 and 49.8% over Sakha-61 and Maryout-5 under WS and WW, respectively.

Molecular Characterization of Best Mutants and their Parents

In this study, SSR analysis was conducted to characterize the genetic markers associated with drought tolerance and identify the differences among the seven putative mutants of bread wheat and their four parents on a molecular DNA level. Moreover, genetic relationships among them were performed.

Genetic Diversity among 11 Wheat Genotypes

Fifteen SSR primers revealed discernible amplification profiles, therefore were employed to investigate the genetic polymorphism among the 11 wheat genotypes [Table-6] and [Fig-4], [Fig-5].

Table 6- Total No. of amplicons, number of monomorphic and polymorphic amplicons and percentage of polymorphism, as revealed by SSR primers for 7 selected families and their 4 parents.

Primers	Total no of Amplicons	No of Mono-morphic Amplicons	No of poly-morphic Amplicons	Polymorphism
WMS 06	1	0	1	100.00
WMS 30	1	0	1	100.00
WMS 108	6	0	6	100.00
WMS 118	3	0	3	100.00
WMS 149	3	0	3	100.00
WMS169	2	0	2	100.00
WMC 177	2	0	2	100.00
WMC 179	10	0	10	100.00
WMS 198	5	0	5	100.00
WMC 235	1	1	0	0.00
WMS 304	1	1	0	0.00
WMC 307	2	0	2	100.00
WMC 322	2	0	2	100.00
WMS 375	2	0	2	100.00
WMC 445	4	0	4	100.00
Total	45	2	43	
Average	3	0.13	2.87	86.67

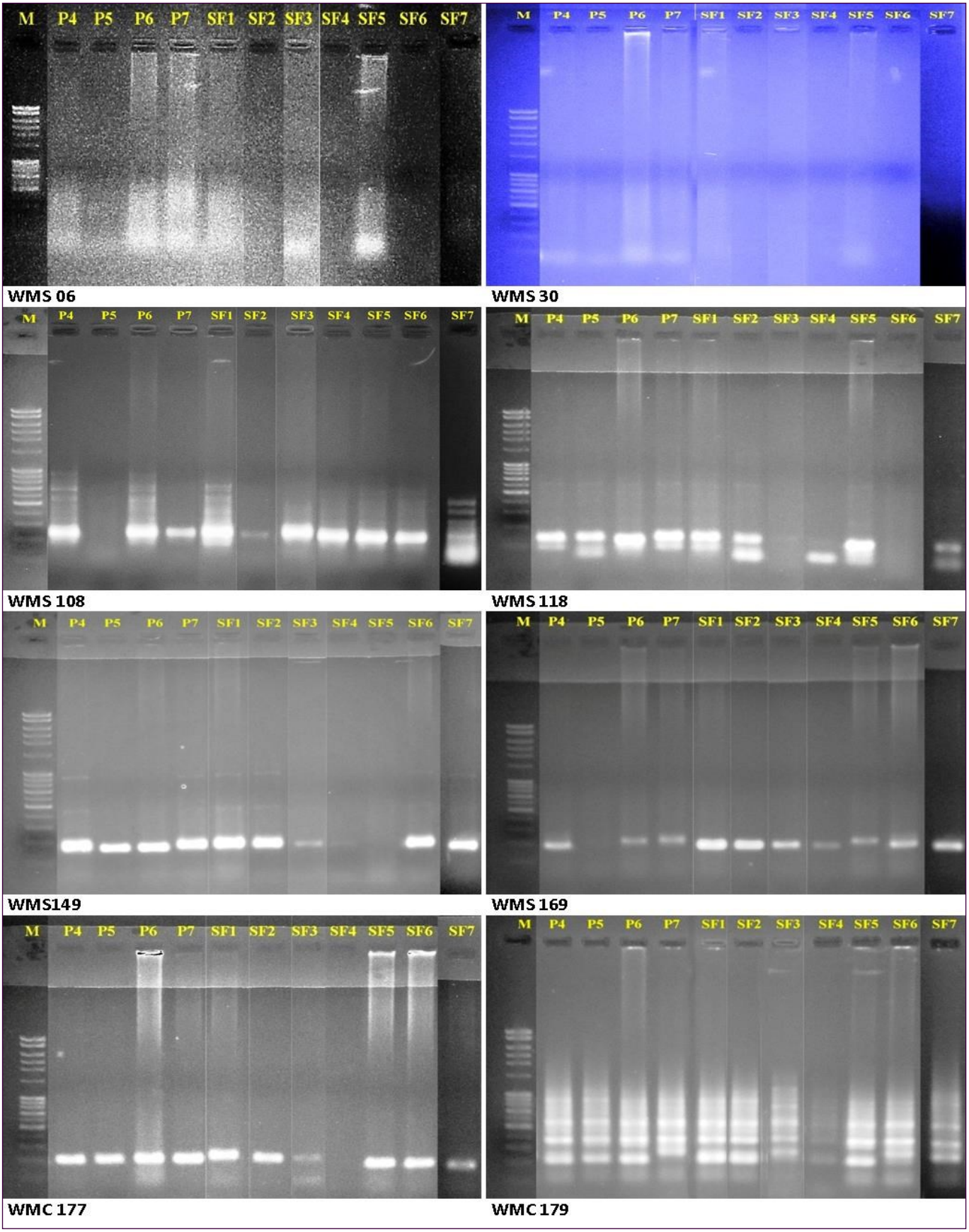

Fig. 4- Banding patterns of eleven bread wheat genotypes amplified with the SSR primers WMS 06, WMS 30, WMS 108, WMS 118, WMS 149, WMS 169 and WMC 177 and WMC 179. M: 100bp DNA ladder, Lane 1: As-5, Lane 2: Sk-93, Lane 3: Gz-168, Lane 4: Sh-1, Lanes 5 to 11: selected families from SF1 to SF7.

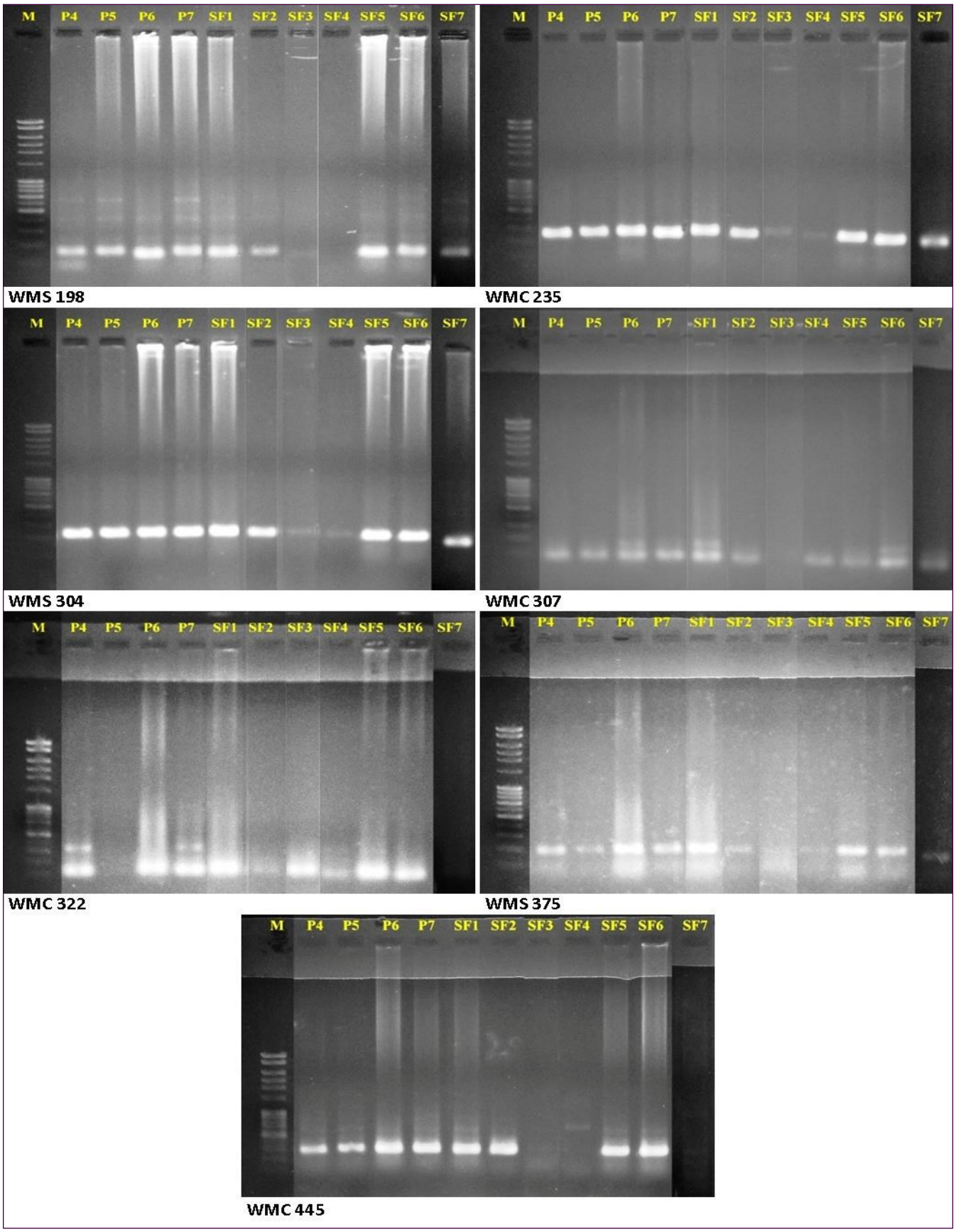

Fig. 5- Banding patterns of eleven bread wheat genotypes amplified with the SSR primers WMS 198, WMC 235, WMS 304, WMC 307, WMC 322, WMS 375 and WMC 445. M: 100bp DNA ladder, Lane 1: As-5, Lane 2: Sk-93, Lane 3: Gz-168, Lane 4: Sh-1, Lanes 5 to 11: selected families from SF1 to SF7.

The 15 SSR primers produced 45 amplicons, 43 and 2 of them were polymorphic and monomorphic, respectively, and the average percentage of polymorphic was 86.67%. The number of amplicons per primer ranged from 1 (WMS 06, WMS 30, WMC 235 and WMS 304) to 10 (WMC 179) with an average of 3.00 amplicons / primer. The average number of polymorphic amplicons was 2.87 fragments /primer. Thirteen out of 15 primers exhibited 100% polymorphism. While, two primers (WMC 235 and WMS 304) showed no polymorphism. The size of amplified fragments varied with the different primers, ranging from 50 to 1500 bp [Fig-4] and [Fig-5].

In this respect, [14] investigated the genetic diversity among three durum wheat cultivars and their six selected drought tolerant lines using ISSR analysis. He reported that out of 99 amplified DNA fragments, 70 were polymorphic, representing a level of 71.42% polymorphism. Moreover, [19] 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 after subject to gamma rays as in the case of this study.

SSR Characterization of Seven Mutants and Four Parents

The genotype specific SSR markers for the different eleven wheat genotypes used in this study are presented in [Table-7]. One hundred four amplicons were useful genotype-specific markers for the studied 11 wheat genotypes, in which 62 of them were positive, while 42 were negative markers. The highest number of genotype-specific markers (17 markers) was recorded for SF3, while the lowest number (4 markers) was recorded for SF7. The primer WMC 179 generated the highest number of genotype-specific markers (10 markers), while the primers WMS 06 and WMS 30 generated the lowest number (one marker).

The selected seven putative mutants (M_3 families) SF1 through SF7 exhibited 5, 2, 5, 3, 6, 5 and 4 positive markers and 0, 4, 12, 12, 4, 1 and 0 negative markers, respectively. The four parents Aseel-5, Sakha-93, Giza-168 and Sahel-1 exhibited 8, 9, 4 and 11 positive and 1, 3, 3 and 2 negative markers respectively [Table-7].

Table 7- Positive and negative SSR markers generated for 11 wheat genotypes (7 mutants and their 4 parents), marker size (bp) and total number of markers identifying each genotype

Genotype	Primer Size/bp	Total No.	Primer Size/bp	Total No.	Grand Total
Aseel-5	WMS 30 (100), WMS 108 (300), WMS118 (100), WMS 149 (400), WMS 169 (220), WMS 198 (100, 700), WMC 322 (400)	8	WMC 445 (750)	1	9
Sakha-93	WMS 30 (100), WMS 108 (150, 200, 250, 300, 400), WMS118 (75), WMC 445 (500, 1500)	9	WMS 149 (900), WMS 169 (200), WMC 322 (100)	3	12
Giza-168	WMS 30 (100), WMS 169 (220), WMC 307 (150), WMS 375 (100)	4	WMS 108 (100), WMS 149 (900), WMS 169 (200	3	7
Sahel-1	WMS 30 (100), WMS 108 (150, 200, 250, 300, 400),WMS118 (100), WMS169 (220), WMC 179 (250), WMS 198 (700),WMC 322 (400)	11	WMS 169 (200), WMC 179 (200)	2	13
SF1	WMS 118 (100), WMS 149 (400), WMS 198 (700), WMC 307 (150), WMS 375 (100)	5			5
SF2	WMS 118 (75),WMS 149 (400)	2	WMS 198 (200, 400), WMC 322 (100), WMC 445 (750)	4	6
SF3	WMS 06 (100), WMC 177 (100), WMC 179 (250, 800, 1000)	5	WMS 149 (900), WMC 179 (100, 200, 900), WMS 198 (150, 200, 400), WMC 307 (100), WMS 375 (200), WMC 445 (300, 750)	12	17
SF4	WMS 118 (75), WMC 179 (50), WMC 445 (1500)	3	WMS 149 (180, 900), WMC 177 (200), WMC 179 (100, 400, 480, 550, 900), WMS 198 (150, 200, 400), WMC 445 (300)	12	15
SF5	WMS 06 (100), WMS 118 (75, 100), WMS 169 (220), WMS 375 (100), WMC 445 (500)	6	WMS 118 (180, 900), WMS 169 (200), WMC 179 (480)	4	10
SF6	WMS 108 (150), WMS 149 (400), WMC 179 (250), WMC 307 (150), WMC 445 (500)	5	WMC 179 (480)	1	6
SF7	WMS 118 (75), WMS 149 (400), WMS 198 (700), WMC 445 (500)	4			4
Total		62		42	104

The drought tolerant mutant SF1 is characterized by seven positive markers amplified by the primers WMS 118 (100 bp), WMS 149 (400 and 900 bp), MWS 169 (200 bp), WMS 198(700 bp), WMS 307(150 bp) and WMS 375(100 bp). Four positive markers generated by the primers WMS 118 (75 bp), WMS149 (440 and 900 bp) and MWS 169 (200 bp) and four negative markers amplified by the primers WMS 198 (200 and 400 bp), WMC 322 (100 bp) and WMC 445 (750 bp) characterized the putative M_3 family SF2.

The drought tolerant M_3 family SF3 is characterized by six positive markers amplified by the primers WMS 06 (100 bp), WMS 169 (200 bp), WMS 177 (100 bp) and WMC 179 (250, 800 and 1000 bp) and twelve negative markers generated by the primers WMS 118 (150 bp), WMC 179 (100, 200, 480 and 900 bp), WMS 198 (150, 200 and 400 bp), WMC 307 (100 bp), WMS 375 (200 bp) and WMC 445 (300 and 750 bp). Four positive markers amplified by the primers WMS 118 (75 bp), WMS 169 (200 bp), WMC179 (50 bp) and WMC 445 (1500 bp) and twelve negative markers generated by the primers WMS 118 (150 bp), WMS 149 (180 bp), WMS 177 (200 bp), WMC 179 (100, 400, 480, 550 and 900 bp), WMS 198 (150, 200 and 400 bp) and WMC 445 (300 bp) characterized the putative drought tolerant mutant SF4.

The drought tolerant M_3 family SF5 is characterized by six positive markers amplified by the primers WMS 06 (100 bp), WMS 118 (75 and 100 bp), WMS 169 (220 bp), WMS 375 (100 bp) and WMC 445 (500 bp and two negative markers generated by the primers WMS 149 (180 bp) and WMC 179 (480 bp). Seven positive markers am-

plified by the primers WMS 108 (150 bp), WMC149 (400 and 900 bp), WMS 169 (200 bp), WMC 179 (250 bp), WMS 307(150 bp) and WMC 445 (500 bp) and two negative markers generated by the primers WMS118 (150 bp) and WMC 179 (480 bp) characterized the drought tolerant M_3 family SF6. The last drought tolerant putative mutant SF7 is characterized by six positive markers amplified by the primers WMS 118 (75 bp), WMS 149 (400 and 900 bp), WMS 169 (200 bp), WMS 198 (700 bp) and WMC 445 (500 bp) and one negative marker generated by the primer WMC 179 (480 bp).

Genotype Identification by Unique SSR Markers

Unique markers are defined as bands that specifically identify an accession from the other by their presence or absence. The bands that are present in one accession but not found in the others are termed positive unique markers (PUM), in contrast with the negative unique markers (NUM), which are absent in a specific genotype. These bands could be used for genotype identification [22].

Table 8- Unique positive and negative SSR markers generated for 11 wheat genotypes (7 selected M_3 families and their 4 parents), marker size (bp) and total number of unique markers identifying each genotype.

Genotype	Positive Unique Markers Primer (Size/bp)	Total No.	Negative Unique Markers Primer (Size/bp)	Total No.	Grand Total
Asseel-5	WMS 198 (100)	1	-		1
Sakha-93	-		-		
Giza-168	-		WMS 108 (100)	1	1
Sahel-1	-		-		
SF1	-		-		
SF2	-		-		
SF3	WMC 177 (100), WMC 179 (800, 1000)	3	WMC 307 (100) WMS 375 (200)	2	5
SF4	WMC 179 (50)	1	WMC 177 (200), WMC 179 (400, 550)	3	4
SF5	-		-		
SF6	-		-		
SF7	-		-		
Total		5		6	11

As shown in [Table-8], the SSR assay permitted the identification of four out of eleven wheat genotypes by unique positive and / or negative markers. Three genotypes, *i.e*., Aseel-5, SF3 and SF4 were characterized by five positive unique markers, while three genotypes, *i.e*., Giza -168, SF3 and SF4 were characterized by six negative unique markers.

The selected drought tolerant mutant SF3 was characterized by three unique positive markers amplified by the primers WMC 177 (100 bp) and WMC 179 (800 and 1000 bp) and two unique negative markers amplified by the primers WMC 307 (100 bp) and MWS 375 (200 bp). The drought tolerant putative mutant SF4 was characterized by one unique positive marker amplified by the primer WMC 179 (50 bp) and three unique negative markers amplified by the primers WMC 177 (200 bp) and WMC 179 (400 and 550 bp).

The drought tolerant Syrian parent (Aseel-5 cultivar) was characterized by one unique positive marker amplified by the primer WMS 198 (100 bp). The Egyptian cultivar Giza -168 (high-yielding) was characterized by one unique negative marker amplified by the primer WMS 108 (100 bp). While, the remaining seven wheat genotypes did not exhibit any unique marker. The highest number of unique markers (five) was amplified by the primer WMC 179 (3 positive and two negative) followed by the primer WMC 177 (two unique markers, one positive and one negative). The size of these unique markers ranged from 50 to 1000 bp.

In this context, [14] 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.

Using SSR analysis, we were able to identify eleven unique bands associated with drought tolerance in wheat. These bands can be verified as markers associated with drought tolerance in durum wheat breeding programs.

Further 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.

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 11 Wheat Genotypes

The recorded data from the SSR analysis in this study were used to compute the similarity matrices according to Dice coefficient [21]. As shown in [Table-9] the genetic similarity coefficient (GS) ranged from 33% (between SF4 and Sahel-1) to 88% (between SF7 and each of SF1 and SF6). High GS between SF6 and SF7 is attributed to that both of them were derived from Sahel -1 cultivar irradiated by 350 Gy gamma rays.

The results of this investigation indicated that all the seven 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 66%.

Table 9- Genetic similarity (GS) matrices among the eleven wheat genotypes (7 drought tolerant mutants and their 4 parents).

	Aseel-5	Sakha-93	Giza-168	Sahel-1	SF1	SF2	SF3	SF4	SF5	SF6
Aseel-5	1									
Sakha-93	0.72	1								
Giza-168	0.77	0.73	1							
Sahel-1	0.83	0.83	0.74	1						
SF1	0.86	0.71	0.84	0.76	1					
SF2	0.78	0.69	0.7	0.63	0.81	1				
SF3	0.45	0.36	0.42	0.43	0.48	0.51	1			
SF4	0.39	0.44	0.4	0.33	0.37	0.46	0.5	1		
SF5	0.72	0.72	0.81	0.69	0.41	0.78	0.68	0.5	1	
SF6	0.74	0.7	0.75	0.71	0.5	0.85	0.76	0.49	0.73	
SF7	0.81	0.78	0.75	0.71	0.45	0.88	0.84	0.54	0.82	0.88

The mutants SF3 and SF4 exhibited very low genetic similarity with their common parent Giza-168 (42 and 40%, respectively), indicating that gamma rays were very effective in changing the genetic background of Giza-168 in the positive direction, *i.e.*, towards high GYPP under WS conditions. In this context, [14] 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, [9] reported that genetic similarity coefficients for SSR markers between 18 salt tolerant wheat accessions ranged from 0.45 to 0.95.

Cluster Analysis as Revealed by SSR

The Dice SSR-based coefficient of genetic similarity among the 11 wheat genotypes was employed to develop a dendrogram using the UPGMA method [Fig-6]. The dendrogram separated the putative mutants SF3 and SF4 from the other nine genotypes. This demonstrates the distinctiveness of the genetic background of these two mutants (SF3 and SF4) from all the other genotypes including their parents. The remaining genotypes were divided into two clusters, the first cluster included the two cultivars Sahel-1 and Sakh-93, while the second was divided into three clusters, the 1st class included the mutant SF2, the 2nd included the Syrian cultivar Aseel-5 and the 3rd class was divided into two sub-classes. The first sub-class included SF1 and SF7, while the 2nd sub-class included SF6.

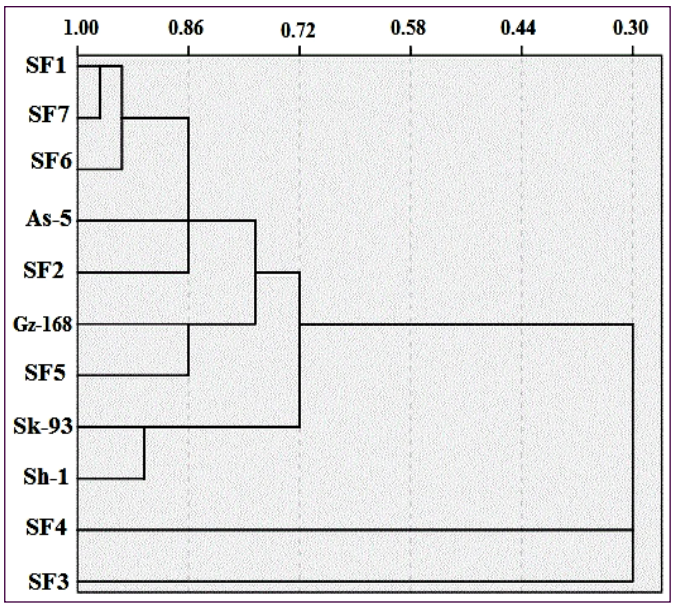

Fig. 6- Dendrogram for the eleven wheat genotypes (7 selected families and 4 parents) constructed from SSR data using (UPGMA) according to Dice coefficients.

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 procedures.

Conflicts of Interest: None declared.

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