RAPD Analysis of Stripe Rust Resistant Synthetic Hexaploid of Wheat

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Abstract

In this study 33 lines of synthetic hexaploid (SH) of wheat were used for RAPD analysis. These 33 SH were found resistant for stripe rust during the two years of screening at seedling and adult plant stage in field and glasshouse. Total 50 primers were screened and 10 primers of OPA series which were giving the reproducible band were selected for the study of genetic diversity. The result showed the stripe rust resistant synthetic hexaploid of wheat are highly diverse. A total of 108 bands were generated by the ten selected primers, 88 of which were polymorphic. Maximum level of polymorphism (93%) which was observed for the primers OPA-01 while minimum level of polymorphism (36%) was observed by OPA-19 primers. Cluster analysis using the unweighted pair group method of arithmetic averages (UPGMA) clustered the 33 lines into two groups. Eight lines did not form cluster with any of the genetypes and found to be very diverse. These lines can be used by the plant breeders of the country to improve the stripe rust status of the susceptible wheat varieties.

Key Words. Stripe rust, synthetic hexaploid RAPDs marker

1. Introduction

Stripe rust, caused by *Puccinia striiformis f. sp. tritici*, is one of the major limitations for wheat production throughout the world as it is damaging to the wheat grain (Singh *et al*., 2000).. More efforts have gone in fighting rusts than any other disease because of the destructive nature of the rust disease. Cultivars are known to loose their resistance after short period following their release. (Kilpatrick, 1975).

Pakistan is the 8th largest wheat producer, more than 40% of the cultivated area (8.307 million hector) is covered by wheat crop, with an average production of 2,615 kg/hectare (Jamali et al., 2007). Although during the year 2006-07 a bumper crop was harvested (23.5 million tons) but the average production is quite low in comparison with other agricultural countries due to lot of biotic and a-biotic stresses. Like many countries Stripe rust, caused by *Puccinia striiformis f. sp. tritici*, is one of the major limitations for wheat production in Pakistan.Thus production and supply of transgenic plants resistant to biotic and abiotic stresses is an essential element for sustainable production. Increased production will significantly contribute to food supply for the rapidly increasing population as well as in the foreign exchange earning for the country.

To date more than sixty stripe rust resistant genes at different loci have been designated and mapped to different wheat chromosomes However, most of these genes are race specific and have become ineffective in a period of time when extensively used in production due to appearance of different virulent races (Daolin et al., 2009).
Although fungicides are available for fighting against the stripe rust pathogen, but the use of fungicide is known to cause many health problems and burden on the economy of developing countries like Pakistan. Growing wheat varieties with stripe rust resistant genes seems to be best strategy for fighting against stripe rust. (Chen et al., 2003). Best strategy is to look for the new genes in three wheat gene pools and of these a focus on the primary gene pool holds priority. (Mujeeb-Kazi and Hettel, 1995). Many agronomically important genes have been introgressed from the wheat gene pools.

Knowledge of genetic diversity among the elite germplasm with high disease resistance and high yield capacity , has a significant impact on the improvement of wheat crop Molecular markers provide the best estimate of genetic diversity since they are independent of the confounding effect of environmental factors. When the genes are introgressed from the other varieties , the potential of successful marker identification is great, as conventional plant phenotype selection is a time consuming. Molecular markers are powerful tools to identify gene of interest and can provide a spectacular improvement in the efficiency and sophistication of plant breeding The development of molecular markers for mapping resistance genes to stripe rust and of marker-assisted selection has been among the most active areas of research on stripe rust. Molecular markers have been identified for most of the stripe rust resistant genes. Depending upon the closeness of markers to the genes, these markers should be useful in marker-assisted selection. Most of the markers were identified using RAPD, SSR, and AFLP techniques.
The main objective of this study was to identify the most genetically diverse stripe rust resistant synthetic hexaploid (SH) of wheat, which can be used by the plant breeders.

2. MATERIAL AND METHODS

2.1 Plant materials/wheat germplasm

Five to ten seeds of 33 synthetic hexaploid of wheat, which were found resistant at seedling and adult plant stages (Sumaira et al., 2007) (Table 1)during the two year of the study were sown in plant growth chamber at the Crop Disease Research Institute, Islamabad, under controlled conditions of temperature, humidity and light. Young leaf tissue was harvested for DNA extraction.

2.2 RAPD Analysis

Genomic DNA of 33 synthetic hexaploid of wheat was extracted from fresh leaves, according to the method described by (Weining and Langridge, 1991).. The quality and concentration of extracted DNA were estimated with a spectrophotometer. For RAPD analysis, a total of 50 screened primers were used for polymerase chain reaction (PCR) For each primer the PCR reaction was carried out in 25 ul reaction volume containing 50-100 ng of total genomic DNA template, 0.25 uM of each primer, 200 uM of each dNTPs, 50 mM NH_4SO_2 buffer, 1.5 mM $MgCl_2$ and 2.5 units of Taq DNA polymerase. The amplification condition were as follow; initial step of denaturation for 1 minute at 94^{o}C followed by 50 cycles of each consisting of a denaturation step of 1 minute at 94^{o}C, an annealing step of 1 minute at 34^{o}C and extension step of 2 minutes at 72^{o}C. Seven minutes were given after the last cycle to the extension step at 72^{o}C to ensure the completion of the primer extension reaction. GeneAmp PCR system 2700 was used for all amplification reactions.

The PCR products were electrophoresed on 3% agarose gels containing 7 µL ethidium bromide, at 80 V for 1 h, and observed under a UV trans illuminator.DNA amplifications with each RAPD primer were repeated at least twice to ensure reproducibility. The bands were considered scorable only after observing and comparing them in two separate amplifications for each primer. Bands were counted and the presence and absence of bands were scored as 1 and 0, respectively. The data were collected and aligned for the construction of cluster analysis. The cluster analysis of 33 synthetic hexaplois of wheat was performed using the POP Gen software version 1.32 (Yeh et al., 2000) to determine genetic diversity and similarity among accessions.

3. Result

A total of 50 RAPD primers of were initially tested. All of them produced amplifications but out 50 tested primers only 10 primers of OPA series generated polymorphic banding patterns. So 10 primers, of OPA series OPA-01,OPA-04,OPA-05,OPA-09,OPA-10,OPA-14,OPA-15,OPA-18,OPA-19, and OPA-20 were selected for the analysis of genetic diversity on the basis of polymorphism obtained. Different primers showed variation in their ability to detect polymorphism..A total of 108 bands were generated by the ten selected primers, 88 of which were polymorphic (Table 2). The number of fragments for these primers ranged between 8 and 15 with size ranging from 250bp to 2500bp. Individual primers, within the set of 20 used in this study, resolved varying degrees of polymorphism. Maximum level of polymorphism (93%) which was observed for the primers OPA-01 while minimum level of polymorphism (36%) was observed by OPA-19 primers.

3.1 Genetic diversity and clustering pattern of 33 synthetic hexaploid of wheat based on RAPD data

Multivariate analysis was conducted to generate a similarity matrix using Nei and Li,s (1979) coefficient to estimate genetic diversity and relatedness among 33 synthetic hexaploid wheats which were resistant during the two years of screening. Genetic similarity coefficients grouped the 33 synthetic hexaploid of wheat into 2 clusters (Figure 1). Cluster A include 26 synthetic hexaploid wheats genotypes The cluster B include 7 genotypes . Coefficients ranged from 94% to 62% (Table 3).The most similar lines were 68.111/RGB-U//WARD.ESEL/3/STIL/4/AE.SQUARROSA(385) and DOY1/AE.SQUARROSA(534) (94%) while 68.111/RGB-U//WARD/3/AE.SQUARROSA(511) and STY-US/CETA//PALS/3/SRN_5/4/AE.SQUARROSA(502 were most genetically dissimilar (62%).

4. Discussion

In this study we used 33 lines of synthetic hexaploid (SH) of wheat which were found resistant to stripe rust caused by *Puccinia striiformis* at the both stages of their growth. The main objective was to assess RAPD diversity among SH found resistant for stripe rust. RAPD analysis revealed polymorphism between the SH genotypes. RAPDs have been used for a variety of purposes, including the construction of genetic linkage maps (Reiter et al. 1992) gene tagging, identification of cultivars (Nybom, 1994) assessment of genetic variation in populations and species (Nesbitt et al., 1995), study of phylogenetic relationships among species, subspecies and cultivars (Landry et al., 1994), and for many other purposes in a large number of plant species including wheat. These applications have led to the development of species-specific (Chen et al., 1998), genome-specific and chromosome-specific markers (Wang et al., 1995) and, more importantly, to the development of molecular markers for identification and selection of the desired genotypes (for a variety of traits of economic importance) in segregating populations during breeding programmes. For the efficient use of RAPDs markers identified, these markers are converted to PCR based markers. This strategy involves the identification of polymorphic and reproducible bands generated in RAPD assay, cloning the fragment, sequencing the clone and design the PCR primers. RAPD markers have shown to be associated with various traits such as leaf rust resistance gene *Lr28* in wheat (Naik et al., 1998), various traits contributing to kernel hardness in bread and cadmium intake in durum wheat (Penner et al., 1995).

In this study we screened 50 primers and use only 10 RAPDs primers of the operone series for the study of genetic diversity Out of 50 primers screened, the capability of theses 10 primers to generate RAPDs markers ranged from 10 to 32 genotypes. Range of scorable bands was from 250 bp to 2500 bp. The degree of genetic polymorphism detected by the primers ranged from 36% to 93 % indicating that these synthetic lines are genetically diverse and possess polymorphism. Out of 10 OPA primers only very only 5 primers gave reproducible bands.

Lintott et al, (1998) used 1100 primers and found only one (UBC868) with reproducible bands. Autreque et al, (1995) used 296 primers and found no primer with reproducibility. In a study Fahima et al, (1999) screened more than 100 primers and identified only 10 primers which yielded reproducible and polymorphic information. The problem of poor reproducibility believed to be associated with the large genome size and the high proportion of repetitive DNA characteristic of the wheat genome (Devos and Gale, 1992; Joshi and Nguyen, 1993; Koebner, 1995).

In the present study RAPD primers amplified between 10-20 band however, all of them were not scorable only 8 and 10 bands per genotype were scorable.. It is well documented in wheat and in other plant species, that not all of the amplified RAPD bands are scorable and useful as markers. This problem may be, in part, due to the relatively low resolving power of the agarose gels commonly used for RAPD analysis (Penner 1996). He et a.l, (1992) observed a higher level of scorable and reproducible polymorphisms in wheat when PCR samples were subjected to denaturing gradient- gel electrophoresis. Penner and Bezt, (1994) observed more scorable bands using temperature sweep gel electrophoresis (TSGE) more as compared to agarose gels.

The genotypes were found in two major clusters, but within the clusters high sub-clustering was observed. The high sub-clustering formed in this study indicated high genetic variability. The mean similarity indices for the 33 SHs ranged from the 62% to 94%, indicating medium to high polymorphism at the DNA level among the resistant accessions. This medium to high level of polynorphsim could be due to the different accession of of *Ae.squarosa* which were used for crossing with the durum wheat.

In this study 8synthetic hexaploid of wheat (DOY 1/AE.SQUAROSA(333), CETA/AE.SQUARROSA(1031), DOY1/AE.SQUARROSA(1027), SCA/AE.SQUARROSA(518), ALTAR 84/AE.SQUARROSA (192), GARZA/BOY//AE.SQUAROSA(311), CETA/AE.SQUARROSA(1024), and 68.111/RGB-U//WARD/3/AE.SQUARROSA(511) were separated from others and did not fall into any cluster.
These lines were found highly diverse as compared to other genotypes. These lines can be used in for further molecular analysis using SSRs and can be used in the development of mapping population for the estimation of number of stripe rust resistant genes and can be use by wheat breeders in breeding programmes.

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Table 1 Lines of synthetic hexaploid wheats from Elite I, and II found resistant to stripe rust at the seedling and adult plant stages

No.	accession No.	Genotype
Synthetic Hexaploid Wheats: Elite I		
1	2	DOY1/AE.SQUARROSA (188) *
2	3	ALTAR 84/AE.SQUARROSA (192)
3	5	ALTAR 84/AE.SQUAROSA(198)
4	27	GARZA/BOY//AE.SQUAROSA(311)
5	32	DOY 1/AE.SQUAROSA(447)
6	34	DOY1/AE.SQUARROSA (511)
7	35	68.111/RGB-U//WARD/3/AE.SQUARROSA(511)
8	42	YAR/AE.SQUARROSA(783)
9	47	68.111/RGB-U//WARD/3/FGO/4/RABI/5/ AE.SQUARROSA(882)
10	52	ALTAR 84/AE.SQUARROSA (BANGOR)
11	62	SCA/AE.SQUARROSA(518)
12	73	GAN/AE.SQUARROSSA(897)
13	76	FALCIN/AE.SQUAROSA(312)
14	79	DOY 1/AE.SQUAROSA(333)
15	80	DOY1/AE.SQUARROSA (428)
16	81	68.111/RGB-U//WARD/3/AE.SQUARROSA(452)
17	87	SCA/AE.SQUARROSSA(409)
18	88	CPI/GEDIZ/3/GOO//J069/CRA/4/AE.SQUARROSA(409)
19	89	STY-US/CETA//PALS/3/SRN_5/4/AE.SQUARROSA(502)
20	90	ALTAR 84/AE.SQUARROSA (502)
21	91	CROC-1/AE.SQUARROSA(517)
22	92	CETA/AE.SQUARROSA(1024)
23	93	DVERD-2/AE.SQUARROSA(1027)
24	94	CETA/AE.SQUARROSA(1027)
1	9	STYUS/CELTA//PALS.3/SRM_5/4/AE.SQUARROSA(431)
2	11	SKARV_2/AE.SQUARROSA(304)\
3	13	DOY1/AE.SQUARROSA(1027)\
4	17	CPI/GEDIZ/3/GOO/JO/CRA/4/AE.SQUARROSA(1018\
5	18	CETA/AE.SQUARROSA(1031)
6	30	68.111/RGB-U//WARD RESEL/3/STIL/4/AE.SQUARROSA (385)
7	31	CETA/AE.SQUARROSA(417)
8	32	68.111/RGB-U//WARD RESEL/3/STIL/4/AE.SQUARROSA (431)
9	33	DOY1/AE.SQUARROSA(534)

* = *Ae. tauschii* accession entry in the Wheat Wide Crossing Programmes working collection at CIMMYT, Mexico. *Ae. squarrosa* is syn *Ae. tauschii* or *T. Tauschii*

Table 2 Polymorphism exhibited by RAPD primers in stripe rust resistant synthetic hexaploid wheats

Sr. No	Primer	5' to 3'	Total loci	Polymorphic loci	%polymorphism
1	OPA-01	CAGGCCCTTC	15	14	93%
2	OPA-04	AATCGGGCTG	13	12	92%
3	OPA-05	AGGGGTCTTG	6	4	66%
4	OPA-09	GGGTAACGCC	10	10	100%
5	OPA-10	GTGATCGCAG	9	6	66%
6	OPA-14	TCTGTGCTGG	11	9	81%
7	OPA-15	TTCCGAACCC	11	10	90%
8	OPA-18	AGGTGACCGT	11	8	72%
9	OPA-19	CAAACGTCGG	11	4	36%
10	OPA-20	GTTGCGATCC	11	10	90%
			108	88	

Table 3 RAPD similarity metrix for the stripe rust resistance genotypes

ID	2/95	3/95	5/95	27/95	32/95	34/95	35/95	42/95	47/95	52/95	62/95	73/95	76/95	79/95	80/95	81/95	87/95	88/95	89/95	90/95	91/95	92/95	93/95	94/95	9/33	11/33	13/33	17/33	18/33	30/33	31/33	32/33	33/33
2/95	0	0.7553	0.7766	0.7447	0.7447	0.7021	0.7234	0.7660	0.7660	0.8404	0.8404	0.8511	0.8511	0.8085	0.8511	0.8511	0.8511	0.8085	0.7766	0.7872	0.8085	0.8298	0.8085	0.8404	0.8723	0.7872	0.8298	0.8298	0.8511	0.8404	0.8511	0.8723	0.8404
3/95		0	0.7021	0.6915	0.6489	0.6489	0.6489	0.7766	0.7340	0.7872	0.6809	0.7340	0.7553	0.7128	0.7766	0.7766	0.7766	0.7553	0.7234	0.7128	0.7128	0.6915	0.7128	0.7447	0.7979	0.6915	0.7128	0.7340	0.7553	0.7660	0.7766	0.7553	0.7447
5/95			0	0.6915	0.6489	0.6489	0.6277	0.6915	0.7766	0.7660	0.7021	0.7340	0.7766	0.7128	0.7340	0.7340	0.7340	0.7340	0.6383	0.6915	0.7340	0.7128	0.7340	0.7447	0.7766	0.7128	0.7766	0.7340	0.7553	0.7447	0.7553	0.7766	0.7447	
27/95				0	0.7021	0.5957	0.6170	0.7234	0.7021	0.7766	0.7128	0.7447	0.7447	0.7447	0.7447	0.7660	0.7660	0.7660	0.7340	0.7021	0.7447	0.7234	0.7021	0.7128	0.7234	0.6596	0.7234	0.7234	0.7234	0.7128	0.7660	0.7872	0.7340		
32/95					0	0.7021	0.6809	0.6596	0.6596	0.7128	0.7553	0.7234	0.7447	0.7021	0.7660	0.7872	0.7447	0.7234	0.7128	0.7021	0.7447	0.7234	0.7447	0.6915	0.7021	0.6809	0.7234	0.7447	0.7234	0.7766	0.7872	0.7872	0.7553		
34/95						0	0.7021	0.6596	0.6383	0.6489	0.7128	0.7234	0.7021	0.6596	0.7872	0.7447	0.7234	0.6596	0.6277	0.7021	0.7447	0.7447	0.6809	0.7128	0.7021	0.6170	0.7021	0.7234	0.6809	0.7553	0.7447	0.7447	0.7128		
35/95							0	0.6383	0.5957	0.6702	0.6702	0.7234	0.7021	0.6596	0.7447	0.7234	0.7021	0.7447	0.6277	0.6596	0.6596	0.7021	0.7021	0.6702	0.6596	0.7021	0.7447	0.7021	0.7021	0.7128	0.7234	0.7447	0.7553		
42/95								0	0.7021	0.7340	0.6915	0.7447	0.7660	0.7234	0.7872	0.7872	0.7872	0.7660	0.7128	0.7447	0.7447	0.7234	0.7447	0.7766	0.7660	0.7021	0.7447	0.7660	0.7447	0.7553	0.7660	0.7872	0.7766		
47/95									0	0.7340	0.6915	0.7021	0.7447	0.7234	0.7234	0.7234	0.7447	0.7447	0.6915	0.6809	0.7021	0.7021	0.7021	0.7766	0.7872	0.6596	0.7234	0.7234	0.7447	0.7553	0.7447	0.7660	0.7553		
52/95										0	0.7872	0.7979	0.7979	0.8191	0.8191	0.7766	0.7979	0.7979	0.7660	0.7553	0.7553	0.8191	0.7766	0.7660	0.8617	0.7979	0.7553	0.7766	0.7766	0.8085	0.8191	0.8191	0.8085		
62/95											0	0.7979	0.7553	0.7553	0.7766	0.7766	0.7979	0.7340	0.7021	0.7553	0.7766	0.7553	0.7766	0.7234	0.7766	0.7128	0.7553	0.7766	0.7340	0.7660	0.7766	0.7979	0.7660		
73/95												0	0.8085	0.7660	0.8085	0.7872	0.8511	0.7872	0.6915	0.7660	0.7872	0.7872	0.7660	0.7766	0.8298	0.8085	0.8298	0.8298	0.7660	0.7979	0.8085	0.8511	0.7979		
76/95													0	0.7660	0.8085	0.8085	0.7872	0.7660	0.7553	0.7447	0.8085	0.8085	0.7872	0.7553	0.7872	0.7447	0.7872	0.8298	0.8085	0.8191	0.8298	0.8511	0.7979		
79/95														0	0.7872	0.7872	0.8085	0.8085	0.7553	0.7660	0.7660	0.8085	0.8085	0.7979	0.8085	0.7447	0.7660	0.7872	0.7872	0.8404	0.8298	0.8298	0.8191		
80/95															0	0.8298	0.8085	0.7872	0.7766	0.7872	0.8511	0.8085	0.7872	0.7979	0.8085	0.7234	0.8085	0.8298	0.8298	0.8191	0.8298	0.8511	0.8404		
81/95																0	0.8298	0.7872	0.7766	0.7660	0.7872	0.7872	0.8085	0.8191	0.8298	0.7447	0.7660	0.8085	0.7872	0.8191	0.8723	0.8511	0.8191		
87/95																	0	0.8085	0.7128	0.7872	0.8085	0.7872	0.7872	0.8191	0.8511	0.7234	0.8085	0.8085	0.7872	0.8191	0.8298	0.8511	0.8191		
88/95																		0	0.7553	0.7447	0.7447	0.7660	0.7872	0.7766	0.8298	0.7021	0.8085	0.7660	0.7660	0.7979	0.8085	0.8085	0.7979		
89/95																			0	0.7340	0.7340	0.7553	0.7553	0.7234	0.7340	0.7340	0.6702	0.6915	0.7340	0.7234	0.7553	0.7553	0.7447		
90/95																				0	0.7872	0.7234	0.7021	0.7553	0.7872	0.7021	0.7447	0.7447	0.7447	0.7766	0.7872	0.7660	0.7766		
91/95																					0	0.8298	0.7660	0.7766	0.7660	0.6809	0.7872	0.8085	0.7447	0.7766	0.8085	0.8085	0.8191		
92/95																						0	0.8085	0.7979	0.7872	0.7447	0.7660	0.8085	0.7447	0.7766	0.7872	0.8085	0.8191		
93/95																							0	0.7979	0.7872	0.7021	0.7447	0.8085	0.7660	0.7979	0.7872	0.8085	0.7979		
94/95																								0	0.8404	0.6915	0.7553	0.7553	0.7766	0.8085	0.7766	0.8191	0.8298		
9/33																									0	0.7872	0.7872	0.7872	0.8085	0.8191	0.8298	0.8298	0.7979		
11/33																										0	0.7447	0.7660	0.7021	0.7766	0.8085	0.8085	0.7553		
13/33																											0	0.8298	0.7872	0.7766	0.7872	0.8511	0.7979		
17/33																												0	0.7872	0.7979	0.8085	0.8511	0.8191		
18/33																													0	0.8191	0.7872	0.8298	0.7766		
30/33																														0	0.9255	0.8830	0.8085		
31/33																															0	0.8936	0.8191		
32/33																																0	0.8830		
33/33																																	0		

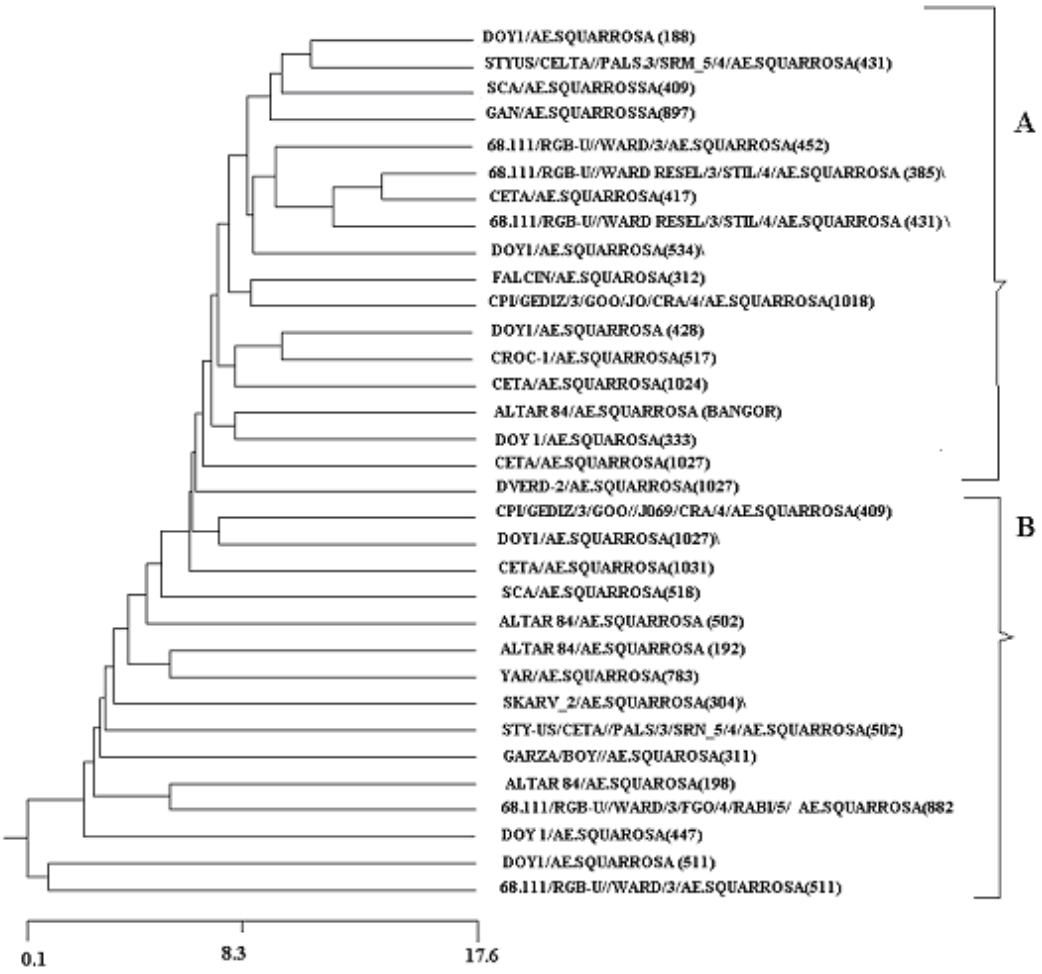

Figure 1 Clustering patterns revealed by 33 stripe rust resistant genotypes of synthetic hexaploid wheats using RAPD data.