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ASSESSMENT OF GENETIC DIVERSITY AND VALIDATING RUST RESISTANCE GENE SOURCES USING MOLECULAR MARKERS IN WHEAT (*Triticum aestivum* L.)

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SUMMARY

Seven hundred ninety-three microsatellite markers were employed for polymorphism, of which 104 markers were identified as polymorphic and were used for assessing genetic diversity in 6 wheat genotypes. Two hundred seventy-eight alleles were identified across the genotypes. Alleles per marker varied from 2 to 5 with an average of 2.67 per locus. Cluster analysis revealed maximum genetic distance (GD = 0.843) between FLW13 and FLW20, depicting their distant relatedness, while cultivars HS240 and HS295 showed least genetic distance (GD = 0.624) suggesting their relatedness. The rust resistance genes present in FLW20 (*Lr*19) and HS424 (*Lr*24) were validated using SCAR markers $SCS265_{512}$ and $SCS1302_{607}$, respectively and in FLW13 (*Yr*15) using microsatellite marker X*gwm* 273. These genotypes showed high genetic diversity and good agronomic features proving their usefulness in wheat improvement program.

Key words: wheat, genetic diversity, molecular markers, rust resistance

Key findings: Gene sources, FLW13, FLW20, HS424 would prove as useful donors for developing improved rust resistance wheat breeding materials.

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INTRODUCTION

Genetic diversity in crop species is essential to breed buffered genotypes capable to withstand under biotic and abiotic stress conditions. Morphological traits can be used to characterize genetic diversity, but are often influenced by the environmental factors (Spanic *et al*., 2012). Today, a variety of DNA markers have been used for estimation of genetic diversity in wheat. Among DNA markers, simple sequence repeats (SSR's) are suitable markers for dissecting genetic diversity particularly in cereals and appear to be more informative in wheat than any other marker system as these markers show high polymorphism, co-dominant inheritance and good reproducibility (Plaschke *et al*., 1995, Roeder *et al*., 1995; Ma *et al*., 1996; Bryan *et* *al*., 1997). Though co-dominant markers SSR's are preferred, yet dominant markers can also be employed successfully for marker assisted selection following simultaneous screening for disease reaction and genotyping (Chhuneja *et al*., 2011).

About 73 *Lr* genes for leaf rust resistance and about 67 *Yr* genes conditioning resistance against stripe rust have been documented (McIntosh *et al*., 2013, personal communication). The *Agropyron elongatum* derived *Lr*19 and *Lr*24 can play an important role for building rust resistance gene pools in Indian wheat against leaf rust. The gene *Lr*24 deployed in Central India through the cultivar HW 2004 has been providing durable resistance to leaf rust for more than a decade (Revathi *et al*., 2010). The wheat variety Kurinji (*Lr*19) in Southern Hills Zone; Kaushambli (*Lr*19) and HW 2034 (*Lr*28) in Northern Eastern Plains Zone showing freedom from leaf rust are the best examples of strategic deployment of race specific alien leaf rust resistance genes, which have helped in curtailing the disease spread in the country (Tomar and Menon, 2001). The evolution of stripe rust pathotype (pt) 49S119 virulent on *Yr*2 and *Yr*9 (Prashar *et al*., 2007) and new variant 78S84 virulent on *Yr*27 has forced the plant breeders to re-orient their breeding strategies to avoid stripe rust epidemics in the Indian Sub-continent. The *Triticum dicoccoides* derived *Yr*15 (Gerechter-Amitai *et al*., 1989) can effectively be used to cope up the threat posed by these new variants of stripe rust. Therefore, information on gene sources of rust resistance and genetic diversity status is most vital for developing pre-breeding potential lines and or high yielding rust resistant varieties in Northern India. Finally, the strategic use of resistance would not only reduce the initial inoculum but would also discourage the selection of new pathotypes (pts). The gene specific rust resistant wheat varieties so developed and deployed would not only reduce cost of cultivation spent on fungicides but would also save environment from pollution and its hazardous effects. The research reported in this paper is a step in this direction.

MATERIALS AND METHODS

Materials

Seven hundred ninety-three microsatellite markers available at Division of Genetics, IARI, New Delhi, were employed for detecting polymorphism in wheat genotypes. One hundred and four polymorphic markers were used for assessing genetic diversity in 6 wheat genotypes *viz*., HS240, HS295, FLW20, HS424, FLW13, Avocet *Yr*15 (pedigree details given in Table 4).

Methods

Amplification profile of SSR markers was recorded with each band representing a different allele, with a particular primer pair. Alleles were calculated on the basis of presence of band (scored as 1) and its absence (scored as 0) for generating binary matrix which was further used to calculate for Jaccard's similarity coefficient for each pair of parents following NTSYS-PC program (Rohlf, 2000). A dendrogram was constructed using genetic similarity matrix (GS). Cluster analysis using an un-weighted pair group method with arithmetic averages (UPGMA) was used to group the genotypes. Genetic diversity index value was calculated as: (1- GS). Genetic distances (GD = 1-GS) were calculated for paired comparison of genotypes (Zhuang *et al*., 2011). Polymorphic information content (PIC) was calculated using the formula suggested by Anderson *et al*., 1993. PIC = $1 - \sum p^2 i$, where *pi* is the frequency of i^{th} allele of each SSR marker. PIC value ranged from 0 to 1. The PIC value 0 is an indication for a marker that has only one allele whereas, PIC value 1 indicating to indefinite number of alleles. It provides useful information about allele diversity and allele frequency among the cultivars. A PIC value > 0.7 is considered as highly informative for distinguishing the genotypes and establishing genetic relationship among them.

The SCAR markers $SCS265_{512}$ (Gupta *et al*., 2006a) and $SCS1302_{607}$ (Gupta *et al*., 2006b), were used to validate FLW20 and HS424 as carrier of *Lr*19 and *Lr*24, respectively. The stripe rust resistance gene *Yr*15 present in

FLW13 and Avocet (Avst-*Yr*15) was validated using SSR molecular marker Xgwm 273 (Peng *et al*., 2000). DNA was extracted by CTAB method as earlier suggested (Rogers and Bendich, 1985). PCR amplification with the SCAR primer for *Lr*19 and *Lr*24 was performed in a 25µl reaction mixture containing 10mM Tris-HCL (pH 8.8), 50mM KCl, 2mM $MgCl_2$, 0.1mM of each dNTPs, 0.75 U *Taq* DNA Polymerase, 22 ng of each of SCAR primers and 40 ng genomic DNA. PCR amplification was performed in a Thermal Cycler, Model-PTC-200 (MJ research, San Fransisco, USA) programmed for 2 min at 95 ºC, 35 cycles [94 ºC 1 min, 60 ºC 1 min for *Lr*19 and 61ºC 1 min for *Lr*24, 72 ºC 1 min] followed by final extension for 7 min at 72 ºC. PCR amplification reaction for SSR primers was performed in a 20µl reaction mixture containing 50-100 ng template DNA, 0.2 mM of each dNTPs, 10 mM Tris-HCL (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.25 mM of each forward and reverse primers and 1U of *Taq* DNA Polymerase. PCR amplification was programmed for 4 min at 94 ºC; 35 cycles [94 ºC 30 s, 55-60 ºC 30 sec depending upon the annealing temperature of SSR, 72 ºC 30 s] followed by final extension for 7 min at 72 ºC. The amplified products were separated on 3.5% high resolution agarose gel, stained with Ethidium Bromide and visualized in gel documentation system.

Seedling rust resistance test

Two wheat cultivars HS240, HS295 (susceptible to leaf and stripe rusts), 4 genetic stocks, FLW20 except pt 253R31, HS424 (resistant to leaf rust and susceptible to stripe rust), FLW13, Avocet *Yr*15 (resistant to stripe rust and susceptible to leaf rust), along with sets of differentials were raised in aluminum trays containing a mixture of loam soil and farm yard manure in the ratio of 3:1. The trays were kept in a spore proof glass house having 12-16 hours day light and watered regularly to keep the soil moist for proper germination of seeds. Seven day-old seedlings were inoculated with urediniospores of leaf rust pt121R63-1 and stripe rust pts 46S119 and 78S84 as per the method suggested by Stakman*et al*., 1962. The inoculated seedlings were atomized with fine mist of water and placed in dew chambers for 48 hrs at 15$\pm$2 ^{0}C temperature for stripe rust, 20$\pm$2 ^{0}C for leaf rust with 12-16 hours daylight for germination of spores and initiation of infection. The seedlings were then shifted on to the glass house benches till recording of infection types (IT). The seedling reaction against 121R63-1, 46S119 and 78S84 pts was recorded after a fortnight according to Stakman *et al*., 1962. The seedlings under seedling resistance test (SRT) showing infection type 0; (naught fleck), ; (fleck), ;1, and ;2 were classified as resistant whereas, infection type 3, 33+ and 3+ were categorized as susceptible. Agronomic observations on 10 randomly selected plants were recorded for growth class (Grw-Cls: SS- semi spreading, SE-semi erect), ear length (Ear-Lt), spikelets /ear (Spkt/ear), plant height (Pt. Ht.), ear color (Ear-Clr), awn length (Awn-Lt), maturity class (Mat-Cls: ML- medium late, ME- medium early), thousand grain weight (TGW) and grain yield/plant (GY/Pt.) (Table 4).

RESULTS AND DISCUSSION

Genetic diversity analysis using SSR markers

Seven hundred ninety-three microsatellite markers were used for polymorphic survey, of which 104 markers were polymorphic for distinguishing the genotypes. Diversity analysis showed the presence of wide genetic diversity among the genotypes. A gel picture depicting allelic variation among the genotypes is represented in Figure 1.

A total of 278 alleles were amplified across 6 genotypes and the number of alleles per marker varied from 2-5 with an average of 2.67 per locus (Table 1). A similar pattern of allelic variation was also observed earlier Salem *et al*., 2008 in 7 wheat varieties using satellite markers. The PIC values (Table 1) for different loci ranged from 0.03 (Wmc611) to 0.83 (Xgwm582) with an average value of 0.45. It was 0.72 to 0.83 for markers Xgwm582, Wmc219, Xgwm11, Xbarc193, Xgwm437, Xgwm369, Wmc70 and Cfd29 and hence, considered as informative for distinguishing the genotypes and establishing genetic relationship among them. PIC values are in agreement with the findings of Banyai *et al*., 2006.

The UPGMA cluster dendrogram (Figure 2) showed 2 major clusters (A & B). Cluster A comprised of 5 genotypes *viz*., HS240, HS295, HS424, Avst-*Yr*15 and FLW13 while cluster B has 1 genotype FLW20. The cluster A is further subdivided into 2 subgroups (A_1 & A_2). Subgroup A_1 possessed wheat cultivars *viz*., HS240 and HS295 and subgroup A_2 possessed 3 genotypes namely HS424, Avst-*Yr*15 and FLW13. Genetic distance was highest (GD = 0.843) among FLW13 and FLW20, depicting their distant relationship, whereas it was lowest (GD = 0.624) among wheat cultivars HS240 and HS295, suggesting their relatedness, which might be due to repeated selection for similar agronomic and grain yield attributes or due to sharing common pedigrees (Table 2). However, the available pedigree details of these varieties do not share common parents, but these varieties may have been derived from common donors at initial stages of their development (Table 4). The genetic stock FLW20 was the most distinct among the parents used in the present study.

Validation of rust resistance stocks through host-pathogen interaction and molecular markers

The genetic stocks (FLW20, HS424 and FLW13) were validated for resistance against most virulent leaf and stripe rust pts *viz*., 121R63-1, 46S119 and 78S84 through host-pathogen interaction (Table 3). However, the conventional host-pathogen based gene matching technique alone is not effective for identifying plant genetic resources carrying rust resistance genes especially when differential host pathogen is not available. Therefore, SCAR markers $SCS265_{512}$ and $SCS1302_{607}$ were employed and proved effective for validating the presence of *Lr*19 and *Lr*24 in FLW20 and HS424, respectively. The SCAR markers $SCS265_{512}$ and $SCS1302_{607}$ were polymorphic among the carrier and non- carrier genotypes and a typical 512 bp fragment in FLW20 (*Lr*19) (Figure 3a) and 607 bp fragment in HS424 (*Lr*24) (Figure 3b) were amplified. STS markers for identification of *Lr*19 and *Lr*24 in different genetic backgrounds have also been reported by Singh *et al*., 2004. In Avst -*Yr*15, the microsatellite marker Xgwm273 gave typical band (185bp) and the same base-pair band was seen in FLW13, confirming the presence of *Yr*15 gene in this genetic stock (Figure 3c). The source genotype (FLW13) has given 185bp band instead of 156bp product (Peng *et al*., 2000) which might be due to variation in repeat units of SSR marker.

Stripe rust resistance genetic stocks, FLW13 and Avst-*Yr*15 were resistant against pts 46S119 and 78S84, whereas, FLW20 carrier of *Lr*19 and HS424 carrier of *Lr*24 were resistant to pt. 121R63-1. HS240 and HS295 were susceptible to leaf and stripe rusts. However, it is important to mention that the wheat variety HS240 render stripe rust resistance to pt. 78S84. Validation of the genetic stocks carrying effective rust resistance genes with good agronomic backgrounds (Table 4) using conventional host-pathogen based gene matching technique and molecular markers offers opportunities for utilizing them in multiple gene pyramiding for rust resistance. These type of studies to validate the presence of rust resistance genes with molecular markers have also been conducted by other workers (Singh *et al*., 2003; Singh *et al*., 2004; Datta *et al*., 2011). The leaf rust resistance gene *Lr*24 known to be linked to *Sr*24 gene for resistance to stem rust, is apparently effective against all the races of stem rust (Knott, 1989) and in India the virulent pathotype is restricted to Nilgiri hills only (Bhardwaj *et al*., 2006). Another leaf rust resistance gene *Lr*19 linked to stem rust resistance gene *Sr*25 and also enhancing the grain yield by 10% (Singh *et al*., 1998) would play an important role if pyramided with stripe rust resistance gene *Yr*15 in wheat improvement program to combat all the 3 rusts. Involving FLW13, FLW20 and HS424 in hybridization is expected to yield transgressive segregants for developing improved rust resistant wheat varieties for Indian Sub-continent. The rust resistance present in FLW20, HS424 for prevalent pts of leaf rust and in FLW13 for stripe rust could be strategically utilized in wheat improvement program for developing improved wheat varieties for North-Western Himalaya, not only to reduce initial inoculum for Indo-Gangetic plains but also to discourage the selection of new virulent pathotypes.

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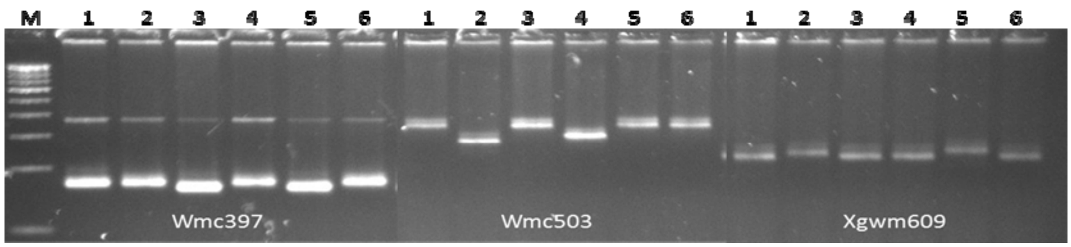

Figure 1. Representative amplification profile of SSR markers for six wheat genotypes: 1. HS240; 2. HS424; 3. FLW20; 4. FLW13; 5. Avst-*Yr*15; 6. HS295.

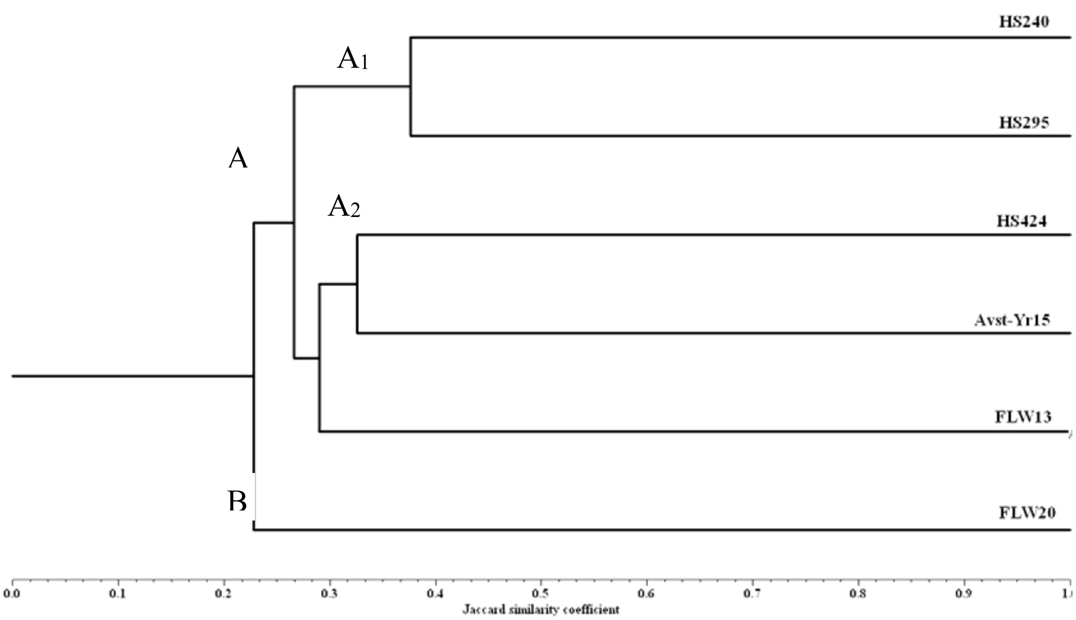

Figure 2. Dendrogram depicting genetic relationship among 6 genotypes of wheat using Jaccard's similarity coefficient.

Table 1. SSR marker, chromosome location, number of alleles & polymorphism information content (PIC).

Marker	Ch. Location	No of alleles with PIC	Marker	Ch. Location	No of alleles with PIC	Marker	Ch. Location	No of alleles with PIC
Xgwm372	2A	2 (0.50)	Wmc503	2D	3(0.50)	Xgwm533	3B,3D	2(0.19)
Xgwm275	2A	2(0.64)	Xgwm539	2D	3(0.50)	Barc109	4B,5B	3(0.25)
Xgwm339	2A	2(0.19)	Xgwm484	2D	2(0.28)	Cfa2070	5B	2(0.44)
Xgwm249	2A, 2D	2(0.50)	Wmc112	2D	2(0.28)	Xgwm499	5B	4(0.31)
Xgwm356	2A, 6A, 7A	4(0.36)	Cfd43	2D	4(0.47)	Xgwm443	5B	4(0.72)
Wmc407	2A	2(0.50)	Cfd44	2D	2(0.06)	Wmc75	5B	2(0.28)
Wmc170	2A, 2D	2(0.28)	Cfd51	2D	3(0.61)	Wmc773	5B,6D	3(0.25)
Wmc453	2A, 2B, 2D	4(0.39)	Wmc805	5A,5D	2(0.28)	Wmc397	6B	2(0.44)
Wmc455	2A	2(0.44)	Xgwm459	6A	3(0.50)	Xgwm219	6B	3(0.50)
Cfd62	3A,7A, 2D, 3D	2(0.44)	Wmc363	6A	2(0.28)	Xgwm626	6B	2(0.50)
Cfa2170	3A,3B,1D	2(0.44)	Wmc753	6A,6D	2(0.28)	Wmc396	7B	2(0.50)
Cfa2037	3A	2(0.28)	Cfa2019	7A	2(0.28)	Wmc758	7B	2(0.50)
Cfa2183	3A	2(0.44)	Wmc809	7A	3(0.61)	Xgwm333	7B	2(0.28)
Wmc388	3A,5A,6A, 7A, 6B	3(0.42)	Wmc479	7A	2(0.44)	Xgwm577	7B	3(0.50)
Xgwm369	3A, 4B	4(0.72)	Cfa2257	7A, 7B	3(0.61)	Wmc70	7B	2(0.72)
Xbarc193	3A,4B	3(0.75)	Wmc83	7A, 2B,6B	3(0.61)	Wmc323	7B	2(0.28)
Xgwm247	3A,3B	4(0.17)	Xbarc121	7A, 6D,7D	2(0.06)	Xgwm111	7B, 7D	5(0.11)
Wmc269	3A,1B	3(0.67)	Wmc273	7A,7B, 7D	2(0.44)	Cfd61	1D	2(0.28)
Wmc532	3A	3(0.67)	Xgwm11	1B	2(0.78)	Wmc609	1D	2(0.44)
Xbarc170	4A	4(0.22)	Xgwm582	1B	3(0.83)	Wmc85	1D	3(0.61)
Xgwm350	4A	2(0.44)	Xgwm268	1B	4(0.58)	Cfd27	1D	3(0.33)
Wmc219	4A	5(0.78)	Wmc406	1B	3(0.69)	Cfd55	3D	2(0.28)
Cfa2173	4A,6A,4D	2(0.28)	Cfa2147	1B,1D	2(0.44)	Xbarc71	3D	4(0.64)
Wmc48	4A,4B,4D	3(0.50)	Wmc419	1B,4B, 6B	2(0.28)	Wmc720	4D	2(0.44)
Wmc89	4A,4B	2(0.44)	Xgwm604	1B,5B	3(0.61)	Xgwm624	4D	2(0.64)
Wmc617	4A,4B,4D	5(0.47)	Cfa2278	2B	2(0.28)	Xgwm190	5D	2(0.28)
Xgdm14	4A,7A,5B,6B,1D,2D, 6D	2 (0.44)	Xgwm501	2B	3(0.61)	Xgwm174	5D	3(0.61)
Wmc491	4A,4B	3(0.53)	Wmc317	2B	2(0.50)	Cfd18	5D	2(0.50)
Cfa2190	5A	3(0.06)	Wmc154	2B	3(0.61)	Cfd29	5D	4(0.72)
Wmc654	5A	4(072)	Wmc332	2B	3(0.61)	Cfd42	6D	3(0.50)
Xgwm186	5A	3(0.61)	Cfd143	3B	2(0.19)	Wmc634	7D	3(0.67)
Xgwm639	5A	4(0.36)	Xgwm340	3B	3(0.42)	Xgwm437	7D	3(0.75)
Xbarc56	5A	2(0.44)	Xgwm493	3B	2(0.28)	Wmc45	7D	2(0.44)
Wmc524	5A	2(0.44)	Xgwm181	3B	3(0.19)	Wmc611	1A,1B	3(0.03)
Cfd19	1D,5D, 6D	2(0.53)	Wmc78	3B	3(0.42)			

PIC value is given in parenthesis.

Table 2. SSR markers based genetic similarity matrix of 6 wheat genotypes.

Genotype	HS240	HS424	FLW20	FLW13	Avst-*Yr*15	HS295
HS240	-					
HS424	0.299(0.701)	-				
FLW20	0.223(0.777)	0.238(0.762)	-			
FLW13	0.247(0.753)	0.326(0.674)	0.157(0.843)	-		
Avst-*Yr*15	0.256(0.744)	0.313(0.687)	0.250(0.750)	0.267(0.733)	-	
HS295	0.376(0.624)	0.291(0.709)	0.272(0.728)	0.275(0.725)	0.226(0.774)	-

Genetic distance (GD) values are given in parenthesis.

Table 3. Seedling response of wheat to leaf and stripe rust.

Cultivar/ genetic stock	Seedling response to Leaf rust (IT)	Stripe rust (IT)	
	pt121R63-1	pt 46S119	pt78S84
HS240	S	S	R
HS295	S	S	S
FLW20	R	S	S
HS424	R	S	S
FLW13	S	R	R
Avst-*Yr*15	S	R	R

IT= Infection type, R= Resistant, S= Susceptible

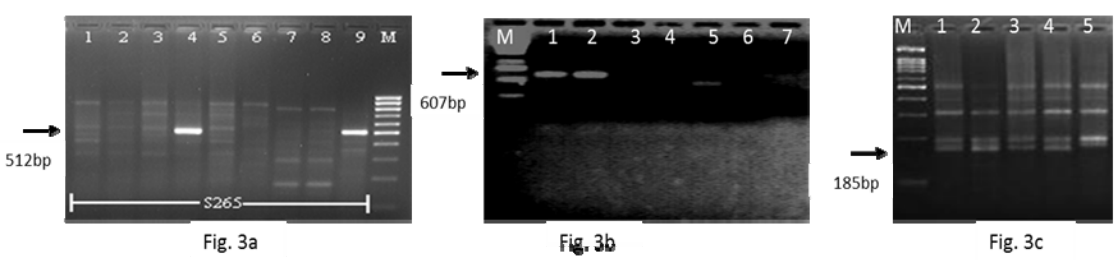

Figure 3a. M: Marker; 1: HS424; 2: HS295; 3: FLW13; 4: FLW20; 5: HS420; 6: HS240; 7: FLO; 8: H-86; 9: Cook+*Lr19*

Figure 3b. M:Marker lane; 1.HS424; 2.*Lr*24; 3.FLW20; 4.*Lr*19; 5.FLW13;6.HS240; 7.HS295.

Figure 3c. M: Marker lane; 1. FLW30; 2. FLW13; 3. Avocet (S); 4. Avst -*Yr*15; 5. Agra local.

Table 4. Pedigree/cross details and agronomic features of wheat cultivars and genetic stocks.

Genotype	Pedigree/Cross	@ Agronomic features								
		Grw- Cls	Ear-Lt (cm)	Spkt/ ear	Pt. -Ht	Ear- Clr	Awn- Lt (cm)	Mat-Cls	TGW (g)	GY/Pt. (g)
FLW13	WH542/Avocet-*Yr*15	SS	11.7	22.6	104.9	Cream	5.02	ML	42	8.0
FLW20	PBW 343/ Agatha=T4=Tc+*Lr*19//FLW6	SE	13.9	20.8	102.1	White	3.39	ML	44	5.9
HS424	CPAN3004/HPW(DL)30/ HS286	SE	10.4	16.4	110.6	Brown	5.89	ME	38	8.8
HS240	BOW “S”/PAVON “S”	SS	12.1	21.4	106.2	Cream	6.26	ML	36	8.4
HS295	CQT/AZ//IAS55/ALD/3/ ALD/ NAFN/4/PJN/PEL SL	SE	10.4	17.6	95.8	Cream	6.63	ME	46	10.6

@ Abbreviated in the text.

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