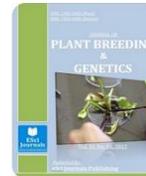




Available Online at ESci Journals

Journal of Plant Breeding and Genetics

ISSN: 2305-297X (Online), 2308-121X (Print)
<http://www.escijournals.net/JPBG>



VALIDATION OF SSR MOLECULAR MARKERS LINKED TO DROUGHT TOLERANT IN SOME WHEAT CULTIVARS

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ABSTRACT

The present study was carried out to conduct drought tolerance in three wheat cultivars including susceptible (Gemmiza7) and tolerant (Sakha93 and Sahel1). Molecular characterization was done by 26 SSR markers located on chromosome7 which was associated with drought tolerance in many previous studies. 26 SSR markers were polymorphic and thus showed 100% polymorphism. The number of alleles per locus varied from 2 to 3 alleles with an average (2.62). The polymorphism information content (PIC) value ranged from 0.34 to 0.59, with a mean of 0.51. The discrimination power (Dp) value ranged between 0.67 and 0.78 with an average of 0.71 per locus and Heterozygosity (He) value varied from 0.44 to 0.67 with an average of 0.59. The genetic relationships estimated by the polymorphism of SSR markers revealed a greater level of genetic variability in wheat cultivars of wide adaptability and applicability. Whereas an average of combined probability value for the SSR markers was 6.15×10^{-16} , suggests the capability of the marker system to distinguish identity and purity of wheat cultivars. In addition to the SSR markers revealed various bands that were either absent or present within tolerant cultivars (Sakha93 and Sahel1) which were altogether absent in susceptible cultivar (Gemmiza7). Also, SSRs of diagnostic and curatorial importance were discerned as 'stand-alone' molecular descriptors for barcoding the application of DNA sequences of standardized genetic markers for the identification of wheat cultivars. However, the genetic information in this study could provide useful information to address breeding programs and germplasm resource management.

Keywords: Wheat, SSR markers, polymorphism, genetic relationships.

INTRODUCTION

Bread wheat (*Triticum aestivum* L.) is a staple food crop that feeds about 30% of the world population and provides over 20% of the calories consumed by humans (FAO, 2015). Due to a rapidly growing world population and climate changes, breeders and farmers are facing the challenge of increasing wheat production up to 70% by 2050 to meet future demands (FAO, 2009; Marcussen *et al.*, 2014), which needs a 2.4% of yield increase yearly. However, the current global average rate of crop yield increase is only 0.9% per year, which is far slower than the desired rate (Ray *et al.*, 2013). Since, wheat is the most important and widely adapted food cereal in Egypt. Therefore, it is necessary to increase wheat production in Egypt by raising the wheat grain yield. Currently, Egypt produces only 40% of its annual domestic demand

for wheat (Maha *et al.*, 2017).

However, despite the fact that wheat has a high socio-economic impact, bread wheat is one of the last major crops lacking a high-quality reference genome sequence and is a difficult material for genome-wide studies due to its hexaploid nature, combining of three ancestral diploid grass species, the A-genome of *Triticum urartu*, the B-genome from a species related to *Aegilops speltoides* and the D-genome of *Aegilops tauschii* (Dvořák and Zhang, 1992). That makes it largest genome (17Gbp) in plant kingdom with 80% repetitive DNA (Bennett, 1995). The complete genome sequence will provide a gene catalogue and be an essential step in understanding the biology of this important crop. Moreover, the availability of a reference genome is expected to allow for discovery of new genes and regulatory sequences and will serve as a foundation for marker development to facilitate trait mapping and make marker-assisted selection in wheat more feasible

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(Perez-de-Castro *et al.*, 2012).

Despite its hexaploid nature with three sets of 7 chromosomes, bread wheat behaves as a diploid, undergoing bivalent chromosome pairing during meiosis. Due to all these constraints, it is also difficult to establish genetic diversity successfully among wheat genotypes. Due to genomic complications, only a few studies have been carried out successfully to establish genetic diversity among wheat genotypes using molecular markers.

However, global wheat production is negatively affected by both biotic and abiotic stresses and its potential yield is rarely achieved. Drought is one of the major abiotic constraints reducing wheat productivity (Nouri *et al.*, 2011). The anticipated world-wide climate change will elevate temperature, which will further aggravate the situation by accelerating evapotranspiration losses during the day and increased photorespiration at night (Mir *et al.*, 2012; Rajaram *et al.*, 1996).

It is difficult to make progress for grain yield and yield components under drought as they are complex characters influenced by many environmental factors and are characterized by low heritabilities and large genotype environment interactions under drought conditions (Smith *et al.*, 1990). It is therefore necessary to improve drought tolerance for sustainable wheat production. Genetic improvement in drought tolerance requires identification of traits associated with drought tolerance and introgression of genes underlying the target traits. Latest high-throughput genotyping and phenotyping have helped to understand the physiological and molecular bases of complex traits including drought tolerance (Mir *et al.*, 2012; Sinclair, 2012).

One important tool for breeders to be able to meet production demands is the deployment of molecular breeding methods that allow for faster development of higher yielding and better-adapted varieties. Having a physically ordered genome sequence allows the development of molecular markers for marker-assisted selection (MAS) and precision breeding. Over the past two decades, many markers technique have been employed for genetic mapping of economically important traits in wheat (Cattivelli *et al.*, 2002; Quarrie, 2006; Röder *et al.*, 1998). Among these, simple sequence repeats (SSRs) also known as microsatellites are widely used for molecular analysis of plants due to their multi-allelic nature, codominant inheritance, high reproducibility and simple assay method and widely distributed along

the genome, and their analysis may be automated (Rafalski *et al.*, 1996). Additionally, it has multiallelic nature, chromosome specificity, high polymorphism ratio and wide distribution throughout the wheat genome, all these make it a suitable molecular marker for genetic characterization studies in wheat (Bousba *et al.*, 2012; Dodig *et al.*, 2010).

Generally, microsatellites are considered to be a 'junk' portion of genomes and have been primarily used to understand evolutionary relationships and characterizing variation among natural populations of plant species (Sharopova, 2008; You *et al.*, 2008; Zhang *et al.*, 2006). Therefore, SSR markers from genic regions are not just a valuable genomic resource for molecular analysis and trait mapping but maybe also a target of selection in future crop breeding programmes. In spite of the higher polymorphism, SSRs could not reflect agronomic differences in the genetic resource evaluation, which could be explained by the fact that all SSR markers were not found in the transcribed regions (Li *et al.*, 2006; Yıldırım *et al.*, 2009). Tomar *et al.* (2016a) made a correlation analysis of morphological and agronomic characters in drought stress conditions and determined that the phylogenetic relationship between 31 wheat genotypes through SSR markers exists. Faheem *et al.* (2015) studied D genome-based genetic diversity research in terms of tolerance to drought using SSR markers. SSRs are becoming the markers of choice in many plant breeding programs because they are transferable, multi-allelic codominant markers, PCR-based, easily reproducible, randomly and widely distributed along the genome, and their analysis may be automated (Rafalski *et al.*, 1996).

SSRs have been used as the marker backbone for the localization of individual genes onto the 21 bread wheat chromosomes, including genes affecting traits of economic importance and have been widely used in marker-assisted selection in wheat (Ganal and Röder, 2007). Furthermore, wheat SSR markers have been used for the localization of a large set of QTLs for morphologically and agronomically important traits (Huang *et al.*, 2004). After the initial work on the use of SSR markers for kernel traits in hexaploid wheat (Snape *et al.*, 2006), various marker-trait associations were identified for disease resistance and yield traits (Peng *et al.*, 2008; Zwart *et al.*, 2008).

Several studies associated chromosome 7 with drought resistance in wheat (Cattivelli *et al.*, 2002; Galiba, 2002;

Morgan and Tan, 1996). Morgan (1991) located a gene for osmoregulation ("or") on chromosome 7A, and later Morgan and Tan (1996), using RFLP analysis, established the location of this gene on the short arm, at about 13 cM distances from the centromere. Applying genetic markers and recognition of polymorphic nucleotide sequences dispersed throughout the genome have provided a new possibility for evaluating genetic diversity and determining of inter- and intra-species genetic relationships (Gostimsky *et al.*, 2005). Genetic markers that are located in close proximity to genes (i.e. tightly linked) may be referred to as gene 'tags'. Such markers themselves do not affect the phenotype of the traits of interest because they are located only near or 'linked' to genes controlling the trait.

In the present research looking for the following aims:

- 1- To use SSR markers to estimate the level of polymorphism on chromosomes 7A, 7B and 7D and to identify the relationships among three wheat cultivars in Egypt.
- 2- To conducted drought stress tolerance in three wheat genotypes including susceptible and tolerant using SSR and functional markers based on genome-specific markers for each of on chromosomes 7A, 7B and 7D.
- 3- To distinctive power of DNA fingerprinting to each cultivar was used in this study.

MATERIALS AND METHODS

Plant material and drought evaluation: Three bread wheat (*Triticum aestivum* L.) cultivars (i.e. Gemmiza 7, Sakha93 and Sahel1) have been selected depending on their background concerning the drought tolerance,

whereas they included susceptible (Gemmiza7), and tolerant (Sakha93 and Sahel1) cultivars to drought stress. Different sensitivities of these genotypes to drought have been determined during few years in different regions of Egypt based on grain yield (El-Fadly *et al.*, 2007; ElSayed and Rafudeen, 2012; Mousa *et al.*, 2016).

In addition to preliminary *in vitro* experiment performed with three wheat cultivars under water deficit stress exerting by PEG6000 for the capacity of drought evaluation, (Bayoumi *et al.*, 2008). The data (not shown) confirmed the types of the wheat cultivars are presented in Table (1).

Grains of these cultivars were kindly obtained from Field Crops Research Institute, ARC, and Giza, Egypt.

DNA Extraction

Plant collection: The seedlings of three wheat cultivars were raised in pots under greenhouse condition at the Experimental Farm, Faculty of Agriculture Suez Canal University, Ismailia, Egypt on October 2017. Young, vigorously growing fresh leaf samples from these seedlings were collected from 21 days old seedlings to extract genomic DNA. Initially, a healthy portion of the youngest leaf of the tiller were cut apart with sterilized scissors and washed in distilled water and ethanol and dried on fresh tissue paper to remove spore of microorganisms and any other sources of foreign DNA. The collected leaf samples (Ten samples for each cultivar) were then kept in polythene bags and for avoiding any damage of the leaf tissues the bags were placed in an ice box to carry it in Lab. and finally, the samples were stored in - 80 °C freezer.

Table 1. Pedigree and the origin of three bread wheat cultivars.

Number	genotype	Pedigree	Tolerant	Origin
1	Gemmiza7	CMH74A.630/5x//Seri82/3/Agent CGM4611-2GM-3GM-1GM-OGM	Susceptible	Egypt
2	Sakha93	Sakha92/TR810328 s 8871-15-25-15-05	Tolerance	Egypt
3	Sahel1	N.S.732/Plm/veery "S" D735-4Sd-1Sd-OSd	Tolerance	Egypt

DNA Isolation: Total genomic DNA was extracted according to the basic DNA extraction protocol of Dellaporta *et al.* (1983) with slight modifications by Porebski *et al.* (1997). A weight (0.2 g) from young leaves were ground in liquid nitrogen to fine powder and extracted using 10 ml preheated (65°C) cetylhexadecyl-trimethyl ammonium bromide (CTAB)

extraction buffer [3% CTAB (w/v), 100 mMTris- HCl, pH 8.0, 20 mM EDTA, 1.4 M NaCl, 2% (w/v) PVP (Polyvinylpyrrolidone)], then 1% (v/v) of β -mercaptoethanol (15 mM) with further grinding. The mixture was incubated at 65°C for 60 min, followed by two extractions with chloroform/isoamyl alcohol (24:1). The nucleic acids were precipitated with cold

isopropanol, and the pellet was dissolved in 1 mL TE 0.1X (Tris-EDTA) buffer (10 mM Tris-HCl, pH = 8 and 1 mM EDTA, pH = 8). Co-precipitated RNA was removed by digestion with RNAase A. 4 µl (10 mg/mL). The DNA was further purified by 300 µl phenol: chloroform: isoamyl alcohol (25:24:1), then left overnight at (-20°C) using 1/10 vol. from 2 M sodium acetate (pH = 8.0) and one volume of cold isopropanol alcohol. The precipitate was washed twice with 10 mM ammonium acetate in 76 % ethanol, and the pellet was dissolved in 0.1 XTE buffer. The purified total DNA was quantified by gel electrophoresis, and its quality verified by Nanodrop spectrophotometer model ND1000. DNA samples were then stored at 4°C. DNA samples of each cultivar were analyzed individually to detect intra-cultivar variations and bulked to detect inter-cultivar variations.

Allele-specific SSRs analysis: Twenty six SSR markers (Table 2) were selected based on their location on chromosome 7, as previous information associated this chromosome with drought resistance in wheat (Cattivelli *et al.*, 2002; Galiba, 2002; Morgan and Tan, 1996; Quarrie, 2006). However, these SSRs used were previously described: *barc* (Song *et al.*, 2002; Song *et al.*, 2005), *cfa* (Sourdille *et al.*, 2003), *cfb* (Guyomarc'h *et al.*, 2002), *gwm* (Röder *et al.*, 1998) and *wmc* (Gupta and Huang, 2014).

The PCR reaction mixture (25 µl total) consisted of 50 mM KCl and 10 mM Tris-HCl (pH 8.8), 2 mM MgCl₂, 125 mM of dNTP, 50 ng of each primer, 1.0 unit of Taq polymerase and 20 ng of genomic DNA. Amplification was carried out in The amplification was carried out in a

thermocycler (Eppendorf Master Cycler Gradient Eppendorf, Hamburg, and Germany) that consisted of initial denaturation for 1 min at 94 °C, followed by 32 cycles of 30 s at 94 °C, 50 s at 53 °C, 50 s at 72 °C, and final extension for 5 min at 72 °C. The analyses were repeated at least twice to assure the reproducibility of the results. PCR products were separated on 2 % agarose gel and stained with ethidium bromide to check the PCR amplification and determine approximately the size of the amplified fragments. After that, The PCR products of the Microsatellite were detected by electrophoresis on Polyacrylamide non-denaturing gels, because Microsatellite alleles may vary in length by only a few base pairs. Therefore, 7 % Polyacrylamide gels were used to exact allele sizing of the SSR loci, and then stained with ethidium bromide solution and documented by gel documentation model. Quantity one software was used to estimate the sizes of the products by comparison to size marker.

SSRs data scoring and analysis: The simple sequence repeat (SSR) bands were scored visually on the basis of their presence (1) or absence (0), separately for each cultivar of wheat and each SSR marker. The scores obtained using all polymorphic markers in the SSR analysis were then calculated for a number of alleles, the effective number of allele, the frequency of allele, observed heterozygosity (*Ho*) and expected heterozygosity (*He*), using Pop gene 1.31 (Yeh, 1999). Power Marker version 3.25 was used to determine the polymorphism information content (PIC) (Liu and Muse, 2005).

Table 2. List of SSR markers used, their map position, according to Somers *et al.* (2004).

Sr.	Marker (locus)	Chromosomal location	Marker sequence
1	Cfa2049	7A	ACGGCATCACAGGTTAAAGG GGTCTTTGCACTGCTAGCCT
2	Wmc83	7A	TggAggAAAcAcAATggATgcc gAgTATcgccgAcgAAAgggAA
3	Wmc479	7A	gAccTAAGcccAgTgTcATcAg AgAcTcTTggcTTTggATAcgg
4	Wmc488	7A	AAAgcAcAAccAgTTATgccAc gAAccATAgTcAcATATcAcgAgg
5	Wmc525	7A	gTTTgAcgTgTTTgcTgcTTAc cTAcggATAATgATTgcTggcT
6	Wmc790	7A	AATTAAGATAGACCGTCCATATCATCCA CGACAACGTACGCGCC
7	Gwm333	7B	GCCCGTCATGTAAAACG

8	Gwm400	7B	TTTCAGTTTGCCTTAAGCTTTG GTGCTGCCACTTGC TGTAGGCACTGCTTGGGAG (CA)21
9	Gwm537	7B	AAGAGATAACATGCAAGAAA TTCAAATATGTGGGAACACTAC
10	Wmc396	7B	TgcAcTgTTTTAccTTcAcggA cAAAgcAAgAAccAgAgccAcT
11	Wmc517	7B	ATccTgAcgTTAcAcgcAcc AccTggAAcAccAcgAcAAA
12	Barc11	7D	5' GCGATGCGTGTAAGTCTGAAGATGA 3' 5' GCGTCCATGGAGCTCTGTTTTATCTGA 3
13	Barc126	7D	GCG CCG TGT AAA TAG TTT TGT TTA CTT GCA CAG CCA AAT AGT GTG GAT AA
14	Barc154	7D	GTAATTCCGGTTCCACTTGACATT GGATGGGCAGCTTCAAGGTATGTT
15	Barc172	7D	GCGAAATGTGATGGGGTTTATCTA GCGATTTGATTTAACTTTAGCAGTGAG
16	Cfd14	7D	5' CCACCGCCAGAGTAGTATT 3' 5' TCCTGGTCTAACAACGAGAAGA 3'
17	Cfd66	7D	5' AGGTCTTGGTGGTTTTGGTG 3' 5' TTTTCACATGCCACAGTTG 3'
18	Cfd69	7D	'AAATACCTTGAATTGTGAGCTGC TCTGTTTCATCCCCAAAGTCC
19	Gwm111	7D	TCTGTAGGCTCTCTCCGACTG ACCTGATCAGATCCCCTCG
20	Gwm130	7D	AGCTCTGCTTCACGAGGAAG CTCCTCTTTATATCGCGTCCC
21	Gwm295	7D	GTGAAGCAGACCCACAACAC (GA)25 GACGGCTGCGACGTAGAG
22	Gwm428	7D	CGA GGC AGC GAG GAT TT (GA)22 TTC TCC ACT AGC CCC GC
23	Wmc14	7D	AcccgTcAccggTTTATggATg TccAcTTcAAgATggAgggcAg
24	Wmc121	7D	ggcTgTggTcTcccgATcATTc AcTggAcTTgAggAggcTggcA
25	Wmc463	7D	gATTgTATAgTcggTTAccccT ATTAgtgcccTccATAATTgTg
26	Wmc702	7D	GAATCACATCGAATGGATCTCA GAGGCCTTTTTCGATATTCTGC

Genetic diversity was calculated using Shannon's diversity index (Shannon and Weaver, 1949). The discrimination power (D) is an estimation of the probability that two randomly sampled accessions could be distinguished by their SSR profiles (Jones, 1972; Lamboy, 1998). This parameter was calculated as $D = 1 -$

C , where C is the probability of coincidence, ($C = \sum p_i^2$, where p_i is the frequency of different genotypes for a given locus).

The probability of identity (PI) was calculated for each marker. PI is the average probability of two random individuals that shared the same genotype, and the

calculation formula of PI is the following: $PI = 2(\sum p_i^2)^2 - \sum p_i^4$, where p_i represents the frequency of the i th allele at a locus. For multiple loci combination, PI was calculated as the product of individual locus PIs while assuming that all loci segregate independently (Peakall and Smouse, 2012).

Genetic similarity (GS) between two genotypes I and j were calculated for each marker and across markers according to the formula given by Nei and Li (1979).

DNA barcode was constructed by uncoupling the allele size and the corresponding SSR locus information and then sorting the allele size data from lowest to highest (Galbács *et al.*, 2015).

RESULTS AND DISCUSSION

I-SSR Marker Informative and Genetic Relationships:

Microsatellite (SSR) markers are recognized as combining a number of advantages for use in breeding: they are codominant and multi-allelic, they are highly variable, being in most cases able to detect a higher level of polymorphism per locus than RFLP or AFLP markers, and they are amenable to high throughput analysis (Röder *et al.*, 1998). This is why it was considered desirable to identify SSR markers associated with the drought tolerance genes in wheat (Quarrie, 2006), that would be easier to use in marker-assisted selection.

However, the informativeness of the SSR markers was represented by various parameters such as the number of alleles, the effective number of allele, the frequency of allele, polymorphic information content, expected heterozygosity, and Shannon diversity index and discrimination power (Table 3). All of 26 SSR loci produced 68 alleles with a high level of Polymorphism (~100 percent).

The number of alleles per locus varied from 2 to 3 alleles (Table 3). The low number of alleles indicated in part the narrow genetic basis in the three wheat cultivars used in this study (Salem *et al.*, 2008). The variability in the number of alleles per locus may results from different locus-specific mutation rates and reflects strong differences in allelic diversity between SSRs loci (Piyusha and Singh, 2018). The mean number of alleles estimated in the three wheat genotypes (2.62 alleles per locus) is consistent with a study conducted by Phougat *et al.* (2018) that found an average of 2.585 alleles per locus in 44 bread wheat genotypes using 100 SSR markers. Moreover, higher allele means numbers (5.9 and 13) were detected in 10 and 40 wheat genotypes by Ateş Sönmezoğlu and Terzi (2017) and Bousba *et al.*

(2012), respectively. It's very important to remember that the comparisons with the allelic diversities reported by other studies should be regarded with caution and take into consideration the different sample sizes used; in addition, the same mean number of alleles may not indicate the same amount of variability.

Effective number of alleles is the measure of allelic evenness. The total number of effective alleles produced by the 26 SSR loci was 67.24 and ranged between 1.78 and 3, with an average value of 2.59 per locus. While, Novoselović *et al.* (2016) reported that the effective number of alleles per locus was 1.64. However, these results imply that abundant genetic polymorphism exists in wheat cultivars.

The frequency of allele at each locus ranged from 33.00% to 55.00% with an average value of 41%. According to Huang *et al.* (2004) it appears that the alleles with higher frequencies might be selected and kept for adaptational reasons. The results in this study is in contrary with Soriano *et al.* (2016) that reported the frequency of allele ranged from 0.003 to 0.857, with a mean of 0.098. This variation in genetic values may be indicated either to the disparity in a number of genotypes or SSR markers used to detect DNA variegation. The PIC value can be used to evaluate the level of genetic variation in a plant and estimate the informativeness of each polymorphic locus, varied from 0.34 to 0.59, with a mean of 0.51 (Table 3). Since, the PIC value is >0.5 the locus is considered to be of high diversity (Ramadugu *et al.*, 2015). In this study, approximately 65% of microsatellite markers that permeate chromosomes 7 A, B and D genomes had a PIC value greater than 0.50, which indicates that the majority of markers enabled a high level of polymorphism. The results that used SSRs are potential markers that could be used as a marker to assist in selection for drought stress tolerance by molecular plant breeding. Moreover, the results are in agreement with those reported Faheem *et al.* (2015) and Tomar *et al.* (2016b), who assigned SSR markers to drought tolerance in wheat genotypes using molecular markers. Subsequently, PIC values correlate positively with the number of alleles for all genotypes. It was determined that the markers that had 2 alleles also had lower PIC values (0.34 and 0.38) whereas the markers had 3 alleles had high PIC values (0.59). These results did not agree with those of Prasad *et al.* (2000) who reported that the PIC value was not correlated with the number of alleles.

Large sample size is necessary for the characterization of a reliable correlation coefficient. The sample size in their study was only three cultivars.

Table 3. Molecular characteristics of the 26 SSR markers used to analyze the three wheat cultivars.

Sr.	Indexes	Total	Minimum	Maximum	Average
1	Number of markers used in this study	26	-	-	-
2	Number of markers amplified fragments	26	-	-	-
3	Percentage of polymorphism	100	-	-	-
4	Number of polymorphic alleles	68	2	3	2.615
5	Number of allele effective	67.24	1.78	3	2.586
6	Frequency of Allele	10.56	0.33	0.55	0.406
7	Polymorphic information content (PIC)	13.130	0.340	0.590	0.505
8	Discrimination power (D)	18.410	0.670	0.780	0.710
9	Expected heterozygosity (He)	15.410	0.440	0.670	0.592
10	Shannon diversity index (I)	24.670	0.640	1.110	0.949
11	Probability (Pi)	6.942	0.182	0.403	0.267
12	Size of base pairs of amplified fragments	-	102pb	411pb	-

The discrimination power (Dp) was found to be high in the majority of the 26 SSR markers, ranged between 0.67 and 0.78 with an average of 0.71 per locus. However, the discrimination power is an extension of the polymorphism information content (PIC), which actually describes the efficiency of a given marker to discriminate between genotypes, i.e., the probability that two randomly selected individuals have different arrays (Anderson *et al.*, 1993). Thus, high PIC coupled with Dp values exhibited that these markers have the potential to disclose allelic variation and each of these markers had a greater affinity towards discriminating between two genotypes (Ashraf *et al.*, 2016).

Heterozygosity (He) refers to the presence of different alleles at one or more loci on homologous chromosomes. Heterozygosity per locus varied from 0.44 to 0.67 with an average of 0.59 (Table 3). The heterozygosity observed at some of the loci could also be due to high mutational rate and mutational bias at SSR loci. The loci with a large number of repeat units (SSR units) tend to show a high mutational rate. As a result, any mutations in any one of the alleles may create a heterozygous condition (Bharathi, 2011). The measure of the level of heterozygosity across loci can be used as an indicator of the amount of genetic variability (Zulkifli *et al.*, 2012). However, Allelic diversity and heterozygosity are important features for the establishment of microsatellite markers for linkage studies (Chiaromonte *et al.*, 2002).

Shannon diversity Index revealed that the genetic diversity ranged from 0.64 to 1.11 with an average of

value 0.95. The minimum value of genetic diversity (0.64) linked with 2 alleles per locus whereas the maximum value (1.11) linked with 3 alleles per locus (Table 3). According to Nei (1973), It was observed that marker detecting the lower number of alleles showed lower genetic diversity than those detected the higher number of alleles showed higher genetic diversity. The high level value of an average genetic diversity found in this study may be due to the presence of many unique alleles in wheat cultivars (Nazco *et al.*, 2014), so it will be essential to assess the genetic structure of the population in future study.

The allele size becomes important information in order to know which fragment maps to which locus when many of the microsatellite markers amplified complex, multilocus profiles (Somers *et al.*, 2004). For the three cultivars the 26 SSR primer pairs produced a total of 68 allele sizes. The overall size of amplified PCR products ranged from 102-411bp (Table 3).

Salem *et al.* (2008) obtained an allelic size range between 77 to 266 bp on using 15 microsatellite markers on some wheat genotypes. While, Nader and Abdelsalam (2014) obtained an allelic size range between 59 to 635bp using 20 SSR markers on five Egyptian bread wheat genotypes and one wild wheat. However, slippage of the polymerase during the amplification of the repeat is believed to be responsible for the production of fragments that are reduced in length by a multiple of the repeat units (Smeets *et al.*, 1989).

Genetic Similarity (GS) matrices constructed on shared allele bases varied from 0.039 to 0.192 with an average of 0.116, which means that the three wheat cultivars share on average 11.6 % of their marker alleles at the investigated SSR loci. The lower estimates of an average genetic similarity indicated a high polymorphism at the DNA level among these cultivars and, therefore, a large amount of genetic variation among homologous DNA sequences (Medini *et al.*, 2005).

Moreover, it might be expectedly found the highest value of genetic similarity (0.192) between Gemmiza7 cultivar (susceptible) and Sakha93cultivar (tolerant). It is attributed that the response to the drought stress appears to be genotype-dependent in experimental fields, each genotype showing a peculiar expression pattern. Additionally, the tolerant and susceptible genotypes can be correlated to the molecular behaviour of the various genes respond to drought stress, even if tolerant genotypes do not correspond to a unique molecular behaviour, suggesting that alternative molecular pathways can be activated to respond to the water stress (Cantale *et al.*, 2007). Following by value (0.117) was recorded between Sakha93and Sahel1, whereas, the lowest GS was found between Gimmeza7 and Sahel1 (0.039).

In meantime, the average direct count of heterozygosity overall loci in the three cultivars is zero (data not shown). Since, wheat is a self-pollinating species; the explanation could be due to segregation of non-amplifying (null) alleles and/or selection against heterozygotes or inbreeding (Salem *et al.*, 2008). However, microsatellite markers have been recommended to be ideal markers for characterizing genetic diversity at the intra-species level by Olufowote *et al.* (1997). Surprisingly, three wheat genotypes produced only one allele was visible; its size is reported twice since the genotypes presumed to be homozygous with all twenty-six primer pairs. It might indicate the abundance of homozygous and/or null alleles (Rafalski *et al.*, 1996). Or else, it was just a coincidence, since in another study (Salem *et al.*, 2008) that using 15 SSR markers produced different alleles in wheat. However, the information about the genetic relationships of wheat cultivars in this study could provide useful information to address breeding programs and germplasm resource management.

II-Genome Wide Allelic Pattern: In the present study, characterization of three cultivars (Table 4) at the molecular level was done by using twenty-six SSRs.

These markers were located on wheat genome A, B and D genome (chromosome7). A genome- six SSR markers amplified 16 alleles (2.6 per locus) with polymorphic (100%),), whereas, B genome- five SSR markers were polymorphic (100 %), with 13 alleles (2.6 alleles per locus), and D genome- fifteen SSR markers were polymorphic (100%), with 39 alleles (2.6 alleles per locus). A genome was detected for the highest value of average PIC (0.51) with an average of He (0.59). Whereas, the variabilities obtained in B and D genomes in were almost similar for average PIC value (0.49) and average He (0.58). The results showed that an average Dp was similar (0.71) for three genomes.

Moreover, average genetic diversity (average Shannon diversity index) revealed that analyses of individual genomes of chromosome 7 in the bread wheat (Table 4). Genome A was the most diverse (1.03) followed by genome B and genome D (0.92). As a tribute to Quarrie *et al.* (2005) that 7A can be considered an important chromosome for yield and yield component QTLs under drought stress. The result is in agreement with Li *et al.* (2013) who indicated that the genetic diversity ranked as genome A > genome B > genome D in 62 Sichuan wheat landraces accession using 114 SSR markers. Therefore, the low genetic diversity of genome D might cause an accurate genetic basis for cultivated wheat (Chen and Li, 2007). This was expected, since hexaploid wheat gathered a larger proportion of genetic diversity from its tetraploid ancestors than from *Aegilops. tauschii* (containing the D genome) during domestication, resulting in a higher number of effective recombination in the A and B genomes relative to the D genome (Wang *et al.*, 2007).

In addition to Quarrie *et al.* (2005) that the D genome gave the lowest number of polymorphic markers in hexaploid wheat maps, although the map length of the D genome was similar to those of the A and B genomes. The results of the present study along with other studies discussed above clearly demonstrate the utility of microsatellite markers in fast and high throughput fingerprinting of numbers of genotypes for detecting polymorphism and estimation of genetic diversity (Han *et al.*, 2015; Karima *et al.*, 2017; Kumar *et al.*, 2016; Phougat *et al.*, 2018).

On other hand, chromosome 7 is conserving the most important chromosome harbinger QTL for drought (Morgan and Tan, 1996). The data in Table (5) identified Gemmiza (susceptible) and Sakha 93(tolerant) sharing

identical alleles in Cfa2049 and Wmc 790 Markers at loci 171pb and 146pb respectively in the 7A genome. Although, several studies associated chromosome 7A with drought resistance and play a major role with respect to productivity and stress responses in wheat (Cattivelli *et al.*, 2002; Galiba, 2002; Merchuk-Ovnat *et al.*, 2016). This suggests that other alleles at the marker loci might also be associated with a better response to drought, or that other mechanisms for resistance to drought are present in wheat (Ciucă *et al.*, 2009). Moreover, Younes (2009) found that none of the studied Algerian wheat cultivars characterized as having good levels of drought resistance but identified a large polymorphism for markers located on chromosome 7A. Up to this point, it is needed to expand the scope of this region for improving abiotic-stress resistance in wheat in further studies.

Regarding chromosome 7B in this study, similar, Wmc396 marker produced sharing band between Gemmiza (susceptible) and Sahel1 (tolerant) at 173pb. Whereas, Wmc 517 amplified sharing bands between Sakha93 (tolerant) and Sahel1 (tolerant) at loci 206 pb. In addition to Chromosome 7B was shown by (Quarrie, 2006; Quarrie *et al.*, 2005) to be the main region for yield QTLs under non-drought condition. Farshadfar *et al.* (2012) reported that most of the genes controlling quantitative criteria of drought resistance are distributed in the 7 B genome.

In genome D, find three loci shared Gemmiza (susceptible) and Sakha 93(tolerant) at Barc111marker - 193pb, Barc154 marker-263pb and Gwm428 marker-159pb. On other side, two loci sharing Sakha 93(tolerant) and Sahel1 (tolerant) at Cfd0016 and gwm111 marker at 141 pb and158 pb respectively. This way a genetic relationship can be assumed between them, providing the bases of further pedigree studies.

Therefore, Cfa2049, Wmc 790, Wmc396, Barc111, Barc154 and gwm428 which sharing loci between susceptible and tolerant genotypes may be explained on the basis that drought tolerant genotypes may adopt different drought tolerance mechanisms such as, osmotic adjustment, accumulation and remobilization of stem reserves, superior photosynthesis, heat- and desiccation-tolerant enzymes, canopy temperature, and root system architecture each controlled by different set of genes, however, the SSR analyzed may not necessarily represent all of these genes (Gupta and Huang, 2014; Reynolds and Langridge, 2016). In future studies, wheat researchers may compare these markers and genome-wide SSR markers in a large set of drought susceptible and tolerant wheat genotypes to see whether the separation pattern observed with SSR markers are not confounded with any other trait. Defiantly, the findings in this investigation, which were based on a relatively small sample, are of a preliminary nature and require confirmation with a larger set of genetic stocks.

Table 4. The amplified number of alleles, genetic variation and the number of used microsatellites through genomes.

Genome	number of used microsatellites	Na	Polymeric	An average per genome				
				Allele/ locus	PIC	He	D	Genetic index
A	6	16	100	2.6	0.51	0.59	0.71	1.03
B	5	13	100	2.6	0.49	0.58	0.71	0.92
D	15	39	100	2.6	0.49	0.58	0.71	0.92

Table 5. Band-Sharing among 26 SSR markers through genomes of three wheat cultivars.

Cultivars	A		B		D	
	Gemmiza- Sakha 93	Gemmiza – Sahel1	Sakha 93-Sahel1	Gemmiza- Sakha 93	Sakha 93-Sahel1	
Marker and size of sharing band	Cfa2049-171pb Wmc 790-146pb	wmc396-173 pb	Wmc 517-206 pb	Barc111 -193pb Barc154 -263pb gwm428-159pb	cfcd0016-141 pb gwm111-158 pb	
Total bands=9	2	1	1	3	2	

III-Power of DNA Fingerprinting: The microsatellite profiles, represented with 26 polymorphic markers, showed that the loci employed delineated all the 3 cultivars. Thus, these markers can be used to distinguish the genetic profile of each cultivar. These genetic profiles, or fingerprints, may be useful in the protection of cultivars, for ensuring genetic purity and for creating further information to support breeding programs (Bertini *et al.*, 2006).

The Probability Value: The parameter of probability (PI) has been extensively used for identifying the fingerprinting power of molecular markers (Tan *et al.*, 2015; Waits *et al.*, 2001). PI expresses the probability of a band present in one genotype for being present in the other genotype by chance per primer (Ramakishana *et al.*, 1994). The minimum value for Pi was 0.182 whereas Maximum value for Pi was 0.403. The average Pi for SSR markers was 0.267 (Table 3). Assuming that all SSR marker loci segregate independently, the probability of identifying two random individuals, sharing the same genotypes at all the 26 loci, was estimated to be 6.15×10^{-16} . It is worth noting that the combined PI for the 26 SSR markers was 6.15×10^{-16} , implying that it is almost impossible to find two distinct Wheat genotypes with the same SSR fingerprinting profile. In fact, it has been suggested that the theoretical PI can be overestimated due to the assumption of independent segregation among loci is not authentic (Waits *et al.*, 2001). On the other hand, the probability of random two markers are linked together in the large wheat genome (17GB) (Abebe and Léon, 2012; Bennett, 1995; Faheem *et al.*, 2015), which is extremely low, thereby; the impact of non-authentic assumption to theoretical PI can be neglected. However, significantly, a low average of combined Pi for the SSR marker system (6.15×10^{-16}) suggests the capability of the marker system to distinguish identity and purity of wheat cultivars.

Wheat Cultivar-Specific Allele: All Twenty six SSR markers used to identify drought tolerant and susceptible wheat cultivars, revealed polymorphism 100 % clearly separating drought tolerant and drought susceptible cultivars. This high level of polymorphism is to be expected because of the unique mechanism responsible for generating SSR allelic diversity by replication slippage (Rafalski *et al.*, 1996). In addition to the data presented in Table 5 that showed the nine sharing bands out the seventy-seven bands. The rest of sixty eight bands are specific bands, though varying

between drought tolerant genotypes, were present only in drought tolerant cultivars, which are either directly or indirectly contributing to the drought tolerant loci. Even the SSR markers revealed various bands that were either absent or present within tolerant cultivars (Sakha93 and Sahel1) which were altogether absent in susceptible cultivar (Gimmeza7). These identified polymorphic bands can be considered as potential markers to identify drought tolerant cultivars for marker-assisted selection (MAS) in wheat breeding programs. Though drought tolerance is a polygenic character, there are examples (Ivandić *et al.*, 2002; Liviero *et al.*, 2002) where specific alleles at some loci have been found to be associated with ecotypes better adapted to drought environments. These markers bind somewhere in the sequence, eventually helping at least in gathering information of trait related regions. Sequence selection is an important part of it, as different sequences produce many times entirely different banding pattern that allows more specific recognition of individual genotype. Drought tolerance in wheat is a quantitatively inherited trait controlled by several genetic loci which their genetic components are difficult to measure (Forster *et al.*, 2000).

Constriction of DNA Barcode: In addition to the high level of polymorphism and the reliability of analysis, the advantages of the widespread use of SSR markers include a possibility for the quantification of the exact fragment sizes. One of the greatest advantages of SSR allele size data is that they can be easily digitized (Galbács *et al.*, 2015; Jeffreys *et al.*, 1985). So that the data were used for the construction of DNA barcode to visibly displays the similarities and also the indiscriminability of three genotypes.

For the three cultivars the 26 SSR primer pairs produced a total of 68 allele sizes. The overall size of amplified PCR products ranged from 102-411bp, with most of the sizes between 135 and 238 bp (Figure1) that indicating wide genetic diversity and it may be used in wheat hybridization program for improving grain yield. Moreover, figure 1 showed each bar on the DNA barcode corresponds to a certain allele size. Whereas, a lower index (2 or 3) was used on the barcode to indicate the overlaps i.e. where the same value was found in two different loci or three different loci (141 pb in Wmc and Cfd14 marker). Also, the data were used for the construction of cultivar-specific barcodes allowing seeing at first sight that the microsatellite markers do really produce the unique DNA fingerprints of the genotypes (Figure1).

Intervals of alleles/ Cultivars	100		120		140		160		180		200		220		240		260		280		300		320		340		360		380		400		420		
	00	10	20	30	40	50	60	70	80	90	00	10	20	30	40	50	60	70	80	90	00	10	20	3	4	5	6	7	8	9	0	1	2		
Gemm1za																																			
Sakha93																																			
Sahel1																																			

Figure 1. Microsatellites based barcodes for three wheat cultivars (FAO).

The resulting barcode system is a visual representation of the data, allowing easy detection of genotypic differences.

All three wheat cultivars were distinguishable by their band patterns. Polymorphism between genotypes can arise through nucleotide changes that prevent amplification by introducing a mismatch at one priming site, deletion of a priming site, insertions that render priming sites too distant to support amplification and insertions or deletions that change the size of the amplified product (Williams *et al.*, 1990). Ultimately, the established fingerprinting profiles can serve as a database for the wheat cultivars or landraces and can provide outstanding tools for plant variety certification and protection.

CONCLUSION

The present study not only revealed the means for identification of SSR markers associated with drought tolerant loci but also defined polymorphism between susceptible and drought tolerant wheat cultivars. Moreover, the polymorphism found among the wheat cultivars for markers located on chromosome 7 offer chances of finding useful associations between

these markers and traits that can contribute to improved drought tolerance. Though some more sophisticated methodologies are required to generate greater specificity and tight associations with drought tolerant loci, the SSR markers have nevertheless proved to be an easy, fast, simple and efficient means to identify such regions. Present effort have provided a base to critically judge these characters further by utilizing more advanced techniques wherein the marker termini will be sequenced to design longer primers such as SCAR for specific amplification of a particular locus. The identification of such loci may be helpful to find the genes involved in drought tolerance. These genes will be helpful to tailor drought resistant varieties of wheat through transgenic approaches.

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