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IDENTIFICATION OF ALLELIC VARIANTS OF XGWM261 LOCUS FOR *RHT8* DWARFING GENE IN PAKISTANI WHEAT GENETIC RESOURCE

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Abstract

Semi dwarfing wheat gene (*Rht8*) is among the important Green Revolution genes to increase productivity by modifying plant stature. Plant height is among one of the important constituent of plant architecture that determines the stability, harvest index and yield of crop plants. Strong linkage of Xgwm261, a wheat microsatellite marker with the dwarfing gene (*Rht8)* resulting in reduced height makes it well known marker among the breeders. Using this functional SSR marker, Pakistani wheat varieties and land races were analyzed to access the allelic distribution/ variation at microsatellite Xgwm261 locus. The 165-bp and 174-bp alleles of Xgwm261 were most frequent (38 and 24% respectively) among studied genotypes. A 192-bp diagnostic allele of *Rht8* gene was not detected in tested germplasm. Here we also report novel WMS261 variants of 182-bp, 188-bp, 196-bp and over 200-bp (205 and 215-bp) in some tested genotypes. For this microsatellite Xgwm261 locus the value of polymorphic information content (PIC) was 0.55. Conclusively, a high level of diversity for this locus was observed. Seven different microsatellite alleles were detected and a strong linkage of these variants at the *Rht8* locus if identified will provide a better understanding for gene to environment interaction for designing appropriate marker assisted selection (MAS) for high yielder wheat cultivars. Observed higher frequency of major allele (165-bp) could possibly be due to usage of CIMMYT wheat materials in Pakistani breeding programs. Selection of appropriate alleles will be useful in the varietal improvements breeding programs.

Key words: Semi-dwarf, SSR marker, Wheat, Xgwm261.

Introduction

High and stable yields are the main priority of every crop breeding programs. Wheat is the staple food for more than half of the world population, therefore increased wheat grain production is demanding for ever increasing population (Edgerton, 2009). Plant stature is one of the main contributory factors for enhanced yield and productivity. In bread wheat introduction of dwarfing and semi-dwarfing genes for plant height reductions and yield improvement has been the main breeding strategies. Semi-dwarf wheat cultivars are shorter having lodging resistance and are capable of more assimilate partitioning leading to high grain yields (Waddington *et al*., 1987). Modern high-yielding bread wheat cultivars utilize reduced height genes (*Rht*), or semi dwarfing genes responsible to reduce plant height and simultaneously increases adaptability and grain yield potential (Worland *et al.*, 2001; Reynolds & Borlaug, 2006); (Alghabari *et al*., 2014). Introduction of the reduced height (*Rht*) genes by Norman Borlaug into Mexican wheats dramatically boosted grain yield during the Green Revolution era. Originally, Norin 10 a Japanese wheat genotype caused a widespread utilization of these genes in CIMMYT wheats subsequently shifting them towards modern wheat cultivars. In wheat plant height is considered a complex trait as it is polygenically controlled. Twenty three dwarfing genes (*Rht1–Rht23*) located on different chromosomes have been reported to effect plant height in wheat (McIntosh *et al*., 2015). Based on their response to exogenous gibberellic acid (GA), these genes have been classified into two groups. The gibberellic acid (GA) insensitive dwarfing genes (*Rht-B1b, Rht-D1b*), whose presence reduced (16%) plant height were positively correlated with increased spikelet fertility and yield (24%). These genes are present in the vast majority of the world's semi-dwarf wheat varieties but they reduce early vigor and a negative correlation between these genes and plant fertility leading to low yields, has also been observed in Southern European environments (Worland *et al*., 2001). Therefore, *Rht1* and *Rht2* are not universally beneficial in every environmental condition. The second category comprised of GA responsive dwarfing genes (2A /*Rht7*, (9)/; 2D /*Rht8*, (10)/; 5A /*Rht12*,(11)/; 7B /*Rht9)* among which *Rht8* (located on chromosome 2D) that do not exert negative effect on seedling vigor has been introduced into Italian germplasm with objective of producing short skimmed varieties with early flowering to avoid the heat stress during ear emergence. Widespread utilization of these genes in particular under high temperatures and drought conditions has been reported and shown that *Rht8* gene reduced height (10%) without significant yield reduction in South-Eastern European wheat. *Rht8* gene has been reported to not only decrease the plant height but also significantly increased the total grain weight (Zhang *et al*., 2016).

DNA marker technology has greatly facilitated the identification and classification of the semi-dwarfing genes in wheat varieties worldwide. Due to higher level of polymorphism microsatellites are considered highly suitable genetic markers for gene mapping and diversity analysis in wheat (Röder *et al.*, 1995; Bryan *et al.*, 1997; Korzun *et al.*, 1997; Iqbal *et al.*, 2009). A strong association of *Rht8* dwarfing gene with 192-bp allele located at the microsatellite locus Xgwm261 on chromosome 2D has been reported using chromosome substitution line (CappelleDesprez and the Strampelli) (Korzun *et al*., 1998). *Rht8* has been considered a candidate gene for height manipulation located at short arm of chromosome 2D and less than 10% height

reduction is reported to be associated with the weaker height promoting allele of this gene (Worland *et al*., 1990). *Rht8* was speculated to produce a too strong dwarfing phenotype (Worland *et al*., 1992). The 192-bp alleles have shown to confer the moderate plant height reduction with an additional reduction when combined with the closely linked *Ppd-D1* gene (Worland *et al*., 1998). *Rht8* gene has 10 alleles. Several researchers used microsatellite marker Xgwm261-192bp allele either to know the incidence in wheat cultivars worldwide as diagnostic tool for *Rht8* gene of hexaploid wheat (Korzun *et al.*, 1998; Worland *et al.*, 2001; Ahmad & Sorrells, 2002; Ganeva *et al.*, 2005; Liu *et al.*, 2005; Zheleva *et al.*, 2006; Fayt *et al.*, 2007; Dvojković *et al.*, 2010; Šíp *et al.*, 2010; Weigt *et al.*, 2013) or to determine the consequence of *Rht8* on other important agronomic traits (Rebetzke & Richards, 2000; Bai *et al.*, 2004). The 192-bp allele of Xgwm261 is indicative of the more commercially favorable *Rht8* allele while the other alleles at this locus are considered associated with various levels of height promotion. To our knowledge, allelic variations at *Rht8* locus in Pakistani wheat germplasm have not been yet analyzed. It will be of great importance to check the divergence and then identify Xgwm261 alleles of *Rht8* in wheat genetic resource and its subsequent utilization in wheat breeding programs. The present study was aimed to analyze and to catalogue allelic variants at Xgwm261 locus tightly linked to the semi-dwarfing gene *Rht8* in Pakistani wheat genetic resource.

Materials and Methods

Plant material: Sixty Pakistani bread wheat varieties from all the provinces of Pakistan including landrace were selected for this study (Table 1).

DNA extraction, PCR amplification and molecular marker analysis: Total genomic DNA was extracted from ten days old seedlings by the method of Plaschke *et al*., (1995). The quality and quantity of DNA was determined by spectrophotometry and agarose gel electrophoresis. The primer pair Xgwm261 (GWM series from IPK, Gatersleben, Germany) was used for PCR amplification. PCR reactions were performed according to (Röder *et al*., 1998) in 25µl reaction volume containing the following components: 50 ng of template DNA, 200 µM of each of the four dNTPs, 1x Taq polymerase buffer, 1 unit Taq polymerase, 2 mM $MgCl_2$ and 0.25 µM each of the two primers. Amplifications were performed in a BioRad gradient thermal cycler. The amplification cycles consisted of initial denaturation at 94°C for 4 minutes followed by 35 cycles with denaturation at 94°C for 1 minute, annealing at 50°C for 1 minute and extension at 72°C for 1 min. The final extension was done at 72°C for 7 min. Amplification products were separated on non-denaturing 6% polyacrylamide gels and fragments were detected by ethidium bromide staining. Gels were documented using UVI pro Platinum 1.1 System and UVIBandMap software (UVItec Ltd., Cambridge, UK). Gene diversity, also described as polymorphic information content (PIC) was calculated using the simplified formula of (Nei, 1973) The generated data matrix was subjected to statistical analyses using Statistica software (StatSoft Inc., Tulsa, OK). Dendrogram showing genetic relationships of the varieties/cultivars was constructed using the un-weighted pair-group method on arithmetic averages (UPGMA).

Results

Molecular analysis of sixty Pakistani wheat genotypes revealed that Xgwm261 was highly polymorphic marker and detected various allelic variants of *Rht8* gene in Pakistani wheat germplasm (Table 1). All the samples successfully amplified the marker Xgwm261. In total, 7 different alleles i.e. 165-bp, 174-bp, 182-bp, 188-bp, 196-bp, 205-bp and 215-bp were detected at the Xgwm261 locus in tested wheat genotypes (Table 1). After the allelic identification, estimates of percentage distribution of dwarfing alleles for the microsatellite locus Xgwm261 were calculated. It showed that 165-bp allele was the most dominant allele with the highest frequency (38%) in tested wheat genetic resource (Fig. 1). The second most frequent allele was 174-bp with 24% frequency followed by 182-bp allele that was detected with 14% frequency in studied genotypes. In addition to reported common alleles, the other novel alleles i.e. 188-bp, 196-bp, 205-bp were also found in relatively lower frequencies of 6%, 8% and 8% respectively. One allele of 215-bp was detected with very minor frequency (2%).

Overall, 40% of tested wheat genotypes were found to carry 165-bp allele (Fig. 2). A 174-bp allele was detected in 25% of genotypes. Subsequently, a 182-bp allele was detected in 15% of tested genotypes. The other novel alleles i.e., 188-bp, 196-bp, 205-bp were observed in limited number of genotypes (6.67%, 8.33% and 8.33% respectively). One allele of 215-bp was detected in only one genotype (1.67%). In the present marker analysis, none of the varieties exhibited 192-bp allele. The electrophoretic patterns of microsatellite locus Xgwm261 showed a homozygous pattern for 95% of genotypes used in this study. However, three varieties namely Marwat, Sarsabz and Bakhtawar-93 depicted a heterozygous genotypic pattern for this locus. Among these genotypes, Marwat depicted 165-bp and 182-bp alleles while in Sarsabz, 165-bp and 188-bp allele combination was detected. In third genotype Bakhtawar-93 that was heterozygous genotype for this locus, 174-bp and 205-bp alleles were detected (Fig. 3).

Cluster analysis (Fig. 4) based on presence of different allelic variants of *Rht8*, classified the genotypes into three major groups having most prevalent alleles i.e. 165-bp (24 genotypes), 174-bp (15 genotypes) and 182-bp (9 genotypes) (Fig. 4). Further, three minor groups having 188-bp (4 genotypes), 196-bp (5 genotypes) and 205-bp (5 genotypes) alleles were observed in addition to one clade that contained only one distinct genotype Bhakkar-2002 having 215-bp allele. Diversity index of wheat microsatellite marker Xgwm261 showed an average PIC value of 0.55 confirming that this microsatellite marker is highly informative.

Table 1. Name of genotypes, year of release, pedigree information, origin and allele size at microsatellite locus Xgwm261 in Pakistani wheat varieties.

Sr#	Genotype	Year of release	Pedigree	Origin	Alleles (bp)
1.	HD2204	1975	HD-2092/3/HD-1962//E-4870/K-65	India	182
2.	HD2236	1976	HD-2119/HD-1981	India	165
3.	HD2285	1975	249/HD 2150 //HD 2186	India	165
4.	HD2329	1973	HD 1962/E 4870/3/K 65/5/HD1553/4/UP 262	India	182
5.	C-217	1944	C516/C591	Punjab, No CIMMYT parent	174
6.	C-228	1941	HARD FED/9D	Sindh, No CIMMYT parent	182
7.	C-271	1957	C 230 X IP 165	Punjab	174
8.	WL-711	1978	S308/CHRIS//KAL	Punjab, One CIMMYT parent	165
9.	MH-97	1997	NORD-DESPREZ(ND)/VG-9144// KALYANSONA/ BLUEBIRD/ 3/YACO/4/VEERY-5	Punjab, CIMMYT Advanced line	165
10.	Maxi pak-65	1965	PJ/GB55 or PJ62/GB55	Punjab, CIMMYT Segregating line or population	182
11.	Pak-81	1981	KVZ/BUHO//KAL/BB	Punjab CIMMYT Segregating line or population	165
12.	Inqlab-91	1991	WL 711/CROW "S"	Punjab, One CIMMYT parent	182
13.	Marwat	2001	WL 711/HD 2169//GHSK'S'	Punjab No CIMMYT parent	165,182
14.	Chakwal-86	1986	FORLANI/ACC//ANA or Fln/ACS//ANA	Punjab Segregating line or population CIMMYT	182
15.	Barani-83	1983	BB/GLL/3/GTO/7C//BB/CNO67	Punjab CIMMYT Segregating line or population	165
16.	FD-83	1983	FURY//KAL/BB	Punjab CIMMYT Segregating line or population	165
17.	FD-85	1985	MAYA/MON/KVZ/TRM	Punjab	165
18.	Bhittai	2004	VEE/TRAP//SOGHAT-90 or VEE/TRAP#1//SOGHAT90	Sindh	165
19.	Kohistan-97	1997	V- 1562//CHRC'S'/HORK/3/KUFRA-I/4/CARP'S'/BJY'S'	Punjab One CIMMYT parent	188
20.	Sulaiman-96	1996	F6.74/BUN//SIS/3/VEE#7 or F6-74/BUN//SIS/3/VEE#7	NWFP, CIMMYT Advanced line	165
21.	Rawal-87	1987	MAYA/MON//KVZ/TRM	Punjab CIMMYT Advanced line	165
22.	Marghalla	1999	OPATA/BOW'S'	Punjab CIMMYT Advanced line	188
23.	Wafaq-2001	2001	OPATA/RAYON//KAUZ	Punjab Advanced line CIMMYT	174
24.	Sarsabz	1986	BY/MAYA/4/BB//HD832.5.5/ON/3/CNO67/PJ62 or PITIC-62/FROND //MEXIPAK/3/PITIC-62/MAZOE-79-75-76	Sindh, CIMMYT Segregating line or population	188, 165
25.	Sindh-81	1981	No info	Sindh	165
26.	Mehran-89	1991	KVZ/BUHO//KAL/BB	Sindh CIMMYT Segregating line or population	165
27.	ABADGAR-93	1996	CNO SIB/NO/3/C273//NP875/PI SIB/4/HD1981	Sindh, No CIMMYT parent	165
28.	Kiran-95	1996	WL 711/CROW"S"	Sindh, one CIMMYT parent	188
29.	Khirman	2006	ULC/PVN//TAN/3/BUC	Punjab	165
30.	Soghat- 90	1991	PAVON MUTANT-3	Sindh, One CIMMYT parent	165

Table 1. (Cont'd.).

Sr#	Genotype	Year of release	Pedigree	Origin	Alleles (bp)
31.	Marvi-2000	2002	CMH-77A917/PKV 1600//RL6010/6*SKA	Sindh, No CIMMYT parent	165
32.	Iqbal-2000	2000	BURGUS/SORT 12-13//KAL/BB/3/PAK 81	Punjab, One CIMMYT parent	165
33.	Lu-26	1976	BLS/KHUSHAL	Punjab One CIMMYT parent	196
34.	Nishtar	1995	No info	NWFP	165
35.	Tatara	1996	JUP/ALD'S'//KLT'S'	NWFP CIMMYT Advanced line	165
36.	Bhakkar- 2000	2000	No info	No info	196
37.	Saleem-2000	2000	CHAM6//KITE/PGO	Punjab CIMMYT Advanced line	165
38.	Fakhr e Sarhad	1998	PFAU'S'/SERI//BOW'S'	NWFP	196
39.	Takbeer	2000		NWFP	165
40.	C-518	1933	T9 x 8A	Punjab	196
41.	C-591	1933	T9/8D or T9 X 8A	Sindh	182
42.	AS-2002	2002	KHP/D31708//CM74A370/3/CNO79/4/RL6043/4*NAC or	Punjab No CIMMYT parent	174
43.	SH-2002	2002	INQALAB-91/FINK'S'	Punjab one CIMMYT parent	196
44.	Yecora	1975	CNO67//SON64/KLRE/3/8156	Punjab CIMMYT Advanced line	174
45.	SA-42	1971	C271//LR64/SN64	Punjab One CIMMYT parent	174
46.	Auqab-2000	2000	CROW'S'/NAC//BOW'S'	Punjab One CIMMYT parent	174
47.	Punjab-96	1996	SA42*2/4/CC/INIA//BB/3/INIA/HD832 or SA42 *2/3/CC/INIA4// BB/4/INIA/HD832	Punjab One CIMMYT parent	205
48.	Chakwal-97	1997	BUC'S'/FCT'S'	Punjab CIMMYT Advanced line	205
49.	Pasban-90	1990	INIA F 66/TH.DISTICHUM//INIA F 66/3/GENARO T 81 or INIA F 66/ A.DISTCHUM//INIA66/3/GEN	Punjab	174
50.	Pavan	1976	VCM//CNO/7C/3/KAL/BB	Punjab CIMMYT Segregating line or population	174
51.	Chenab-70	1970	No info	Punjab	182
52.	Sehar-06	2006	CHILL/2 STAR/4/BOW//BUC/PVN/3/2*VEE#10	Punjab CIMMYT Advanced line	174
53.	Shafaq	2006	LU 26/HD 21790/ 2*INQALAB 91	Punjab	205
54.	Bhakkar-2002	2002	No info	Punjab	215
55.	Rohtas-90	1991	INIA F 66/TH.DISTICHUM//INIA F 66/3/GENARO T 81 or INIA F 66/ A.DISTCHUM//INIA66/3/GEN	Punjab CIMMYT Advanced line	174
56.	Sandal-73	1973	CNO67//SN64/KLRE/3/8156	Punjab CIMMYT Segregating line or population	205
57.	Bakhtawar-93	1993	JUP/BJY//URES	Punjab	174, 205
58.	Zardana	1993	CNO67/8156//TOB66/CNO67/4/NO/3/12300//LR64A/8156/5/PVN or CNO67/ 8156//TOB 66/CNO67/4/NOROESTE F66/3/12300//LR64A/8156/5/PVN	CIMMYT Advanced line	174
59.	Raskoh	2005	Kauz/Yaco//Kauz		174
60.	Zarlashta	1999	URES/BOW'S'	CIMMYT Advanced line	174

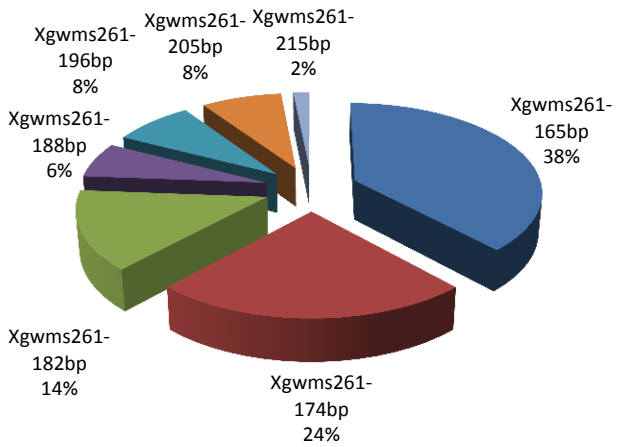

Fig. 1. Percentage distribution of detected alleles for the microsatellite locus Xgwm261 in the Pakistani wheat genotypes.

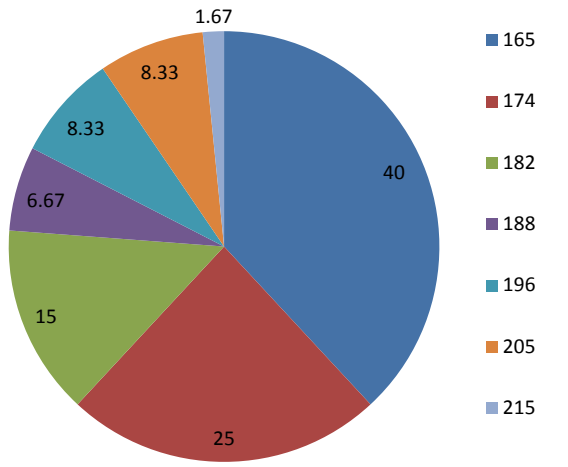

Fig. 2. Percentage of Pakistani wheat genotypes carrying different alleles at microsatellite locus Xgwm261.

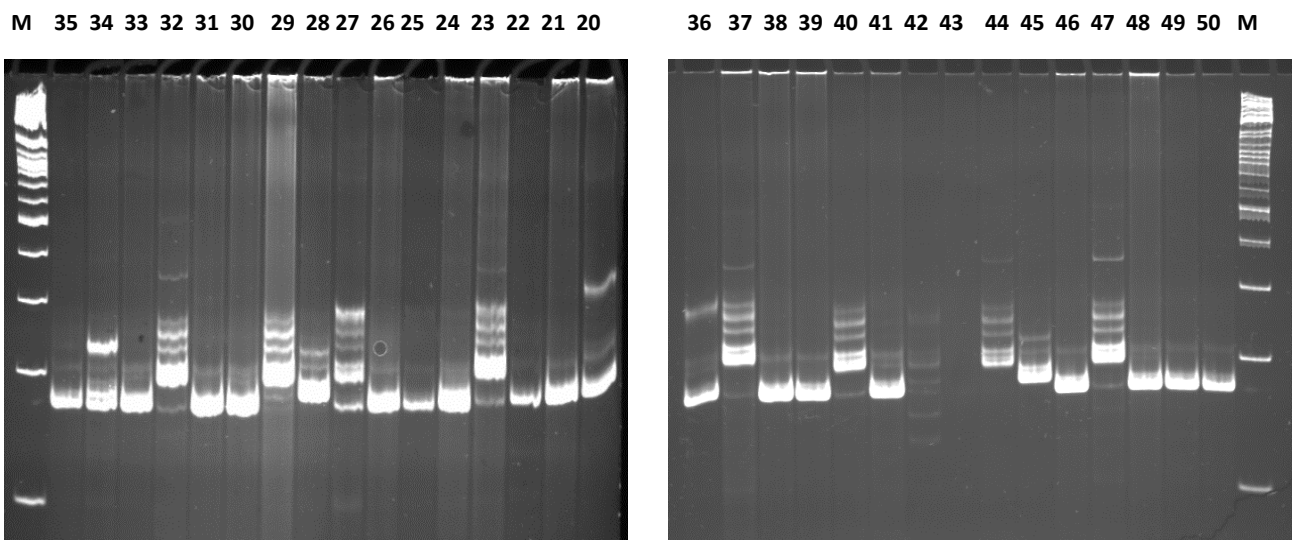

Fig. 3. Xgwm261 maker amplification showing the presence of different allelic variants of *Rht8* in Pakistani wheat germplasm. M= I Kb DNA ladder, lane 20-50 wheat genotypes. Number is in accordance with table 1.

Discussion

Molecular identification of genes involved in crop plant improvement has progressed in modern breeding era (Doebley *et al.*, 2006; Burger *et al.*, 2008). Genetic diversity of world germplasm is routinely being explored to get the insights about the role of genes in plant domestication from traditional to modern agriculture (Iqbal *et al.*, 2009; Khalid & Hameed, 2017). Remarkably increased production of the staple crops i.e. rice, maize and wheat has been achieved after introduction of dwarfing genes during the Green Revolution (Taiz, 2013). The semi-dwarfing wheats have been considered to increase yield through height reduction and lodging resistance. *Rht8* mainly decreases the plant height up to 15.7% (16.0 cm) by shortening peduncle length (Wang *et al.*, 2015). DNA based marker analysis has been extensively used to identify and classify these genes worldwide. The genetic diversity at the microsatellite locus Xgwm261 has been an important diagnostic tool for genotyping the *Rht8* gene and Xgwm261-192 bp allele has been proposed as a diagnostic marker for *Rht8* (Ahmad & Sorrells, 2002; Bai *et al.*, 2004; Ganeva *et al.*, 2005; Liu *et al.*, 2005; Zhang *et al.*, 2006). Worland *et al.*, (1998) explained the role of dwarfing gene *Rht8* in 20th century wheat improvement and geographical distribution of the microsatellite locus Xgwm261 alleles along with their linkage relationship to a photoperiod responsive gene and plant height. The gene *Rht*8 from the Japanese cultivar of the Akakomugi wheat is of the greatest significance for breeding (Gale & Youssefian, 1985). It reduces straw length by ca 10 cm and is widespread among the Yugoslavian cultivars of common wheat (Worland *et al.*, 1998). Grover *et al.*, (2018) reported a 13.3% reduction in plant height due to *Rht8* gene as compared to lines lacking this gene. Presence of allele that provides the product of 192-bp was related to the decrease in plant height by about 7-8 cm (Kowalczyk, 2006). Also starters for marker WMS261 of the gene *Rht*8 generate polymorphic products with the sizes of 165-bp, 174-bp and 192-bp. Correlation has been reported between the presence of bands of certain length and reduction in plant height. The 165 and the 174-bp alleles are associated with a 10 and 8 cm height increase respectively, as compared to the 192-bp allele diagnostic for *Rht8*.

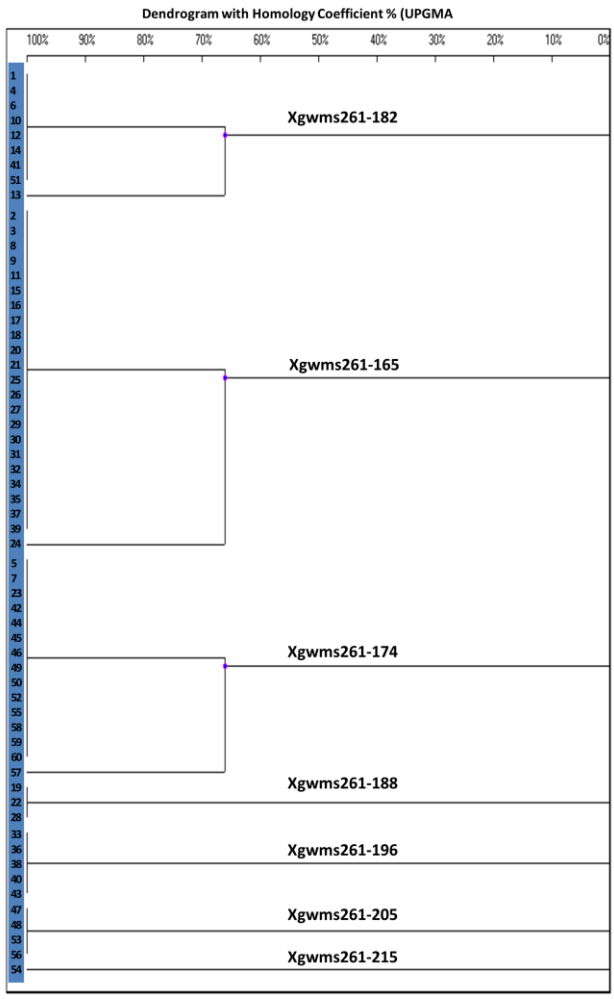

Fig. 4. Dendrogram showing clustering of Pakistani wheat varieties based on allelic distribution at microsatellite locus Xgwm261. Number is in accordance with table 1.

Presently, we analyzed the genetic diversity regarding distribution of allelic variants for *Rht8* gene and molecular analysis revealed diverse nature for this locus in Pakistani wheat germplasm. Majority of Pakistani wheat genotypes were found to carry the 165-bp allele with higher frequency (Table 1). In this connection, it has been reported that the varieties originating before Green Revolution era mostly contained the alleles other than 165-bp while the varieties with Mexcian origin were having the alleles of 165-bp (Worland *et al*., 2001). Overall, majority of the studied Pakistani wheat genotypes carried 165-bp (40%) and 174-bp alleles (25%) with the highest frequencies (38 and 24% respectively) that have been previously described using different sets of wheat germplasm across the globe. Worland *et al*., (1998) categorized the wheat varieties into 3 main groups on the basis of presence of 165-bp fragment (28 lines), 174-bp fragment (25 lines) or the 192-bp fragment (58 lines). Later Worland *et al*., (2001) further analyzed the allelic variability for Xgwm261 locus by the SSR marker in 800 wheat cultivars from twenty countries and reported that 165-bp and 174-bp fragments occur most frequently. The similar was also recorded for present study using Pakistani wheat germplasm. Our results also coincide with the finding of Ahmad and Sorrells, who reported the predominance of 165-bp and 174-bp alleles in the hard red winter cultivars of the US while screening 71 wheat cultivars from 13 countries. In that study, highest allele frequency was observed for a 174-bp fragment (52.11%) followed by a 165-bp fragment (26.76%).

Although Ganeva *et al*., (2005) reported the presence of 165- and 174-bp alleles mostly in Bulgarian cultivars developed by hybridization with foreign cultivars, however, unlike our results only few cultivars were found to have 174-bp allele. Ganeva *et al*., (2005) explained that all cultivars, released before 1960 involved crosses with local landraces carried the Xgwm261 174-bp allele suggesting a selective advantage of this allele as a result of breeding for shorter and more productive genotypes. Earlier, Zheleva *et al*., (2006) studied the wheat germplasm from different gene banks of Bulgaria and described that 174-bp and 164-bp allele variants were observed in majority of wheat varieties. While analyzing the 95 German wheat cultivars using WMS 261 marker (Knopf *et al*., 2008) also observed that about 90% of the studied cultivars had 165-bp and 174-bp fragments. Likewise, Weigt *et al*., (2013) also reported 165-bp and 174-bp alleles the most frequently occurring alleles. Similar to our results, recently Salem, (2015) reported the dominance of Xgwm261-165-bp and Xgwm261-174 bp alleles in Egyptian wheat genotypes with 65.52 and 24.14% respectively. The predominance of the Xgwm261-165 bp allele in the Pakistani bread wheat genotypes in present study suggested that there might be selection for that allele in breeding program. Dry growing conditions, short growing season or other environmental factors might be responsible for that preferential selection. As Xgwm261-174 bp allele is associated more with shorter plant height than the Xgwm261-165 bp allele that may be preferable in areas where ample soil moisture cause lodging problem.

CIMMYT wheat cultivars carry the Xgwm261-165 bp allele while Great Britain, French and German wheat genotypes mostly carry the Xgwm261-174 bp allele (Worland *et al*., 1998). CIMMYT green revolution-wheat improvement programs are the cause of *Rht8* gene spread (Borlaug, 1968). South American cultivar ÔMentanaÕ (carry 165 bp) also being directly used in breeding early CIMMYT varieties was also introduced into Brazil and crossed with Brazilian landraces to develop ÔFrontanaÕ and later ÔMaringaÕ which carry the same WMS 261-165-bp allele. ÔFrontanaÕ were crossed with ÔNorin 10/Brevor 14Õ (carry 174-bp allele) to develop early-maturing semi-dwarf CIMMYT varieties for their worldwide programs of germplasm improvement. All early CIMMYT varieties carry the 165-bp allele and CIMMYT wheats crossed with Russian varieties which lack the microsatellite allele diagnostic for *Rht8* but carry the 165-bp allele suggesting an adaptive significance for this allele in CIMMYT germplasm. The presence of the higher frequency of Xgwm261-165 bp allele in the Pakistani wheat genotypes suggests introduction of this gene from CIMMYT wheat germplasm indicating that wheat breeders subsequently use CIMMYT wheat material in their breeding programs. This may be the reason for highest frequency of Xgwm261-165 bp allele in tested Pakistani wheat germplasm.

In this investigation we could not detect the diagnostic marker allele for *Rht8* (Xgwm261-192 bp) suggesting that our germplasm lack any ancestor having this allele. Similar to present findings, 192-bp fragments could not be detected in any of the 95 German cultivars using similar WMS261 marker (Knopf *et al*., 2008). Similarly, 192-bp allele was not detected in any of the New Zealand and Australian cultivars, however, among 41 analyzed USA cultivars only one cultivar 'Pioneer Var 2510' had 192-bp allele specific to *Rht8* (Ahmad & Sorrells, 2002). In literature, mostly Italian, Russian and Yugoslavian wheat genotypes were found to carry Xgwm261-192 bp allele (Worland *et al*., 1998). Contrary to our finding there are several reports for higher frequency of *Rht8* (Xgwm261-192 bp) allele in Ukrainian (Chebotar *et al*., 2001), US & Chinese (Bai *et al*., 2004), Australian (Schmidt *et al*., 2004), Bulgarian and Belgian (Ganeva *et al*., 2005; Zheleva *et al*., 2006), Chinese (Liu *et al*., 2005; Zhang *et al*., 2006), Croatian (Dvojković *et al*., 2010) and Slovakia and Czech Republic (Šíp *et al*., 2010) wheat materials indicating the adaptive significance of the corresponding allele of *Rht8* gene in these geographical regions.

In the present varietal screen, we have also detected some less common minor alleles i.e., 182-bp, 188-bp and 196-bp including two alleles over 200-bp i.e., 205 and 215-bp (Table 1). Worland *et al*., 1998 also found 7 varieties carrying novel bands with size more than 200-bp as observed in present study. Previously, 4 new alleles in the US and New Zealand wheat cultivars (180-bp, 198-bp, 200-bp and 204-bp) have been reported (Ahmad & Sorrells, 2002). Korzun *et al*., (1998) also detected novel fragments at the WMS261 locus with fragments of 201-bp, 202-bp, 210-bp and 215-bp in six varieties. Some of these alleles (bp) have also been found in Turkish cultivars (Worland *et al*., 2001). It has been reported that the old cultivars carried rare alleles (211 and 215-bp) at Xgwm261 locus that probably trace back to the local landraces as they observed 203-bp allele in 6 modern cultivars (Ganeva *et al*., 2005). Zheleva *et al*., (2006) demonstrated that a limited number of varieties and advanced breeding lines carried 194-bp, 196-bp and over 200-bp. Similar to our results, Asplund *et al*., (2012) also found few accessions (Canadian and German) with a 182-bp allele in their varietal screen. The presence of the rare alleles could possibly be due to seed exchange practice from possible donors. Further, there is possibility that the novel allelic variants with size over 200-bp at the *Rht8* locus might be responsible for unique phenotype that can be used in breeding programs.

In present investigation most of the varieties (95%) exhibited homologues pattern for tested locus with exception of few varieties being heterozygous with more than one WMS 261 allelic variant (Fig. 3). Similar to our findings, high percentage (90%) of homozygosity for WMS 261 locus has been reported (Schmidt *et al*., 2004). They reported that six varieties (25%) carry two or more of the major alleles but it was not clear whether this results from heterozygosity within individual seeds, or from heterogeneity of breeding stocks (Schmidt *et al*., 2004). Even after sequencing the alleles, it remained unclear whether heterozygosity represents a separate allelic lineage or is simply the result of replication error. In another study, Worland *et al*., (1998) also observed heterozygoity however; they did not include those varieties for further analysis. They described that screening individual seed from sample varieties within this group indicated that the pooled DNA used in the original screen carried more than one fragment due to a heterozygous stock indicating either residual heterozygosity in the original variety or contamination problems during stock maintenance. Further, studies may be conducted to get insights into sequence variations leading to observed heterozygosity in present study.

In the present investigation Gatersleben wheat microsatellite Xgwm261 locus analysis showed a PIC value of 0.55 which was almost comparable with previous results (Botstein *et al*., 1980; Salem, 2015) who reported a PIC value > 0.5. These results confirm the significance of this highly informative marker for analyzing the diversity of *Rht8* alleles.

Conclusion

In the aforesaid discussion it is clear that Pakistani wheat has great diversity regarding *Rht8* locus. The present analysis of presence and frequency of *Rht8* gene at DNA level will be helpful in characterizing Pakistani wheat genotypes for accurate selection of parents for wheat breeding program. However, detailed studies are needed to determine the correlation of observed alleles at the Xgwm261 locus with specific effects on plant height and other related agronomic traits. Furthermore, it will also be worth full to study the contribution of uncommon alleles and to investigate the reasons of heterogeneity.

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