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GENETIC DIVERSITY, POPULATION STRUCTURE AND ASSOCIATION MAPPING OF MORPHOLOGICAL TRAITS IN SOME EGYPTIAN BREAD WHEAT LANDRACES UNDER HEAT STRESS

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Abstract

Genetic diversity and association mapping were performed on 70 bread wheat landraces from Upper Egypt which adapted to heat stress using SSR, ISSR and AFLP markers. Thirteen agronomic traits were evaluated over three growing seasons at two locations (New Valley and Luxor) to identify superior wheat lines under heat stress conditions. This collection showed a polymorphism percentage of 79, 75 and 79 for SSR, ISSR and AFLP markers, respectively and PIC was 0.213, 0.187 and 0.189 for SSR, ISSR and AFLP markers, respectively. UPGMA clustering analysis classified the 70 wheat lines into two subpopulations that was further confirmed by population structure that gave subpopulations k=2. The association of genetic markers studied with thirteen agronomic traits was analyzed using a general linear model where population structure was used to avoid spurious marker-trait associations (MTAs). A total of 57 and 60 significant ($P < 0.001$) MTAs were obtained for ten traits in New Valley and Luxor, respectively under heat stress. Spike length (SL) had a maximum (35) of significant MTAs distributed over 23 loci, followed by 27 MTAs for days of heading (DH) and 14 MTAs for number of kernel/spike (NKSp) at both locations. While, Chlorophyll B associated with one marker on chromosome 4A. SSR locus (Xgwm369) on chromosome 3A was pleiotropic with (DH), days of maturity (DM), grain weight/plot (GWP), (SL) and plant height (PH) and common at both locations which are potentially important targets for selection. Five MTAs on chromosomes 3A, 5D, 6A and 6B were co-located at both locations for DH, which seem important for heat stress environments. While, ten significant MTAs associated with maturity were detected in chromosomes 2B, 3A, 3B, 4B,5B,5Dand 6B at both locations. Results indicated that MTAs detected are linked with genes controlling important heat stress-tolerance traits. The detected MTAs can be targeted for future utilization in wheat genetic improvement through integrative strategies and marker assisted selection for existing germplasm.

Keywords : Wheat, Heat-tolerance, SSR, ISSR, AFLP, association mapping.

Introduction

Wheat is the second most produced cereals in the world with over 600 million tones being produced annually. Bread wheat is a hexaploid (6n = 21) with a genome size estimated at ~17 Gb, composed of three closely related and independently maintained genomes (AABBDD). In Egypt, the growing season of wheat extends through the wet and cool seasons at middle and Upper Egypt. More critical is, the period of flowering-to-grain filling in April and May, when high temperatures can dramatically disturbs the duration and rate of grain filling which affect both grain yield and quality (Hamam *et al*., 2015). It is prophesied to face more recurrent exposures to terminal heat stress (>32°C) due to global climate change. Heat tolerance is the main aim of wheat breeders to stabilize and increase food production which is become increasingly crucial with rising worldwide population. Improvement for stress tolerance can be achieved by the introduction of heat related genes and QTLs to desired wheat cultivars. However, targeting stress tolerance genes is a complicated process since these traits are polygenic and influenced by environment. Also, wheat lines have limited polymorphism and narrow genetic base due to genetic bottleneck through historic domestication. So, dissecting the genetic basis of agronomic traits and responses to heat stresses is essential for the improvement of wheat (Li *et al.,* 2019). The choice of germplasm is a key factor determining the resolution of association mapping. Wheat landraces that adapted to heat environments are valuable source of genetic diversity and should possess traits of adaptation. (Gupta *et al.,* 2015). Therefore, LD based-association mapping utilizing diverse landraces is the ideal material for identification of marker-trait associations (MTAs) and exploits the high marker coverage without the time and effort needed to develop biparental mapping populations. (Talukder *et al*., 2014; Lopes *et al*., 2015 and Ogbonnaya *et al*., 2017). LD is the nonrandom association between alleles at different loci, which exploits ancestral recombination events that occurred during the breeding history resulting in much higher mapping resolution (Gupta *et al*., 2015 and Mérida-García *et al*., 2020). LD is affected by population structure admixture, mating system, low allele frequencies and genetic drift and by artificial or natural selection during evolution, domestication, or plant improvement Mérida-García *et al*., 2020. Molecular markers, SSR ISSR and AFLP are powerful tools to study genome characterization, evolutionary studies, detection of quantitative trait loci (QTL) for both abiotic and biotic stresses, and for marker assisted selection (MAS).In bread wheat, a variety of complex traits have been subjected to QTL analysis (Li *et al*., 2019). Moreover, association mapping studies have been performed a better grain yield in wheat (Edae *et al*., 2014; Lopes *et al*., 2015; Ogbonnaya *et al*., 2017 and Tadesse *et al*., 2019).

This study was performed using a collection of 70 bread wheat landraces adapted to heat stress from Upper Egypt to determine the allelic and genetic diversity, to evaluate the population structure, to determine linkage disequilibrium (LD) decay rate and to identify closely associated markers with morphological grain yield, and yield components traits under heat-stressed environments.

Materials and Methods

A total of 70 spring bread wheat landraces adapted to heat stress was collected from different locations in Upper Egypt for heat association analysis. They were evaluated in three field trials at two targeted locations at New Valley (El Khargah) and Luxor (El Mataanh) for two successive seasons, 2016/2017 and 2017/2018. Numbers and locations of the collected landraces are illustrated in Table (1). Each field trial was irrigated five times per season. Data for thirteen morphological and yield components traits; Days to heading (DH), Days to maturity (DM), Plant height(PH), Spike length (SL), Leaf area (LA), Chlorophyll A (Ch A), Chlorophyll B (ChB), Carotene (Cro), Number of spikelets/spike (NSt), Number of spikes/m^2 (NSp), 1000 Kernel-weight (KW), number of Kernel/spike (NKSp), Grain weight/plot (GWP) were recorded on ten guarded plants chosen at random from each plot. The experimental design for the field trials was a randomized complete block design with three replications. Each plot consisted of four rows; each row was 2.5 meters long and 30 cm apart. Plants within rows were 20 cm apart. Analysis of variance of the phenotypic data was performed using the Statistical Analysis System (SAS) software V9.3 (SAS Institute, 2011).

Table 1: Numbers and locations of 70 bread wheat landraces collected.

No	Governorate	District	Village	No	Governorate	District	Village
1	*Aswan*	*Aswan*	*Aswan*	**36**	*Sohag*	*Al Monshaah*	*Nagaa Abd El Azeem Salem*
2	*Aswan*	*Aswan*	*Aswan*	**37**	*Sohag*	*Idfa*	*Idfa*
3	*New Valley*	*Dakhla*	*El Qasr*	**38**	*Sohag*	*Sohag*	*Al Shoryfat*
4	*New Valley*	*Dakhla*	*El Qasr*	**39**	*Qena*	*Nag Hamad*	*Dandrah*
5	*New Valley*	*Dakhla*	*El Qasr*	**40**	*Qena*	*Nag Hamad*	*Dandrah*
6	*New Valley*	*Khargah*	*Khargah*	**41**	*North Sinai*	*Rafah*	*Rafah*
7	*New Vvalley*	*Dakhla*	*El Qasr*	**42**	*New Valley*	*Dakhla*	*Bashandy*
8	*New Valley*	*Dakhla*	*El Qasr*	**43**	*Sohag*	*Sohag*	*Awlaad Naseer*
9	*New Valley*	*Khargah*	*Khargah*	**44**	*Sohag*	*Sohag*	*Awlaad Naseer*
10	*New Valley*	*Dakhla*	*El Qasr*	**45**	*Sohag*	*Akhmim*	*Arab Al Atawla*
11	*Sohag*	*Sohag*	*Jazirat Shandawi*	**46**	*Sohag*	*Idfa*	*Idfa*
12	*Sohag*	*Al Monshaah*	*Kombedar*	**47**	*Sohag*	*Akhmim*	*Al Sawamaa Sharq*
13	*Sohag*	*Al Monshaah*	*Kombedar*	**48**	*BeniSwif*	*BeniSwif*	*Sayan Abu Ali*
14	*Sohag*	*Sohag*	*Jazirat Shandawi*	**49**	*Assiut*	*Assiut*	*Assiut*
15	*Sohag*	*Al Monshaah*	*Kombedar*	**50**	*New valley*	*Farafrah*	*Farafrah*
16	*Sohag*	*Al Monshaah*	*Al Zook Al Gharbyea*	**51**	*New valley*	*Farafrah*	*Farafrah*
17	*Sohag*	*Al Monshaah*	*Kombedar*	**52**	*Sohag*	*Al Monshaah*	*Al Kawamel*
18	*Sohag*	*Gerga*	*Gerga*	**53**	*Sohag*	*Sohag*	*Al Salaah*
19	*Sohag*	*Al Monshaah*	*Al Kawamel*	**54**	*Sohag*	*Sohag*	*Al Salaah*
20	*Sohag*	*Al Monshaah*	*Al Horyzat Al Gharbeya*	**55**	*Sohag*	*Al Monshaah*	*Al Zook Al sharkhya*
21	*Qena*	*Nag Hamad*	*Al HalfayaKebly*	**56**	*Sohag*	*Gerga*	*Gerga*
22	*Sohag*	*Al Monshaah*	*Awlaad Harron*	**57**	*Sohag*	*Gerga*	*Bany Aysh*
23	*Sohag*	*Sohag*	*Jazirat Shandawi*	**58**	*Assiut*	*Abo teeg*	*Al Zaraby*
24	*Sohag*	*Al Monshaah*	*Al Kawamel*	**59**	*Luxor*	*Esna*	*Tuffnice*
25	*Sohag*	*Sohag*	*Shandawi*	**60**	*Luxor*	*Esna*	*Tuffnice*
26	*Sohag*	*Al Monshaah*	*Nagaa Abd El Azeem Salem*	**61**	*Luxor*	*Esna*	*Tuffnice*
27	*Sohag*	*Al Monshaah*	*Kombedar*	**62**	*Luxor*	*Esna*	*Tuffnice*
28	*Qena*	*Qena*	*Al Rahmanya*	**63**	*Luxor*	*Esna*	*Tuffnice*
29	*Sohag*	*Sohag*	*Sohag*	**64**	*Luxor*	*Esna*	*Tuffnice*
30	*Sohag*	*Sohag*	*Sohag*	**65**	*Luxor*	*Esna*	*Tuffnice*
31	*Qena*	*Keft*	*Keft*	**66**	*Luxor*	*Esna*	*Tuffnice*
32	*Sohag*	*Sohag*	*Sohag*	**67**	*Luxor*	*Esna*	*Asfone*
33	*Sohag*	*Sohag*	*Sohag*	**68**	*Luxor*	*Esna*	*Asfone*
34	*Sohag*	*Akhmim*	*Al deyabat*	**69**	*Luxor*	*Esna*	*Asfone*
35	*Aswan*	*Al Noba*	*Al Sayala*	**70**	*Luxor*	*Esna*	*Asfone*

Genotyping

DNA extraction

Genomic DNA was extracted from young wheat leaves using DNeasy Plant MiniKit (QIAGEN, Hilden, Germany). The quantity and quality of the extracted DNA were determined at 260 and 280 nm using Thermo Scientific NanoDrop 2000™ spectrophotometer.

Simple Sequence Repeat (SSR)

Out of the 20 SSR markers, 10 SSR markers were selected based on their polymorphism level to characterize the 70 wheat accessions (Table 3). PCR amplifications were performed in 25µl containing 1 X of green GoTaq® Flexi buffer, 1.5 mM $MgCl_2$, 0.2 mM of dNTP mix, 1 µM of each primer of a primer pair, 1 U GoTaq Flexi polymerase (Promega) and 40 ng genomic DNA. The PCR cycling reaction was as follows: 5 min at 94°C, followed by 40 cycles with 50 sec at 94°C, 1min at 60°C, 1min at 72 °C, a

final extension step for 7 min at 72 °Cand stored at 4 °C. PCR products were separated using 3% agarose gels.

Inter Simple Sequence Repeat (ISSR)

ISSR-PCR was conducted using ten anchored primers (Eurofins, Germany)Table (4). The following reagents were mixed in a final volume of 25 µl: 1 X of green GoTaq® Flexi buffer, 1.5 mM of $MgCl_2$, 200 µM of dNTPs (Promega), 25 µM of primer, 1 U of GoTaq® Flexi DNA Polymerase (Promega), 30 ng of template DNA and up to 25 µl distilled H_2O. Amplification was carried out in a Gene Amp® PCR System 9700 thermal cycler (Applied Biosystems) programmed as follows: 94° C/5 min. for one cycle; followed by 40 cycles of 94°C/45 sec, 45°C/50 sec, and 72° C/1.5 min, a final extension step of 7 min. at 72 °C for 7 min. and stored at 4°C. A volume of 10µl of the ISSR-PCR product was resolved using (1.5%) agarose gel electrophoresis.

Results for SSR and ISSR were visualized on a UV transilluminator and photographed by Molecular Imager® Gel Doc™ System with Image Lab™ Software, Bio-Rad and 100 bp DNA marker (Fermentas) was used as a DNA molecular weight standard. The profiles for both markers were manually scored as present (1) or absent (0).

Automated Amplified Fragment Length Polymorphism (AFLP)

AFLP was carried out according to Vos *et al.* (1995). Genomic DNA was digested using *EcoRI* and *MseI* (New England BioLabs) followed by ligation the adaptors using T4 DNA ligase (New England BioLabs) to produce modified restriction fragments to be used in pre-selective amplification. Selective amplification was performed using two AFLP primers; one Fluorescence-labeled primer: *Eco*RI [Dye-primer-AXX] and unlabeled *Mse*I [Primer–CXX]. Three primer combinations, E-AAG (JOE)/M-CTC, E-ACT (FAM)/M-CTT and AAC (NED)/M-CAG that resulted in good amplifications and showed polymorphisms were chosen (Table 3). One µl of selective amplified product was mixed with 12 µl of Hi-Di formamide and 0.5 µl of GeneScan 500 ROX size standard (Applied Biosystems, Foster City, California, USA) to facilitate fragment sizing. The mixture was denatured and loaded on a single capillary of the Applied Biosystems 310 Genetic Analyzer. Gene Scan Analysis 2.1 Software was used to estimate fragment size (in bp) and binary matrix scored was constructed.

Data analysis

Scored data as present (1) or absent (0) for all the samples under study was used to calculate Gene diversity and polymorphism information content (PIC) using the software package Power Marker V 3. 25 (Liu and Muse, 2005)

Population structure

The Unweighted Pair Group Method with Arithmetic means (UPGMA) and Bayesian clustering method were used to investigate possible structure and genetic relatedness in wheat collection studied. Cluster analysis based on distance using UPGMA with Dice's similarity coefficient implemented in SPSS software. While, Population structure was analyzed using Bayesian-model implemented in the STRUCTURE v2.3.4 software (Pritchard *et al*., 2000). The number of sub-populations (K) was set from 1 to 10 based on admixture and correlated allele frequencies models. For each K, three runs were performed separately. Each run was carried out with 100,000 iteration and 100,000 burn-in period (Chen *et al*., 2012). The true number of clusters was estimated according to the procedure described by Evanno *et al*. (2005) using STRUCTURE HARVESTER (online platform).

In silico PCR analysis

In-silico PCR is used to determine the physical position of loci to use for LD analysis. It is a computational procedure that estimates PCR results theoretically using a given set of primers to amplify DNA sequences from a sequenced genome (Lexa *et al*., 2001). The full genome of wheat with a genome size estimated at ~17 Gb (Rudi *et al.*, 2018) was used as template for in silico PCR analysis against 23 PCR primers (SSR, ISSR and AFLP) used in this study to reveal the possible PCR amplicons with their locations and sizes. Practical Extracting and Reporting Language (PERL) scripts were used for performing in silico PCR analysis. Circos software package (Circos 0.66) was used for visualizing output results in a circular layout (Krzywinski *et al.*, 2009).

Linkage Disequilibrium (LD)

The pairwise linkage disequilibrium between pairs of loci was estimated using the squared correlation coefficient of the allele frequencies (r^2) between bi-allelic combinations using TASSEL software ver. 5.2.50 (Bradbury *et al*., 2007).

Marker trait association

The marker–trait associations among the marker alleles and squares means of 13 phenotypic traits in two different environments (Luxor and New valley) were performed by a general linear model (GLM) with Q model using TASSEL program v 5.2.50. Population structure (Q model) was used as fixed-effect covariate to avoid spurious detection of associations between markers and phenotypes.

Results and Discussion

Phenotypic variations

Agronomic traits under heat-stressed environment has been widely monitored as an indicator to identify heat-tolerant genotypes through wheat breeding programs to maximize yield gains despite heat stress conditions. Phenotypic data of the 70 wheat landraces were calculated for the two locations including, minimum, maximum, mean values and standard deviations (Table 1). Data obtained revealed a wide variation for the thirteen traits in both locations under heat stress, indicating a common genetic background explaining the phenotypic variation (in terms of heat tolerance indices). DH had a mean of 81.46 days (minimum 72.67days and maximum 92.17 days) with 4.11 days standard deviation in Luxor, while, PH ranged from 81.50 cm to 103.04cm with 8.73 cm standard deviation in New Valley. Moreover, GWP had an average of 1.51 and 1.29 in Luxor and New Valley respectively. Furthermore, the mean of KW at Luxor ranged from 35.17 to 53.73 kg with average yield of 44.15. The average Spike length (cm) and Number of spikes/m2 were 11.07 and 297.72 higher in Luxor than New valley, respectively. Mean leaf chlorophyll A content at New Valley was 20.99 µg/g FW with a range from 11.4 to 23.9 while, at Luxor, mean leaf chlorophyll A content was 18.99, ranging from 10.49 to 23.51. The findings of this study agree with that of Hamam *et al*., 2015 and Tadesse *et al*., 2019. On the other hand, Jamil *et al.,* 2019 reported that wheat plants are sensitive to heat stress

induced during the reproductive stage which has a detrimental effect on productivity due to the reduction in both seed development and fertility. Heritability is the percentage of phenotypic variance that is attributed to genetic variance. Hence, in the present study, the high heritability obtained indicates that the environmental influence has a minimal effect on traits. Estimates of broad sense heritability (h^2) of nutritional traits ranged from 0.18 % (Number of spikelets/spike) to 1.00 (Chlorophyll A, B content; µg/g FW) at Luxor and 0.44 % (Grain weight / plot) to 0.98 (Chlorophyll B content; µg/ g FW) at New Valley. Similar results were reported by Ahmed *et al*. (2018), Jamil *et al.* (2019). Thus, results suggested that, the studied germplasm would be useful for wheat breeding programs.

Table 2: Morphological yield and yield component trait variations and the heritability (h^2) of the seventy bread wheat lines evaluated for heat stress tolerance at Luxor and New Valley.

Traits	Code	Location	Min	Max	Sum	Mean	Stand. Dev	Heritability (h2)
Days to Heading (days)	DH	Luxor	72.67	92.17	5702.25	81.46	4.11	0.95
		New Valley	67.50	89.00	5378.50	76.84	4.86	0.69
Days to Maturity (days)	DM	Luxor	126.00	136.67	9189.42	131.28	2.43	0.87
		New Valley	114.50	128.50	8598.50	122.84	2.50	0.68
Plant height (cm)	PH	Luxor	85.83	129.17	7532.67	107.61	9.01	0.91
		New Valley	81.50	124.00	7213.00	103.04	8.73	0.65
Spike length (cm)	SL	Luxor	7.00	15.54	774.79	11.07	1.74	0.78
		New Valley	6.50	16.00	752.25	10.75	1.88	0.73
Leaf Area	LA	Luxor	26.38	67.66	3320.28	47.43	9.03	0.87
		New valley	19.05	63.20	2622.48	37.46	9.99	0.90
Chlorophyll A µg/ g fw	Cho A	Luxor	10.49	23.51	1329.58	18.99	2.83	0.99
		New valley	11.42	23.98	1469.10	20.99	1.91	0.95
Chlorophyll B µg/ g fw	Cho B	Luxor	5.65	25.10	957.20	13.67	4.05	1.00
		New Valley	4.45	29.66	1184.92	16.93	5.96	0.98
Carotene µg/ g fw	Ca	Luxor	981.04	2835.31	142292.10	2032.75	448.73	1.00
		New valley	948.83	3236.00	182324.80	2604.64	489.13	0.96
Number of spikelets/ spike	NSt	Luxor	19.00	24.00	1473.86	21.06	0.99	0.18
		New valley	16.50	25.50	1501.00	21.44	1.94	0.55
Number of spikes/m^2	NSp	Luxor	263.67	330.33	20840.29	297.72	14.80	0.24
		New Valley	182.00	352.00	19628.00	280.40	35.60	0.84
number of Kernel /spike	NKS	Luxor	26.50	52.50	2868.34	40.98	5.94	0.76
		New Valley	25.88	53.00	2576.23	36.80	6.46	0.93
1000- Kernel weight	KW	Luxor	35.17	35.17	3090.18	44.15	4.47	0.48
		New Valley	33.50	51.60	3035.00	43.36	4.11	0.94
Grain weight / plot	GWP	Luxor	0.93	1.93	105.82	1.51	0.25	0.58
		New valley	0.91	1.75	90.54	1.29	0.18	0.44

Genetic diversity

In SSR analysis, out of the 20 SSR primer pairs used, 10 were monomorphic and other 10 polymorphic primer pairs produced 38 (79%) polymorphic alleles among the 70 bread wheat landraces tested. The number of alleles ranged from 2 to 6 with an average of 3.8 alleles per locus with size ranging from 100 to 320 (Table 3). This is similar to the results obtained by Gupta *et al*., 2015who reported a percentage of 79 polymorphic alleles among two wheat parents using 22 SSR loci. Moreover, Chen *et al*., 2003 detected 3.5 alleles per locus in winter wheat of China. Also, in Sichuan wheat landraces, the mean alleles per locus observed were 4.8 by Li *et al*., 2013. Moreover, Kumar *et al*., 2017 obtained an average of 2.71 alleles per locus for wheat lines using 42 primer pairs. On the contrary. These results were lower than that obtained by Sönmezoglu and Terzi 2018; Li *et al*., 2019 and Slim *et al.,* 2019 who detected alleles varying from 2 to 33with an average of 5.9 to 12.41 alleles/SSR loci among bread wheat cultivars using SSR markers. This could be attributed to the multi allelic nature of SSR markers and tested genotypes.

Table 3: Polymorphic SSR primer pairs sequence, Chromosome location, allele's numbers and their molecular weight (MW) range.

Primer	Sequence 5'----3'	Chromosome location	No. of alleles	M .W range
Xgwm181	F - TCATTGGTAATGAGGAGAGA	3B	4	100- 150bp
	R -GAACCATTCATGTGCATGTC			
Xgwm371	F - GACCAAGATATTCAAACTGGCC	5B	5	110- 210
	R - AGCTCAGCTTGCTTGGTACC			
Xgwm219	F - GATGAGCGACACCTAGCCTC	6B	3	160- 200
	R - GGGGTCCGAGTCCACAAC			

Xwmc596	F- TCAGCAACAAACATGCTCGG	7A	5	140- 270
	R-CCCGTGTAGGCGGTAGCTCTT			
Xgwm99	F -AAGATGGACGTATGCATCACA	1A	4	110-170
	R -GCCATATTTGATGACGCATA			
Xgwm369	R - ACCGTGGGTGTTGTGAGC	3A	6	160-320
	F -GAGTCCTGATGTGAAGCTGTTG			
Xgwm234	F -GAGTCCTGATGTGAAGCTGTTG	5A	3	120-160
	R- CTCATTGGGGTGTGTACGTG			
Xgwm205	F- CGACCCGGTTCACTTCAG	5A	3	130-160
	R- AGTCGCCGTTGTATAGTGCC			
Xbarc108	R- GCGGGTCGTTTCCTGGAAATTCATCTAA	7A	3	140-160
	R-GCGAAATGATTGGCGTTACACCTGTTG			
Xgwm124	F- GCCATGGCTATCACCCAG	1B	2	200-210
	R- ACTGTTCGGTGCAATTTGAG			
Total			38	

In ISSR analysis, a total of 136 bands were produced using 10 ISSR primers with an average of 13.6 bands per primer among 70 landraces Table (4). Bands size ranged from 200 to 2500bp. Out of them, 102 bands were polymorphic with a mean of 10.2 polymorphic bands per primer. These results are in agreement with that obtained by Etminan *et al*., 2016 who obtained a mean of 10.2 polymorphic alleles per primer. Moreover, Yadav *et al*., 2018 and Saxena and Khanna 2019 reported average number of bands per primer of 6.64 and 18.25 respectively. This variation in polymorphic alleles produced per primer is correlated to the genetic background of the varieties under study and the primers used. Results showed that the polymorphism percentage ranged from 41 to 100 with a mean percentage of 75. Similarly, Najaphy *et al.,* 2011 obtained a polymorphism percentage of 80 using ten ISSR primers among thirty wheat accessions.

Table 4: ISSR primer sequences, total number of bands, number of polymorphic bands and percentage of polymorphism.

Primer	Sequence 5'----3'	Total Bands	Polymorphic bands	%
ISSR-05	$(GT)_8YG$	13	13	100
ISSR-06	$CGC(GATA)_4$	14	8	57
ISSR-07	$GAC(GATA)_4$	11	8	72
ISSR-08	$(AGACA)_4GC$	14	11	79
ISSR-09	$(GATA)_4GC$	11	10	91
ISSR-14	$(CTC)_5TT$	14	11	79
ISSR-15	$(CT)_8RG$	13	11	85
ISSR-24	$CGA(GATA)_4$	17	7	41
ISSR-26	$GAA(GATA)_4$	30	23	77
ISSR-28	$(GATA)_4CG$	13	11	85
Total		136	102	75

A total of three AFLP primer combinations generated 1409 total peaks with molecular sizes ranging from 55to 500 bp. They produced 1116 (79%) polymorphic peaks with an average of 372 peaks per primer (Table (5). These findings are closely matched with that obtained by Bhattacharyya *et al*., 2017 who obtained 1188 polymorphic peaks across *D. thyrsiflorum* using automated AFLP technique. While, Sciacca *et al*., 2010 used three fluorescence dye-labelled primer combinations and obtained a total of 6630 AFLP peaks, out of which, 2277 (34.3 %) were polymorphic with a mean percentage of 759 polymorphic peaks per primer pair in fifteen Italian cultivars of durum wheat. On the other hand, Altintaşe *et al*., 2008 obtained 344 amplicons among 12 durum and 22 bread wheat using five amplified primer combinations, out of which 214 were polymorphic. Also, Roncallo *et al*., 2019 reported that six AFLP primer combinations used in 119 of durum wheat accessions yielded 402 total bands, 182 (45.3%) were polymorphic with an average of 67 bands/ primer combinations. These results demonstrate that automated AFLP is more accurate and sensitive compared to the conventional AFLP technique because of the difference in the detection and scoring methods (Nahla El-Sherif*et al*., 2014 and Bhattacharyya *et al*., 2017).

Table 5 : AFLP primer combinations, total number of bands, number of polymorphic bands and the polymorphism percentage.

Primer combination	Total number of bands	number of polymorphic bands	Percentage of polymorphism
1. AG/CTC	504	405	80
2. ACT/CTT	371	275	74
3. AAC/CAG	534	436	82
Total	1409	1116	79

In this study, the genetic diversity of the 70 wheat landraces gave an average gene diversity of 0.26 for SSR, 0.23 for ISSR and 0.23for AFLP and the mean of polymorphism information content (PIC) was 0.213, 0.187 and 0.189 for SSR, ISSR and AFLP, respectively. Compared with previous reports, Tadesse *et al.* (2019) obtained an average of 0.30 and 0.24 for gene diversity and PIC values, respectively for a panel of 197 heat association spring wheat genotypes from ICARDA. While, Najaphy *et al*., (2011) reported PIC values that ranged from 0.13 to 0.42 with an average of 0.22 for the ten ISSR primers in thirty wheat genotypes. Also, similar results have been reported by Roncallo *et al*., 2019 that obtained low PIC values across wheat genotypes. The low values of PIC and gene diversity could be attributed to the self-pollinating nature of bread wheat (Holasou *et al*., 2019). These results demonstrated that the markers used are informative and reliable in characterizing the genotypes, determining level of genetic diversity in bread wheat.

Population structure

Structured populations are present in most plant populations as consequences for nonrandom mating, isolation, or artificial selection. Hence, genetic loci might show false positive association. Such results would be reported if population structure is not appropriately accounted for (Pritchard *et al.,* 2000). As a result, assessment of population structure is a critical process for association mapping analysis Alemu *et al.,* 2020. In this study, two different clustering methods, STRUCTURE analysis and UPGMA analysis, were used to assess the genetic relatedness of the 70 bread wheat landraces. Bayesian model based-population structure (Q-matrix) was implemented in the STRUCTURE software and the ΔK were plotted against the K numbers of the sub-groups according to Evanno *et al*. (2005). The highest ΔK was that occurred at K= 2 (Figure 1), the wheat collection was divided into two major groups that composedof40 (group 1) and 30 lines (group 2) respectively based mainly on geographic origins (Figure 2). Group 1 contained 16 lines belonging to Sohag, 12 to Luxor, 4 lines from Qena, 3 to New Valley, two lines from Assuit, one Rafah, one beniswif and one from Aswan. On the other hand, Group 2 contained 19 lines from Sohag, 8 lines from New Valley, two lines from Aswan and one from Qena. Similar results were obtained Holasou *et al*., 2019who reported K=2 as the best fitting K. Moreover, they grouped wheat lines in two main groups according totheir pedigree. Interestingly, the dendrogram based-UPGMA method (Figure 2) delineated the 70 wheat landraces into two major clusters that contained 14 and 56 lines, suggesting a genetic relationship among them. The first cluster had 8 lines from New Valley, two lines from Aswan and 4 lines from Sohag. While, in the second cluster the other 56 wheat samples were split into 3 groups of 5, 16 and 35 lines. However, the distribution of some lines was not completely related to the geographic distribution because these landraces are adapted to a wide range of agro-ecological conditions and the probability of seed exchange between farmers. These findings are in congruence with the previous report of Sonmezoglu and Terzi 2018; Yadav *et al*., 2018 and Slim *et al*., 2019, who reported that the cluster analysis based on different molecular markers (SSR, ISSR and AFLP) distinguished the wheat genotypes or landraces into two major clusters, in agreement with the origins and pedigrees in their respective studies. Also, Roncallo *et al*., 2019 who compared that dendrograms-based AFLP and SNP with the results obtained by STRUCTURE software at a maximum K=2, where both methods gave consistent results and indicated that two subpopulations were appropriate in describing the structure in the association panel for 168 durum wheat accessions.

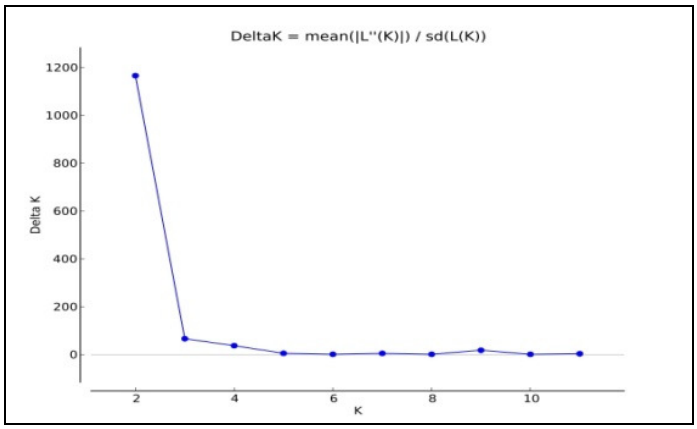

Fig. 1: Calculation of best K of wheat lines, Magnitude of Δk for each K value according to Evanno *et al*. 2005

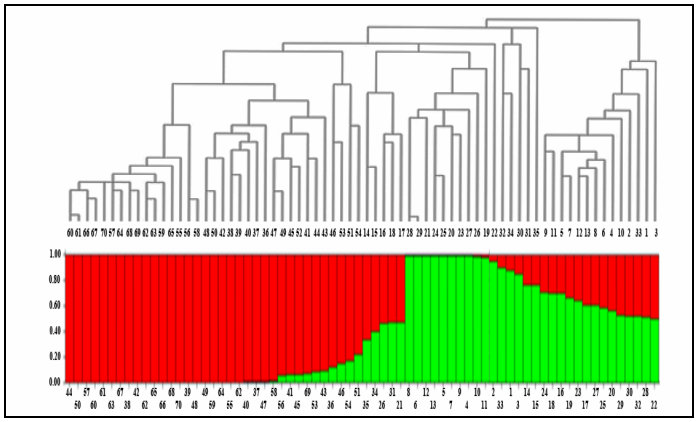

Fig. 2: Population structure and dendrogram of the 70 wheat lines listed in table 1 using combined genetic markers data (SSR, ISSR and AFLP).The red and green colors correspond to G1 and G 2, respectively. The values on the Y-axis correspond to membership coefficients of each genotype and the numbers on the X-axis indicate the code numbers of the genotype.

***In silico* PCR analysis**

Assessment of SSR, ISSR and AFLP techniques included primer selectivity, genome coverage, chromosome position and ability to target genic regions in wheat genome were performed through *in silico* PCR analysis. The total number of in silico amplicons detected by the 23 primers was 682701. The highest number of in silico-amplicons (19158) was revealed by primer combinations AFLP2 and the lowest (1) was that obtained by ISSR-5. The distribution of *in silico* amplicons revealed by SSR, ISSR (Figure 3) showed a total coverage of genomic area using the combined data of198Mbp (3.96%) of the wheat genome. The largest genomic area was that covered by AFLP (0.59%), while the smallest area covered was that covered by SSR (0.04%). The chromosome position for marker tested was further used for LD analysis. Similar results reported by Al samman *et al*., 2017 who obtained 43,432 in Silico amplicons detected by 78 primers for olive genome and the highest number of in silico amplimers (17,632) was revealed by Selective Amplification of Start codon Polymorphic Loci (SASPL) and the lowest (1,024) was that obtained by RAPD. While, the total

coverage of genomic area was 15.9 Mbp (1.21%) using the combined data of the olive genotypes.

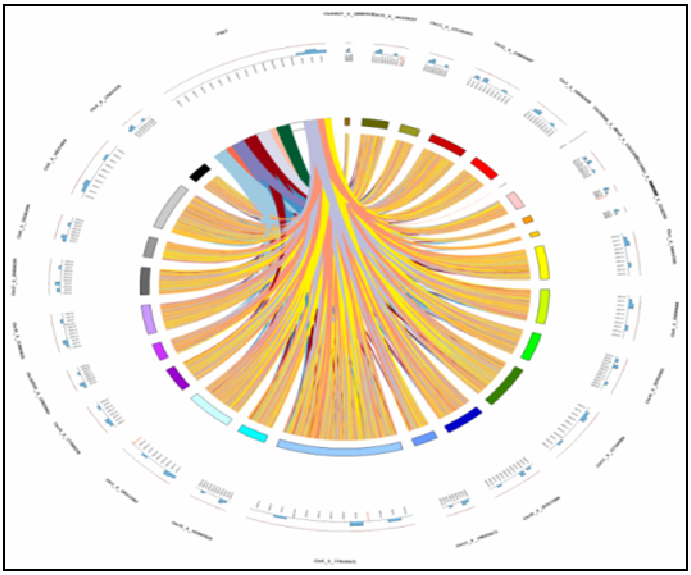

Fig. 3 : The in-silico PCR results for the SSR, ISSR and AFLP against wheat genome.

Linkage Disequilibrium(LD)

In the present study, LD analysis was performed for 1108 markers using the pairwise squared-allele frequency correlations (r^2) and these markers were located on the chromosomes of B genome (38%), followed by the chromosomes of A and D genome; 35% and 27%, respectively.

Among 49538 marker pairs, 2874 (5.8%) locus pairs showed significant LD based on r^2<0.1 for the whole genome (Table 6) and (Fig. 4). At the chromosome level, 36, 36 and 28 % of the locus pairs on A, B and D genome, respectively showed a significant LD (r^2> 0.1; p < 0.05). These findings were higher than that obtained by Saeed *et al.,* 2017 who reported a total of 36046 marker pairs, of which 1670 pairs were linked. Out of the 1670 linked pairs, 19 pairs had an r2 >0.1 using 269 SSR markers with 59 winter wheat genotypes. Moreover, Roncallo *et al*., 2019 found 4.9% of significant LD values were linked with wheat collection. Also, Ballesta *et al*., 2018 revealed that, 29, 27 and 33 % of the SNP pairs on A, B and D genome, respectively, were correlated with LD (r^2> 0.03; p < 0.05). Nevertheless, their results showed 4.1 %significant marker pairs for LD (0.2 > r^2> 0.1). These results are higher than that reported by Somers *et al.,* 2007 who found that only 0.9% and 3.2% locus pairs that were in significant LD with r^2< 0.2 for durum and bread wheat, respectively. The low values of LD be evidence that many of the recombination events in past breeding history have been maintained and fixed in homozygous self-fertilizing bread wheat. LD decay distances vary greatly from one study to the next. These differences could be correlated to both variations in material type and quantity as well as differences in r^2 values (i.e., r^2.0.05, 0.1, or 0.2) used for the estimations and the most LD decay distances were estimated with r^2 = 0.1 (Chen *et al*., 2012).

Table 6: Linkage disequilibrium (LD) estimates.

Number of accessions	70	
Number of markers	1108	
Pairwise measurement	N	%
r^2<0.1	2874	5.8
0.2> r^2>0.1	2018	4.1
0.5> r^2>0.2	753	1.5
r^2>0.5	103	0.2
Total significant pairs(p<0.01)	2275	4.6
Total pairwise combinations	49538	100

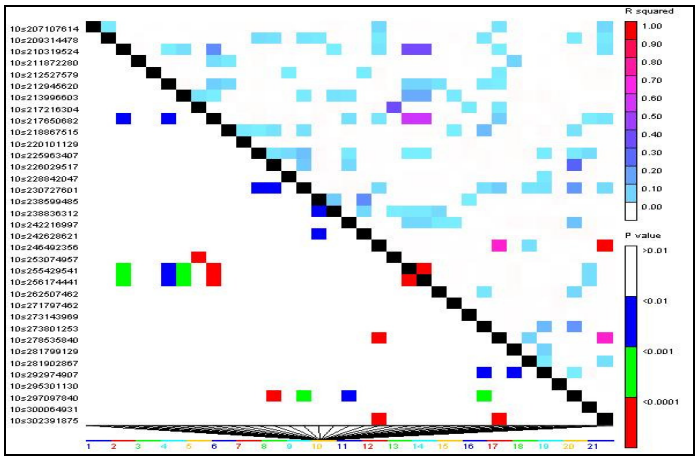

Fig. 4 : LD plot generated by genetic marker pairs. The upper diagonal shows r^2 among each pair of markers. The lower diagonal shows the levels of significance between each pair of markers

Marker-Trait Associations (MTAs)

In this study, General linear Model approach (GLM) was used to identify the genetic regions controlling the response of 10 traits representing the yield and yield components under stress at two locations (Luxor and New Valley) for 70 hexaploid wheat landraces (Table 7). A representative Manhattan plots of the association results for DH, DM, and GWP) on 21 bread wheat chromosomes for 70 wheat lines are shown in Figure 5.

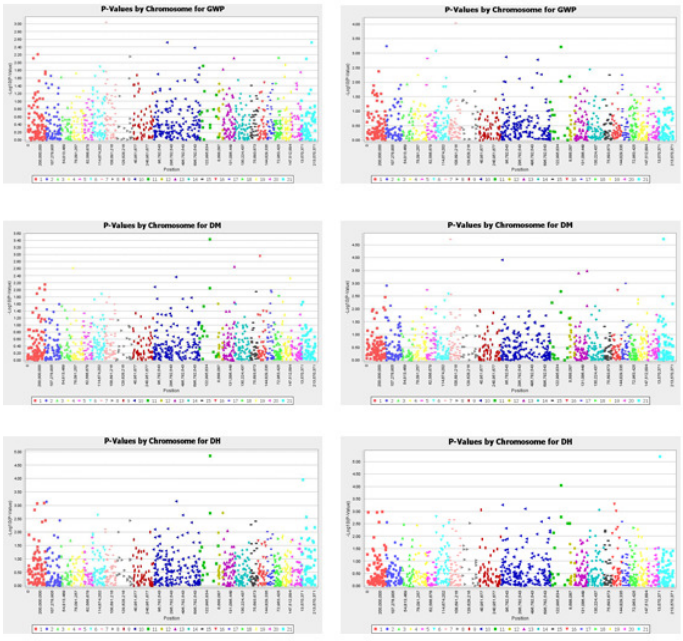

Fig. 5 : Manhattan plots show association result for heading day (DH), Days to heading (DM), Plant height (PH), Spike length (SL), Grain weight/ plot (GWP) and Number of spikelets/ spike (NSt) on 70 bread wheat. The x-axis represents the physical map locations of the molecular markers and the y - axis represents the –log base 10 P - value.

Table 7: Summary of unique loci from the association mapping performed on 10 traits of yield and yield components obtained in New valley and Luxor under heat stress.

New Valley			Luxor		
Trait	**Marker**	**Chr.**	**Trait**	**Marker**	**Chr.**
ChA	ISR6-1500	2D	**Ch A**	AAC- CAG-43	**4A**
ChB	AAC- CAG-261	4A	Ch A	AAG-CTC-186	**2B**
DH	ACT-CTT-186	6B	**DH**	**Xgwm369**-160	**3A**
DH	ACT-CTT-35	7A	DH	AAG-CTC-165	5A
DH	**Xgwm369**-240	3A	DH	ACT-CTT-246	7D
DH	ACT-CTT-101	2D	DH	ACT-CTT-169	7B
DH	ACT-CTT-85	6B	DH	ACT-CTT-186	6B
DH	ACT-CTT-215	1B	DH	ACT-CTT-35	7A
DH	ACT-CTT-82	7B	DH	ACT-CTT-247	2A
DH	AAC- CAG-104	4B	DH	AAG-CTC-73	6A
DH	AAG-CTC-27	5D	DH	AAG-CTC-20	6B
DH	AAC- CAG-320	3B	DH	AAC- CAG-391	3B
DH	AAG-CTC-73	6A	DH	AAG-CTC-27	5D
DH	AAC- CAG-135	2B	DH	ISR7-1400	1A
DH	AAC- CAG-305	6B	DH	AAG-CTC-288	2A
DH	AAC- CAG-48	5D	**DM**	AAG-CTC-191	**3B**
DM	AAG-CTC-109	**2B**	DM	ACT-CTT-37	4B
DM	AAG-CTC-150	2B	DM	AAC- CAG-320	3B
DM	**Xgwm369**-160	3A	DM	AAG-CTC-20	6B
DM	AAG-CTC-62	5B	DM	AAC- CAG-19	2B
LA	ACT-CTT-12	**7B**	DM	AAG-CTC-227	5D
LA	AAC- CAG-77	6A	**GWP**	**Xgwm219-200**	**6B**
LA	AAC- CAG-447	5B	GWP	AAC- CAG-114	7B
LA	AAC- CAG-69	1A	GWP	AAC- CAG-391	3B
NKSp	ACT-CTT-83	3D	GWP	Xgwm369-160	3A
NKSp	AAC- CAG-338	3D	GWP	AAG-CTC-195	7D
NKSp	ACT-CTT-177	7D	GWP	ACT-CTT-77	6A
NKSp	AAC- CAG-320	3B	GWP	ACT-CTT-78	6D
NKSp	AAG-CTC-227	5D	**LA**	AAC- CAG-445	**5 B**
NKSp	ACT-CTT-76	6A	LA	AAC- CAG-439	3D
NKSp	AAG-CTC-65	1A	**NKSp**	AAC- CAG-360	7B
NKSp	ACT-CTT-219	4A	**NSt**	AAG-CTC-294	**7D**
NKSp	AAG-CTC-391	6A	NSt	ACT-CTT-25	3B
NKSp	ACT-CTT-64	7B	NSt	AAG-CTC-114	7D
NKSp	Xwmc596-160	7A	**PH**	**Xgwm369**-240	**3A**
NKSp	AAG-CTC-408	5D	PH	AAG-CTC-326	4A
NSp	**Xgwm369**-240	3A	PH	AAG-CTC-73	6A
NSt	AAC- CAG-98	**6A**	PH	AAC- CAG-267	6B
NSt	ISR14-940	5D	PH	AAG-CTC-7	**3A**
NSt	AAG-CTC-341	5A	**SL**	**Xgwm369**-240	**3A**
PH	**Xgwm369**-240	**3A**	SL	ACT-CTT-38	5D
PH	AAC- CAG-199	4A	SL	AAG-CTC-153	7D
PH	AAC- CAG-161	6A	SL	ACT-CTT-214	2A
SL	**AAG-CTC-153**	7D	SL	ACT-CTT-131	4B
SL	ACT-CTT-214	2A	SL	ISR14-570	3B
SL	Xgwm219-160	6B	SL	ACT-CTT-35	7A
SL	ACT-CTT-35	7A	SL	SSR5-160	6B
SL	ACT-CTT-131	4B	SL	ACT-CTT-162	4D
SL	AAG-CTC-50	6D	SL	H26-1480	7A
SL	Xgwm369-240	3A	SL	AAG-CTC-73	**6A**
SL	ACT-CTT-38	5D	SL	AAG-CTC-50	**6D**
SL	AAG-CTC-85	2D	SL	ACT-CTT-321	**1A**
SL	AAG-CTC-374	7B	SL	AAG-CTC-294	**7D**
SL	ACT-CTT-321	**1A**	SL	AAG-CTC-358	**4B**
SL	AAG-CTC-383	**7D**	SL	ACT-CTT-169	**7B**
SL	AAG-CTC-42	**6D**	SL	**Xgwm369**-160	**3A**
SL	AAG-CTC-358	**4B**	SL	AAG-CTC-208	**5D**

			SL	AAG-CTC-374	**7B**
			SL	ACT-CTT-73	**3B**
			SL	AAG-CTC-33	**7D**
10	57		10	60	

This study showed 14 and 21 MTAs at New Valley and Luxor, respectively that are linked with Spike length (SL), Eleven of them were located on chromosomes 1A, 3A, 4B, 6A, 6B, 6D, 7 A, 7 B and 7D in the two studied locations. Also, Acuna-Galindo *et al*., 2014; Sukumaran *et al*., 2016 and Ogbonnaya *et al.,* 2017 reported QTLs for SL that are located on chromosomes 1A, 4B and 7A suggesting that these chromosomes would play a role in controlling spike length.

For Days to heading (DH), 27 MTAs were identified, 14 of them were in New Valley and 13 were in Luxor. Five MTAs were located on chromosomes 3A, 5D, 6A and 6B both studied locations. On the other hand, the remaining MTAs was recorded on chromosomes 2A, 2B, 2D, 3B, 4B, 5A, 5B, 6D, 7A, 7B and 7D. Nevertheless, MTAs were also reported to be associated with DH on chromosomes 2A, 5A and 6A (Tadesse *et al*., 2019), 5A, 5B, 6A, 7A and 7D (Ogbonnaya *et al*., 2017) 5A (Lopes *et al*., 2015 and Sukumaran *et al*., 2015) and 7A (Acuna-Galindo *et al*., 2014) locus with pleiotropic effect on PH, DM and HD.

For Days to maturity (DM), ten MTAs were detected; four of them were on chromosomes 2B, 3A and 5B in new valley and six MTAs on 2B, 3B, 4B, 5D, and 6B in Luxor. These results are consistent with that reported by Sukumaran *et al*., 2015who detected MTAs on 2B, 3B, 4B, 4D, and 6A for DM. Also, Acuna-Galindo *et al.* (2014) who identified two loci on chromosome 5B that is associated with DM.

For Plant Height (PH), eight loci were significantly linked with PH that were located on chromosomes 3A, 4A, 6A and 6B in both studied locations. Similar results were previously reported by Edae *et al.*, 2014 and Tadesse *et al*., 2019 who identified four loci on chromosomes 6A and 6B that are linked with HD, GY, and PH. A new heat specific locus (Xgwm369) on 3A was pleiotropic on PH, DH, DM, GWP, NSP that could be targeting for QTL pyramiding and further validation.

For Leaf Area (LA), six markers were significantly associated with LA on seven loci, two MTAs on chromosomes 3D and 5B in Luxor and four MTAs on chromosomes 1A, 5B, 6A and 7B. Also, Qaseem *et al*. (2018) recorded significant MTAs that are associated with LA and are located on chromosome 1B, 5A and 6A under heat-stress treatment for 108 spring bread wheat cultivars. Moreover, Qaseem *et al*. (2019) detected MTAs that are associated with LA on chromosomes 3D, 5B, and 7B for 192 elite bread wheat genotypes.

Also, the Number of Kernel per Spike (NKSp) showed polygenic control with 13 MTAs, 12 of them in the New valley lines and were located on chromosomes 1A, 3B, 3D, 4A, 5D, 6A, 7B, and 7D. The remaining MTAs is located on chromosomes 7B in Luxor. Moreover, Number of spikelets/spike (NSt) was controlled by three MTAs foreach location. Three MTAs were located on chromosome 5A, 5D and 6A in the NewValley and the other three MTAs were located on 3B and 7D for Luxor.

Grain weight/plot (GWP) was controlled by seven individual QTLs located on chromosome 3A, 3B, 6B, 7A, 7B and 7D in Luxor Only, these were previously identified by Acuna-Galindo *et al*. (2014) as meta-QTL for GWPS, PL, and SL on 7A. While, Ogbonnaya *et al*. (2017) detected two loci on chromosome 3B with TKW, GY, DM and GWPS.

Finally, Chlorophyll A content was controlled by three MTAs at both locations, they were located on 2B, and 4A at Luxor. While, one marker was located on chromosome2D in New Valley, Chlorophyll B content was associated with MTA that are located on 4A in New Valley only. Interestingly, Maulana *et al*., 2018 reported that leaf chlorophyll content is associated with seven QTLs that were located on chromosomes 2B, 2D, 4A, 4B, and 5B and that they are significantly associated with heat stress response.

Thus, a total of 117 (10.56%) MTAs, 57 for New valley and 60for Luxor, were significantly ($p < 0.001$) associated with ten agronomic traits across two locations (Table 8). MTAs were distributed over 77 loci; of which17 were consistent over two locations. Spike length (SL) had the maximum number (35) of significant associations distributed over 23 loci, followed by DH (27) and NKSp (14) at both locations. One marker was identified as the minimum number for Ch A, on chromosomes 2D. Also, numbers of loci linked to the 10 traits were recorded on A genome (42), followed by B genome (34), and the D genome (31).This is in line with the results obtained by Tadesse *et al.,* 2019 who identified a total of 111 significant MTAs; out of which, thirty-nine MTAs were detected in Egypt and 72 in Sudan under severe heat stress. Also, Ogbonnaya *et al*., 2017 recorded 16 loci as the maximum numbers of MTAs for the days of maturity (DM), followed by grain yield on 13 different loci.

Results showed, several co-localized QTL on chromosomes 3A, 6A and 7D for yield and agronomic traits were detected and consistent at both environments under heat stress. These MTAs that are consistent across locations would be interest and therefore have more probability of being useful in different environments. Also, the QTL on chromosomes 1A, 3A, 5A, 6B, 7A and 7B had pleiotropic effects on DH, DM, SL, PH, Nst and NKSP. Tadesse *et al*., 2019 recorded specific QTL for grain yield on chromosome 5A at Sudan. Also, Talukder *et al.,* 2014 reported five QTLs on chromosomes 1B, 1D, 2B, 6A, and 7A for heat tolerance in wheat using a bi-parental mapping. Moreover, Acuña-Galindo *et al*., 2015 identified major QTLs associated with heat tolerance on chromosomes 1B, 2B, 2D, 4A, 4D, 5A, and 7A using a meta-analysis strategy.

It worth to mention that Xgwm369 locus with two alleles (160 and 240 bp) on chromosome 3A linked to DH, DM, GWP, SL, and PH at both locations is likely to be a new QTL. Also, Loci (AAG-CTC-73) associated with PH, SL, DH were detected on chromosome 6B.

Table 8 : Trait names and their codes with total number of marker trait association in New Valley and Luxor under heat stress for 70wheat landraces.

Traits	Abbreviation	New Valley MTAs	Luxor MTAs
Spike length (cm). (SL)	SL	14	21
Days to heading (days). (DH)	DH	14	13
Days to Maturity (days).	DM	4	6
Plant height (cm). (PH)	PH	3	5
Number of spikelets/ spike	NSt	3	1
number-of--Kernel/spike	NKSp	13	1
Grain weight / plot (g).	GWP	-	6
Leaf area meter (cm^2).	LA	4	2
Chlorophyll A µg/ g fw	ChA	1	2
Chlorophyll B µg/ g fw (Chl B)	ChB	1	-
	Total	57	60

Qaseem *et al*., 2018 detected seven clusters of QTLs associated with more than two traits. Also, Ogbonnaya *et al.,* 2017 who obtained eight out of 27 loci on chromosomes 1A, 1D, 5A, 5D, 6B, 7A, and 7B had pleiotropic effects on DH,TKW, SM−2, and SNS across different environments including heat stress.

Conclusion

In this study, a high level of variation were observed for all of the agronomic traits measured in a collection of wheat landraces evaluated for three years in heat prone locations. Such variation was further confirmed at the molecular level using SSR, ISSR and AFLP markers analysis, which demonstrates the existence of considerable genetic variability among these landraces. Marker trait association identified highly significant and suggestive major QTLs for yield and yield components under heat environments located at 1A, 3A, 4B, 6A, 6B, 6D, 7 A and 7 B. Hence, microsatellite marker, Xgwm369 locus on chromosome 3A liked to DH, DM, GWP, NSP, SL, and PH at both locations could be used for fine mapping and marker assisted selection to discover new genes responsible for heat and drought tolerance in wheat. Finally, the superior lines identified in this study and their respective QTLs can be used in wheat breeding programs and marker assisted approach to improve productivity and stability under heat conditions and, the association mapping analysis can effectively detect stable and environment-specific QTL for multiple agronomic traits

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