

IDENTIFICATION OF GENETIC POLYMORPHISM AND DNA METHYLATION PATTERN IN WHEAT (*Triticum aestivum* L.)

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Received: 02.08.2011

ABSTRACT

SSR, RAPD, ISSR and SRAP markers have been used to examine the genetic and epigenetic diversity of wheat (*Triticum aestivum* L.) germplasm. PCR amplification of the DNA isolated from six wheat genotypes yielded a total of 2118 amplified products, of which 1105 were polymorphic and 1013 were monomorphic. The average PIC value for all molecular marker data was 0.8704 and average percentage of polymorphism was 40.86%. All primers used for RAPD analysis, were also tested by CRED-RA analysis, a method to detect DNA methylation. There were differences between genetic and methylation polymorphism in the studied cultivars. Dendrogram created based on genetic polymorphism showed that PI178383 and Sonmez2001 (0.30) were genetically very different from each other, but in the dendrogram constructed by methylation polymorphism Sonmez2001 and ES14 (0.44) were grouped in one subcluster. Similar to this, in the dendrogram created based on epimarker data Izgi2001 and Harmankaya99 (0.59) were grouped in one subcluster as well as Sonmez2001 and ES14 (0.57) as in the second subcluster. The present study has provided the genetic data that diagnose the level of polymorphisms and epigenetic data which present natural variations of DNA methylation.

Keywords: CRED-RA- epimarker- genetic similarity – molecular markers

INTRODUCTION

Wheat (*Triticum aestivum* L.) is one of the most important food crops in the world, and understanding its genetics and genome organization using molecular markers is of great value for breeding purposes (Galovic *et al.* 2005). The rapidly evolving technology of DNA markers helps to open a real possibility for developing functional markers as reliable genetic markers for genetic improvement (Repkova *et al.* 2009). Genetic diversity is the basis for genetic improvement and knowledge of germplasm diversity has a significant impact on the improvement of crop plants. Due to modern breeding, it has been suggested that genetic diversity in wheat has been increasingly narrowed. Narrow genetic diversity is problematic in breeding for adaptation to biotic stresses, like diseases, and abiotic stresses, such as drought or salt tolerance. Therefore, it is necessary to investigate the genetic diversity in wheat germplasm in order to broaden the genetic variation in future wheat breeding. The use of molecular markers for the evaluation of genetic diversity is receiving much attention (Huang *et al.* 2002). Many scientists have studied genetic diversity in common wheat using different molecular markers such as RAPD (Random Amplified Polymorphic DNA) (Joshi and Nguyen 1993), RFLP (Restriction Fragment Length Polymorphism) (Kim and Ward 2000), AFLP (Amplified Fragment Length Polymorphism) (Barrett and Kidwell 1998), STS (Sequence Tagged Sites) (Chen *et al.* 1994) and ISSR (Inter Simple

Sequence Repeat) (Nagaoka and Ogihara 1997). PCR-based genotyping is favored over other techniques because it is fast, does not require radioactive labeling and can be carried out with very small amounts of genomic DNA. PCR-based genomic polymorphism has been detected in cultivated hexaploid wheat (*Triticum aestivum* L.) by microsatellite markers (Ma *et al.* 1996; Liu *et al.* 2005). ISSR markers are also useful to detect genetic polymorphism in this species (Zietkiewicz *et al.* 1994; Carvalho *et al.* 2005). Furthermore, one of the new types of molecular markers, SRAP (Sequence-Related Amplified Polymorphism) was developed and used in plant genetic studies (Li and Quiros 2001; Wang *et al.* 2005).

DNA methylation variability is important for artificial selection in breeding programs (Marfil *et al.* 2009). In higher plant genome, about 20%-50% cytosines are methylated, among which about 90% methylated sites lie in 'CpG' dinucleotide or 'CpNpG' trinucleotide (Xiao *et al.* 2006). Variation in DNA methylation can lead to alterations in chromatin structure and changes in gene expression. Furthermore, disturbance of intrinsic DNA methylation patterns may have structural and functional consequences to the organisms with this epigenetic code (Dong *et al.* 2006). Cytosine DNA methylation is an important epigenetic modification of the nuclear DNA of many eukaryotes, and plays essential roles in regulating gene activity and maintaining genome integrity (Selker 1997; Rangwala and

Richards 2004). Given that altered DNA methylation patterns in plants often transmit faithfully through organismal generations and, hence, potentially produce heritable new phenotypes, it is conceivable that epigenetic alleles (epialleles) in the form of altered DNA methylation patterns are important in genome evolution (Kalisz and Purugganan 2004; Rapp and Wendel 2005). Alternatively, identical alleles may take on different methylation states. Environmental and genetic perturbations induce the novel genetic and epigenetic changes that trigger methylation (Lukens and Zhan 2007). It has been recognised that the DNA methylation has significant implications in plant breeding (Tsaftaris and Polidoros 2000). In the past, genetic variation was considered only as a result of alterations in the primary nucleotide sequences due to mutations and/or gene recombination. However, it is now established that epigenetic system like DNA methylation could generate epigenetic variation in wheat (Shaked *et al.* 2001, Han *et al.* 2003). CRED-RA (Coupled Restriction Enzyme Digestion-Random Amplification) is a method to detect DNA methylation which is one of the main causes of epigenetic variations (Cai *et al.* 1996). It is used to detect methylation changes between different tissues or different stages of development after restriction digestion of DNA with methylation sensitive enzymes such as *HpaII* and *MspI* (Temel *et al.* 2008). This technique could be used for variation studies in between species and lines. Different studies on DNA methylation were conducted in wheat (Cantu *et al.* 2010), barley (Bednarek *et al.* 2007; Li *et al.* 2007b), maize (Kaeppeler *et al.* 1993), and rye (Ashikawa *et al.* 2001, Takata *et al.* 2005).

In this study RAPD, SSR, ISSR and SRAP markers were used to determine polymorphism between six wheat cultivars and CRED-RA was used to identify polymorphism associated with DNA methylation and epigenetic markers for six wheat cultivars. The aim of using these six cultivars was to improve the competitiveness of public wheat breeding programs through the intensive use of modern, particularly marker-assisted, selection technologies. In our previous studies, the F₂ individuals derived from cultivar crosses (PI178383 x Harmankaya99, Izgi2001 x ES14, Sonmez2001 x Aytin98) were screened for resistance to yellow rust at the seedling stage in greenhouses and adult stage in the field to identify DNA markers genetically linked to resistance (Senturk-Akfirat *et al.* 2010; Ercan *et al.* 2010). Epigenetic alleles or epialleles differ in the number or distribution of methylated nucleotides at specific gene sequences, and plants with different epialleles can exhibit distinct phenotypes. The present study has provided the genetic data that diagnose the high level of polymorphisms by using different types of PCR-based markers and epigenetic data which present natural variations of DNA methylation.

MATERIALS AND METHODS

Plant material and DNA isolation

Six homozygous bread wheat genotypes (three yellow rust-resistant cultivars: PI178383, Izgi01, Sönmez2001, and three yellow rust-susceptible cultivars: Harmankaya99, ES14, Aytin98) were obtained from the Anatolian Agricultural Research Institute, Eskisehir, Turkey. Leaf tissue samples

were collected, frozen in liquid nitrogen, and ground to a powder using the Retsch MM301 system. Extraction of the genomic DNA was carried out as described by Weining and Langridge (1991). DNA concentrations were measured with spectrophotometer and checked with 0.7% agarose gels. A portion of the DNA were diluted to 50 ng/μl with sterile double-distilled water for use in RAPD, SSR, ISSR, SRAP, CRED-RA analysis, the stock and diluted portions were stored at -20°C.

Molecular marker analyses

106 RAPD, 65 SSR, 8 ISSR and 18 SRAP primers were used for molecular analysis to assess genetic diversity and genetic distance among six Turkish wheat genotypes.

RAPD marker analyses

Totally 106 primers (92 OP primers generated by Operon Technologies and 14 UBC primers generated by British Columbia University) were used for screening wheat genotypes. PCR reaction volume was 25 μl, consisting of 800 nmol of primer, 0.2 mM each of the nucleotides, 2.5 mM MgCl₂, 1X PCR buffer, 0.625 units of *Taq*-DNA polymerase (Fermentas), and 100 ng of genomic DNA as a template. A typical PCR procedure was as follows: 3 min at 94°C, then 40 cycles of 1 min at 94°C, 1 min at 30 to 50°C (depending on the primer annealing temperature), 1 min at 72°C, and 10 min at 72°C. PCR products were separated on 2% agarose gel at 80 V for 2 or 3 h, and visualized under UV light.

SSR analyses

65 SSR primers described by Pestsova *et al.* (2000) were used and the PCR reaction volume was 25 μl, consisting of 400 nmol of forward and reverse primers, 0.2 mM each of the nucleotides, 2.5 mM MgCl₂, 1X PCR buffer, 0.625 units of *Taq*-DNA polymerase, and 100 ng of genomic DNA as a template. A typical PCR procedure was as follows: 3 min at 94°C, then 40 cycles of 1 min at 94°C, 1 min at 43-54°C (depending on the primer), 1 min at 72°C, and 10 min at 72°C. PCR products were separated on 2.5% agarose gel at 100 V for 3 or 4 h, and visualized under UV light.

ISSR and SRAP analyses

Totally 26 primers, 7 ISSR and 18 SRAP applied by Li *et al.* (2007b), were used and PCR amplifications with these primers were performed in the reaction mixture volumes of 25 μl, each containing 100 ng of genomic DNA, 1x PCR buffer, 2.5 mM MgCl₂, 200 μM of dNTP mixture, 0.4 μM of each primer, and 0.625 U *Taq* DNA polymerase. Reactions for ISSRs were performed in as follows; 3 min at 94°C, 1 min at 94°C, 1 min at 31°C to 57°C (depending on the annealing temperature), 1 min at 72°C for 40 cycles with 10 min final extension at 72°C before cooling to 4 °C. For SRAP analysis, reactions were conducted as follows; 5 min at 94°C, for first 5 cycles; 1 min at 94°C, 1 min at 35°C, 1 min at 72°C, then 1 min at 94°C, 1 min at 46°C to 51°C (depending on the annealing temperature), 1 min at 72°C for 35 cycles with 7 min final extension at 72°C before cooling to 4°C. All PCR products resulted from ISSR and SRAP analyses were separated on 1.5% agarose gel at 100 V for 2 to 3 h, and visualized under UV light.

CRED-RA analyses

CRED-RA analysis was performed according to the Cai *et al.* (1996) and Leljak-Levanic *et al.* (2004) to determine DNA methylation. In 100 µl reaction mixture, 2.5 µg genomic DNA was separately digested with 10 U/µl HpaII at 37°C and 10 U/µl MspI at 37°C for overnight, then heated to 70°C for 15 min to inactivate the enzyme according to manufacturers' recommendations. After checking digestion on 1% agarose gel, 2 µl of each the digested and undigested DNAs were amplified with 106 RAPD primers. Amplification and visualization conditions for CRED-RA are the same described for RAPD analysis.

Genetic similarity estimation and cluster analyses

The positions of reproducible RAPD, SSR, ISSR, SRAP and CRED-RA bands were scored and compared. Banding patterns were scored conservatively for a presence/absence (1/0) matrix for the different cultivars and the data were entered into a binary matrix as discrete variables ('1' for presence and '0' for absence of a homologous fragment). Only distinct, reproducible, well-resolved fragments were scored and the data were analyzed using MVSP 3.1 software (Kovach 1999). This software package was also used to calculate Jaccard similarity coefficients to construct a dendrogram by a neighbour-joining algorithm. Polymorphic information content (PIC) or average heterozygosity was calculated as per the formula of Roldan-Ruiz *et al.* (2000): $PIC = 2fi(1 - fi)$, where fi is the frequency of the amplified allele and " $1 - fi$ " is the frequency of null allele. Average heterozygosity (H_{av}) is obtained by taking the average of PIC values obtained for all the markers and it is calculated as $H_{av} = \sum [2fi(1-fi)] / N$. N is sample size and multiplex ratio ($MR = m + p/n$) for each assay was estimated by dividing the total number of bands (monomorphic- m , and polymorphic- p) amplified by the total number of assays (primer combinations employed- n), marker index ($MI = H_{av} \times MR$) was obtained by multiplying the average heterozygosity (H_{av}) with MR (Powell *et al.* 1996).

RESULTS

RAPD data

The results obtained herein refer to the amplification product produced by using 106 random decamer primers in six cultivars analyzed in this study. The polymorphic fragments generated by the PCR technique were confirmed from experiments carried out to obtain a genetic profile generated by each primer, in each genotype. A total of 1276 fragments with RAPD analysis were obtained, with molecular weights varying from 100 to 1500 bp. The amplification product resulted in totals of 1109, 1133, 1185, 1192, 1146, and 1129 fragments in PI178383, Harmankaya99, Izgi2001, ES14, Sonmez2001 and Aydin98 respectively. In evaluation of the band number, only those bands with enough intensity and difference in size from neighboring fragments were used. OPE9 primer was the most polymorphic, producing a total of 21 (100%) different bands, while OPB13 and OPM6 primers were the least polymorphic, producing only 1 (10%) different band. Most of the primers

generated polymorphic patterns which means that genetic polymorphism in this cultivar is generally high.

The distinguishable banding patterns for each genotype was obtained by 35 primers (33% of all tested RAPD primers) named OPA04, OPA05, OPA06, OPA09, OPA14, OPA15, OPA16, OPB03, OPB06, OPB11, OPB13, OPC01, OPC05, OPC07, OPE10, OPF14, OPG07, OPG11, OPH05, OPH07, OPJ14, OPJ15, OPK04, OPK11, OPK12, OPK15, OPK17, OPK20, OPL03, OPM05, OPM09, OPM18, UBC212, UBC320, UBC537. The number of primers which gave distinct pattern for genotypes were 13, 7, 7, 12, 12, 5 in PI178383, Izgi2001, Sonmez2001, Harmankaya99, ES14, and Aydin98 respectively (Table 1 and Table 2). The PIC value, a reflection of allele diversity and frequency among the genotypes, ranged from 0.37 (OPC15) to 0.95 (OPA03) with a mean of 0.88.

Table 1. Number of percentage of polymorphic loci across methods in six wheat cultivars

Cultivars	RAPD		SSR		ISSR		SRAP	
	No.	%	No.	%	No.	%	No.	%
PI178383	13	1.17	12	5.02	2	2.50	3	1.49
Izgi2001	7	0.59	12	4.98	2	2.47	5	2.14
Sonmez2001	7	0.61	13	6.05	-	-	1	1.40
Harmankaya99	12	1.06	6	2.55	-	-	4	1.86
ES14	12	1.01	4	1.69	-	-	3	1.40
Ayidin98	5	0.44	27	9.96	3	3.57	8	3.62

Table 2. Total number and percentage of polymorphic loci of six wheat cultivars

Cultivars	No. of polymorphic loci	Percentage of polymorphic loci
PI178383	30	10.18
Izgi2001	26	10.18
Sonmez2001	21	8.06
Harmankaya99	22	5.47
ES14	19	4.10
Ayidin98	43	17.59

SSR data

Sixty-five SSR markers, covering all 21 chromosomes of the hexaploid wheat genome with 1–4 SSR markers per chromosome, were used to characterise the genetic diversity of six winter bread wheat genotypes. Out of 65 SSR primers examined, 37 primers produced polymorphic fragments. A total of 450 fragments with SSR analysis were obtained, with molecular weights varying from 50 to 1200 bp. The amplification product resulted in totals of 239, 235, 241, 236, 215, and 271 fragments in PI178383, Harmankaya99, Izgi2001, ES14, Sonmez2001 and Aydin98 respectively. The distinguishable banding patterns for each genotype was obtained by 25 primers (38% of all SSR primers tested) named XGDM3, XGDM5, XGDM6, XGDM33, XGDM36, XGDM40, XGDM43, XGDM67, XGDM68, XGDM77, XGDM87, XGDM88, XGDM98, XGDM99, XGDM107, XGDM109, XGDM111, XGDM132, XGDM133, XGDM141, XGDM145, XGDM147, XGDM149, XGDM150, XGDM152. The number of primers which gave

distinct pattern for genotypes were 12, 12, 13, 6, 4, 27 in PI178383, Izgi2001, Sonmez2001, Harmankaya99, ES14, Aytin98 respectively (Table 1 and Table 2). All the loci used in this study were multiallelic, ranging from 2 (*Xgdm145* and *Xgdm153*) to 22 (*Xgdm125*) with an average of 6.92 alleles per locus. The PIC values ranged from 0.21 for the *Xgdm145* locus to 0.95 for *Xgdm125* with a mean of 0.77.

ISSR and SRAP data

Out of 7 ISSR primers examined, 6 primers produced polymorphic fragments. The size of ISSR bands was between 1500 to about 80 bp. Out of 18 SRAP primers examined, 15 primers produced polymorphic fragments. A total of 430 fragments with ISSR and SRAP analysis were obtained, with molecular weights varying from 2.50 to 0.30 kb. The distinguishable banding patterns for each genotype was

obtained by 16 primers (64% of all ISSR and SRAP primers tested) named ISSR1, ISSR11, ISSR25, ISSR32, SRAP2, SRAP5, SRAP6, SRAP7, SRAP8, SRAP19, SRAP20, SRAP21, SRAP26, SRAP27, SRAP28, SRAP31. The number of ISSR and SRAP primers which gave distinct pattern for genotypes were 5, 7, 1, 4, 3, 11 in PI178383, Izgi2001, Sonmez2001, Harmankaya99, ES14, Aytin98 respectively (Table 1 and Table 2). The number of percentage of polymorphic loci across methods and total number and percentage of polymorphic loci across genotypes were presented in Table 1 and 2 respectively. It can be seen from these that the highest number of polymorphic loci was in Aytin98 (43, 17.59%) while the lowest was ES14 (19, 4.10%). All the dendrogram confirm the consistency of data. The four molecular marker systems were compared on the basis of different criteria (Table 3).

Table 3. Comparative list showing various molecular markers information in evaluating genetic diversity of six *T. aestivum* cultivars

Primer	RAPD	SSR	ISSR	SRAP	Total
Number of primers used	106	65	7	18	196
Number of primers amplified polymorphic fragments	98	37	6	15	156
Total number of polymorphic bands	678	232	46	149	1105
Total number of monomorphic bands	598	218	57	140	1013
Total number of bands	1276	450	103	289	2118
Average percentage of polymorphism (%)	48.66	36.27	37.4	41.1	40.86
Average number of polymorphic bands/primer	6.4	3.6	6.6	8.3	6.23
Multiplex ratio(MR)-(n/T, total number of bands/primer)	12.03	6.92	14.71	16.05	12.43
Average heterozygosity (<i>Hav</i>)	-	0.7976	-	0.9369	-
PIC	0.8761	0.7727	0.8998	0.9330	0.8704
Marker index (MI) = <i>Hav</i> × MR	-	5.52	-	15.04	-

[(MR): multiple ratio, MR was estimated by dividing the total number of bands; (m): monomorphic and (p): polymorphic amplified by total number of assays, (n): primer combinations employed].

All ISSR loci used in this study were multiallelic, ranging from 6 (ISSR19) to 23 (ISSR22) with an average of 13.38 alleles per locus. All SRAP loci used in this study were also multiallelic, ranging from 11 (*SRAP1*) to 26 (*SRAP8*) with an average of 17.9 alleles per locus. The PIC value for ISSR analysis ranged from 0.80 (ISSR19) to 0.95 (ISSR22) with a mean of 0.90. The PIC value for SRAP analysis ranged from 0.89 (*SRAP1*) to 0.95 (*SRAP2*) with a mean of 0.90.

Phylogenetic analyses

The amplified fragments were scored for the presence and absence of fragments on the gel photographs and these fragments were compared among the genotypes. SSR, ISSR, SRAP and RAPD banding patterns were recorded on spreadsheets, which were used to determine Nei's (1978) gene diversity and to calculate Jaccard similarity coefficients for construction of dendrograms by a neighbour-joining algorithm using MVSP 3.1. software (Table 4).

The analyses were carried out using 1105 fragments obtained by the RAPD, SSR, ISSR and SRAP technique, producing the phylogenetic tree shown in Figure 1. This figure reveals that 'PI178383' constitutes a more peripheral

Table 4. Similarity coefficients among cultivars of *Triticum aestivum* generated through different primer (SSR, ISSR, SRAP, RAPD) data

	1	2	3	4	5	6
PI178383	1.00					
Harmankaya99	0.34	1.00				
Izgi2001	0.34	0.38	1.00			
ES14	0.36	0.39	0.48	1.00		
Sonmez2001	0.30	0.36	0.39	0.42	1.00	
Aytin98	0.32	0.30	0.40	0.45	0.41	1.00

branch, therefore being the most differentiated of the five genotypes. From the analysis of these data, a greater similarity was observed between Izgi2001 and ES14 (0.48), and a lesser one between PI178383 and Sonmez2001 (0.30). A greater genetic distance was determined between Sonmez2001 and PI178383.

Methylation analyses

CRED-RA analysis with 106 RAPD primers were conducted at PI178383, Harmankaya99, Izgi2001, ES14, Sonmez2001, and Aytin98 genotypes. Methylation polymorphism and epimarker data were scored to cutting

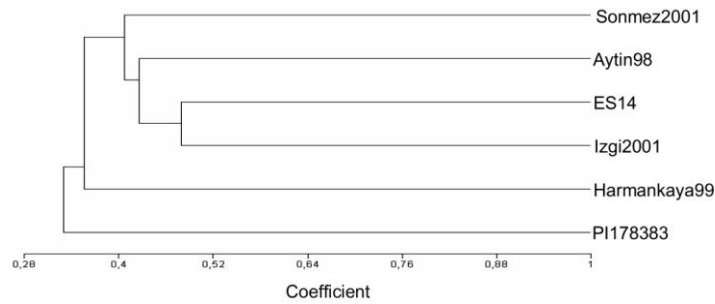


Figure 1. Dendrogram generated using genetic similarity analysis, showing relationships among wheat cultivars, using RAPD, SSR, ISSR and SRAP data

different with *MspI* and *HpaII* at PI178383 x Harmankaya99, Izgi2001 x ES14 and Sonmez2001 x Aytin98 combinations. The DNA methylation polymorphism ratios (%) of PI178383 x Harmankaya99, Izgi2001 x ES14 and Sonmez2001 x Aytin98 were 15.2%, 12.6%, 11%, respectively and the ratios of monomorphic fragments were 61.2%. From the methylation data of CRED-RA analysis, a greater similarity was observed between Sonmez2001 and ES14 (0.44), and the least similar one between PI178383 and Harmankaya99 (0.28). PI178383 was showed the same similarity with Sonmez2001 and ES14 (0.29) (Table 5). Figure 2 shows the dendrogram generated using genetic similarity analysis, for methylation pattern.

Table 5. Similarity coefficients among cultivars of *Triticum aestivum* generated through CRED-RA with methylation patterns

	1	2	3	4	5	6
PI178383	1.00					
Harmankaya99	0.28	1.00				
Izgi2001	0.30	0.39	1.00			
ES14	0.29	0.39	0.41	1.00		
Sonmez2001	0.29	0.37	0.38	0.44	1.00	
Aytin98	0.29	0.33	0.35	0.39	0.39	1.00

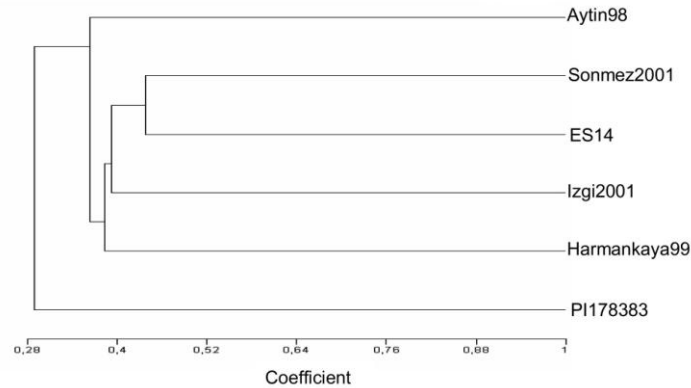


Figure 2. Dendrogram generated using genetic similarity analysis, showing relationships among wheat cultivars for methylation pattern

We detected differential fragments in terms of methylated state, referred to as epigenetic markers (epimarkers). Epimarker refers to a fragment for which the patterns from *HpaII*-digested samples differ between two genotypes. Data generated from CRED-RA was evaluated for epimarker analysis. For this analysis, different fragments between genotypes digested with *HpaII* were counted and datasheet were prepared for dendrogram. The epigenetic marker ratios (%) of PI178383 x Harmankaya99, Izgi2001 x ES14 and Sonmez2001 x Aytin98 combinations were showed 41%, 39%, and 20%, respectively. As a result of epigenetic marker analysis, the matrix based on Nei and Li's (1979) were showed genetic distance between cultivars of *Triticum aestivum* and a dendrogram was drawn by MVSP 3.1.

Similarity coefficients among 6 cultivars of *Triticum aestivum* generated through epimarker data was given in Table 6. From the epimarker data of CRED-RA analysis, a greater similarity was observed between Izgi2001 and Harmankaya99 (0.59), and the least similar one between PI178383 and Sonmez2001 (0.47). ES14 was showed the same similarity with Sonmez2001 and Harmankaya99 (0.57). Figure 3 shows the dendrogram generated using genetic similarity analysis for epimarker analysis.

DISCUSSION

DNA fingerprinting is a routine method employed to study the extent of genetic diversity across a set of germplasm or cultivars and group them into specific

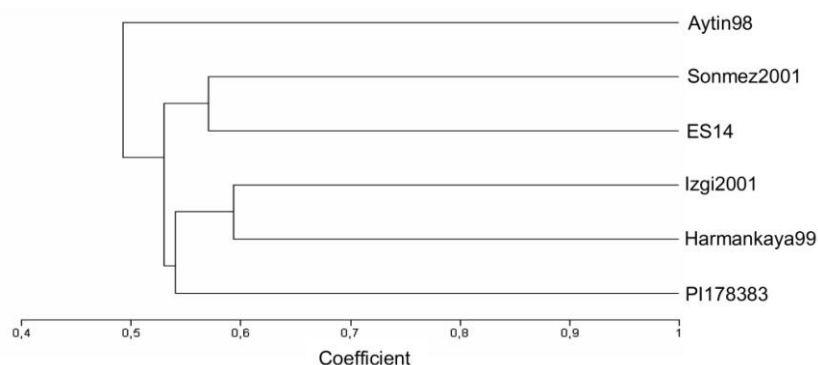


Figure 3. Dendrogram generated using genetic similarity analysis, showing relationships among wheat cultivars for epimarker analysis

Table 6. Similarity coefficients among cultivars of *Triticum aestivum* generated through epimarker data

	1	2	3	4	5	6
PI178383	1.00					
Harmankaya99	0.54	1.00				
Izgi2001	0.54	0.59	1.00			
ES14	0.56	0.57	0.54	1.00		
Sonmez2001	0.47	0.50	0.54	0.57	1.00	
Aytin98	0.50	0.44	0.48	0.55	0.50	1.00

categories. PCR-based markers are useful and valuable tools for cultivar discrimination, and detection of genetic polymorphism for marker assisted selection of new cultivars and clones (Cooke 1995). In this study, four different type of PCR-based molecular marker namely RAPD, SSR, ISSR, and SRAP markers were employed to study the genetic variability in six wheat cultivars of the genus *Triticum*. The number of primers required is dependent on the number of polymorphisms detected per primer and the number of genotypes or accessions to be investigated. The total number of amplified DNA bands varied between 3 (OPC15) and 26 (XGDM145) with an average of 12.43 bands per primer. Polymorphism percentage ranged from as low as 10% (OPM06) to as high as 100% (OPE09, XGDM88, XGDM98, XGDM99, XGDM113, XGDM153). For SSR, ISSR, and SRAP analyses, only the bands with a frequency of at least 10% were considered, in an attempt to avoid including bands with non-specific amplification. Carvalho et al. (2009) used ISSR primers for genetic diversity analyses of 99 old Portuguese wheat collection. Eighteen ISSR primers produced 96.3 and 98.5% of ISSR polymorphism in bread and durum wheat cultivars, respectively. In contrary to this, our study was revealed that seven ISSR primers produced 37.4% polymorphism in six wheat genotypes. Average RAPD polymorphism across the six wheat varieties was found to be 48.66%, which is quite consistent with that (45.3%) obtained by Wenguang et al. (2002) among 29 wheat genotypes, which is close to the present values. Mukhtar et al. (2002) reported high level of polymorphism of 64.3% in 20 wheat genotypes whereas Grewal et al. (2007) and Teshale et al. (2003) observed higher level of polymorphism 86.8%, 79.6% polymorphism in 20 and 27 Indian wheat accessions,

respectively. The PIC value of markers indicates the usefulness of DNA markers for gene mapping, molecular breeding and germplasm evaluation (Peng and Lapitan 2005). In this study, the average PIC value for SSR in wheat genotypes was 0.77, which is higher than that detected by genomic SSR markers (0.54, Roder et al. 1995), and higher than RFLP markers (0.30, Anderson et al. 1993). Similar to our work, the suitability of the RAPD, SSR, ISSR and SRAP techniques for genetic diversity studies and germplasm evaluations has been shown in many studies (Emon *et al.* 2010, Li *et al.* 2007a). Comparative studies in wheat species involving RAPD, RFLP, isozymes and SSR marker systems were successfully used by researchers (Guadagnuolo *et al.* 2001; Nagaoka and Ogihara 1997; Powell *et al.* 1996), as it has been carried out in many other crops. The discriminatory power of DNA markers used as tool to characterize the wheat genotypes is very important because they can be used to assess the genetic diversity among the wheat genotypes.

Information about how DNA methylation is naturally involved in phenotypic variations is limited. It has recently been shown that phenotypic variations can also be potentially caused by epigenetic changes due to aberrant methylation states. Comparison of the fragment patterns among the genotypes revealed polymorphism of the methylation state referred to as epigenetic markers. Epigenetic information might play an important role in maintaining the natural variation of DNA methylation. A phylogenetic tree based on epigenetic markers within nonpolymorphic fragments was analogous to that based on genetic markers (Takata 2005). There were differences between genetic and methylation polymorphism in the studied cultivars. For example, the RAPD, SSR, ISSR and SRAP primers showed that PI178383 and Sonmez2001 were genetically very different from each other, but in the dendrogram created based on methylation polymorphism Sonmez2001 and ES14 were grouped in one subcluster. Similar to this, in the dendrogram created based on epimarker data Izgi2001 and Harmankaya99 were grouped in one subcluster as well as Sonmez2001 and ES14 as in the second sub-cluster.

The same degree (0.30) of genetic and methylation polymorphism was noted for PI178383 and Sonmez2001 and PI178383 and Izgi2001. In epigenetic marker polymorphism, Izgi2001 and Harmankaya99 were similar (0.59) to each

other and Aytin98 and Harmankaya were different (0.44) from each other. Xiong et al. (1999) who examined DNA methylation polymorphisms of an elite rice hybrid and its parental strains by MSAP analyses, detected 46 differential methylation fragments (epimarkers) in a total of 1076 fragments. Ashikawa (2001) surveyed a few representative rice cultivars using MSAP analysis, and showed that the fragments carrying differential methylation states were randomly distributed in the genome. Compared with the methylation-sensitive AFLP technique was used by Xiong et al. (1999), Ashikawa (2001), and Portis et al. (2004). RAPD and ISSR fingerprinting on *HpaII/MspI*-digested DNA represents an alternative and much simpler method to identify DNA segments with differential CG and CNG cytosine methylation patterns among rice cultivars (Wang et al. 2004).

Summarizing all results obtained from RAPD, SSR, ISSR, SRAP methylation polymorphism and epimarker data with CRED-RA analyses it could be concluded that all subgroups derived from 3 dendrograms showed that interestingly one resistant and one susceptible genotype for yellow rust disease was coupled as ES14 and Izgi2001 (susceptible and resistant), ES14 and Sonmez2001 (susceptible and resistant) derived from genetic and methylation polymorphism dendrogram respectively. Furthermore, two subclusters (Harmankaya99 and Izgi2001, ES14 and Sonmez2001) from epimarker data contained one resistant and one susceptible wheat genotype with the consistency of genetic and methylation data. Given the functional roles of DNA methylation in regulating gene expression and maintaining genomic integrity in eukaryotes that possess this epigenetic code (Matzke et al. 1999; Martienssen and Colot (2001), cultivar-specific methylation patterns may contribute to physiologically meaningful inter-cultivar differentiation. Phenotypic differences between cultivars with a genetically close relationship might be partly caused by epialleles. Epialleles mediated by methylation alterations have been translated into evolutionarily conserved new phenotypes in *Linaria vulgaris* (Cubas, 1999) and in *Arabidopsis suecica* (Lee and Chen 2001).

The optimal strategies of breeding system require extensive knowledge of the breeding materials employed. The results presented here will be useful to understand the current status of genetic and epigenetic diversity between genotypes. Because all PCR-based markers applied in the present study were randomly selected from the entire wheat genome, exploring genetic variation for specific traits could not be expected. With advances in mapping of quantitative trait loci (QTL) for many agronomic important traits in wheat (Börner et al. 2002), it becomes possible to detect the allelic variation at these loci among accessions by using marker haplotypes. Such haplotype information will allow the breeder to directly accumulate favorable alleles at multiple loci in a controlled manner leading to superior varieties for parent selection to gain maximum value and practical impact on a breeding program. Unless the identified genes are subsequently cloned, sequenced and tested for methylation states, one cannot distinguish the effects of epialleles from those resulting from base sequence changes between parents.

Currently, applied methods of estimating genetic variance within plant populations do not shed much light on the magnitude or frequency of the contribution of epialleles (Kalisz and Purugganan 2004). In this study, the results derived from the dendrograms showed that both marker alleles and epialleles should take into account for heritable phenotypic variation.

ACKNOWLEDGMENTS

The authors thank Dr. Necmettin Bolat for providing the plant material. This work was supported by TUBITAK KAMAG (Project no. 105G075).

LITERATURE CITED

- Akfirat, F.S., Y. Aydin, F. Ertugrul, S. Hasancebi, K. Kazan, H. Budak, K. Akan, Z. Mert, N. Bolat, O. Yorgancilar, A.A. Uncuoglu, 2010. A Microsatellite Marker for Yellow Rust Resistance in Wheat. *Cereal Res. Commun.* 38, 204-211.
- Anderson, J.A., G.A. Churchill, J.E. Autrique, S.C. Tanksley, M.E. Sorrells, 1993. Optimizing parental selection for genetic linkage maps. *Genome* 36, 181-186.
- Ashikawa, I. 2001. Surveying CpG methylation at 50-CCGG in the genomes of rice cultivars. *Plant Mol. Biol.* 45, 31-39.
- Barrett, B.A., K.K. Kidwell, 1998. AFLP-based genetic diversity assessment among wheat cultivars from the Pacific Northwest. *Crop Sci.* 38, 1261-1271
- Bednarek, P.T., R. Orłowska, R.M. Koebner, J. Zimny, 2007. Quantification of the tissue-culture induced variation in barley (*Hordeum vulgare* L.). *BMC Plant Biol.* 7, 10-19.
- Börner, A., E. Schumann, A. Furst, H. Coster, B. Leithold, S. Roder, E. Weber, 2002. Mapping of quantitative trait loci determining agronomic important characters in hexaploid wheat (*Triticum aestivum* L.). *Theor. Appl. Genet.* 105, 921-936.
- Cantu, D., L.S. Vanzetti, A. Sumner, M. Dubcovsky, M. Matvienko, A. Distelfeld, R.W. Michelmore, J. Dubcovsky, 2010. Small RNAs DNA methylation transposable elements in wheat. *BMC Genomics* 11, 408-423.
- Cai, Q., C.L. Guy, G.A. Moore, 1996. Detection of cytosine methylation mapping of a gene influencing cytosine methylation in the genome of Citrus. *Genome* 39, 235-242.
- Carvalho, A., M. Matos, J. Lima-Brito, H. Guedes-Pinto, C. Benito, 2005. DNA fingerprint to F1 interspecific hybrids from the Triticeae tribe using ISSRs. *Euphytica* 143, 93-99.
- Carvalho, A., J. Lima-Brito, B. Macas, H. Guedes-Pinto, 2009. Genetic diversity variation among botanical varieties of old portuguese wheat cultivars revealed by ISSR assays. *Biochem. Genet.* 47, 276-294.
- Chen, H.B., J.M. Martin, M. Lavin, L.E. Talbert, 1994. Genetic diversity in hard red spring wheat based on sequence-tagged-site PCR markers. *Crop Sci.* 34, 1628-1632.
- Cooke, R.J. 1995. Variety identification of crop plants. In: Skerrit, J.H., Appels, R. (eds) *New diagnostics in crop sciences*. CAB International, Wallingford, UK, pp 33-63.
- Cubas, P., C. Vincent, E. Coen, 1999. An epigenetic mutation responsible for natural variation in floral symmetry. *Nature* 401, 157-161.
- Dong, Z.Y., Y.M. Wang, Z.J. Zhang, Y. Shen, X.Y. Lin, X.F. Ou, F.P. Han, B. Liu, 2006. Extent pattern of DNA methylation alteration in rice lines derived from introgressive hybridization of rice *Zizania latifolia* Griseb. *Theor. Appl. Genet.* 113, 196-205.
- Ercan, S., F. Ertugrul, Y. Aydin, F. Senturk-Akfirat, S. Hasancebi, L. Cetin, K. Akan, Z. Mert, N. Bolat, M. Cakmak, A. Altinkut-Uncuoglu, 2010. An EST-SSR marker linked with yellow rust

- resistance in wheat (*Triticum aestivum* L.). *Biol. Plantarum* 54, 691-696.
- Emon, R.M., J.P. Gustafson, H. Nguyen, T. Musket, M. Jahiruddin, M.A. Islam, M.S. Haque, M. Islam, S.N. Begum, M.M. Hassan, 2010. Molecular marker-based characterization genetic diversity of wheat genotypes in relation to Boron use efficiency. *Indian J. Genet.* 70, 339-348.
- Galovic, V., S. Dengic, D. Jelovac, 2005. Determination of polymorphism of microsatellite primers in hexaploid wheat. *Genetika*, 37, 217-223.
- Grewal, S., P. Kharb, R. Malik, S. Jain, R.K. Jain, 2007. Assessment of genetic diversity among some Indian wheat cultivars using random amplified polymorphic DNA (RAPD) markers. *Indian J. of Biotech.* 6, 18-23.
- Guadagnuolo, R., D.S. Bianchi, F. Felber, 2001. Specific genetic markers for wheat, spelt, four wild relatives: comparison of isozymes, RAPDs, wheat microsatellites. *Genome* 44, 610-621.
- Han, F.P., G. Fedak, T. Ouellet, B. Liu, 2003. Rapid genomic changes in interspecific intergeneric hybrids allopolyploids of *Triticeae*. *Genome* 46, 716-723.
- Huang, X.Q., A. Börner, M.S. Röder, M.W. Ganal, 2002. Assessing genetic diversity of wheat (*Triticum aestivum* L.) germplasm using microsatellite markers. *Theor Appl Genet* 105, 699-707.
- Joshi, C.P., H.T. Nguyen, 1993. RAPD (random amplified polymorphic DNA) analysis based intervarietal genetic relationships among hexaploid wheats. *Plant Sci.* 93, 95-103.
- Kaeppeler, S., R. Phillips, 1993. Tissue culture-induced DNA methylation variation in maize. *Proc. National Acad. Sci. USA.* 90, 8773-8776.
- Kalisz, S., M.D. Purugganan, 2004. Epialleles via DNA methylation: consequences for plant evolution. *Trends Ecol. Evol.* 19, 309-314.
- Kim, H. S., R.W. Ward, 2000. Patterns of RFLP-based genetic diversity in germplasm pools of common wheat with different geographical or breeding program origins. *Euphytica* 115, 197-208.
- Kovach, W.L. 1999. MVSP-A Multivariate Statistical Package for Windows, v. 3.1. Kovach Computing Services, Pentraeth, 133.
- Lee, H.S., Z.J. Chen, 2001. Protein-coding genes are epigenetically regulated in *Arabidopsis* polyploids. *Proc. Natl. Acad. Sci. USA* 98, 6753-6758.
- Leljak-Levanic, D., N. Bauer, S. Mihaljevic, S. Jelaska, 2004. Changes in DNA methylation during somatic embryogenesis in *Cucurbita pepo* L. *Plant Cell Rep.* 23, 120-127.
- Li, G., C.F. Quiros, 2001. Sequence-related amplified polymorphism (SRAP), a new marker system based on a simple PCR reaction: its application to mapping gene tagging in Brassica. *Theor. App. Genet.* 103, 455-461.
- Li, S., J. Jia, X. Wei, X. Zhang, L. Li, H. Chen, Y.Fan, H. Sun, X. Zhao, T. Lei, Y. Xu, F. Jiang, H. Wang, L. Li, 2007a. A intervarietal genetic map QTL analysis for yield traits in wheat. *Mol. Breed.* 20, 167-178.
- Li, X., X. Yu, N. Wang, Q. Feng, Z. Dong, L. Liu, J. Shen, B. Liu, 2007b. Genetic epigenetic instabilities induced by tissue culture in wild barley (*Hordeum brevisubulatum*). *Plant Cell Tissue Organ Cult.* 90, 153-168.
- Liu, Y., D. Liu, H. Zhang, J. Wang, J. Sun, X. Guo, A. Zhang, 2005. Allelic variation, sequence determination microsatellite screening at the XGWM261 locus in Chinese hexaploid wheat (*Triticum aestivum*) varieties. *Euphytica* 145, 103-112.
- Lukens, L.N., S. Zhan, 2007. The plant genome's methylation status response to stress: implications for plant improvement. *Curr. Opin. Plant Biol.* 10, 317-322.
- Ma, Z.Q., M.S. Röder, M.E. Sorrells, 1996. Frequencies sequence characteristics of di-, tri-, tetra-nucleotide microsatellites in wheat. *Genome* 39, 123-130.
- Marfil, C.F., E.L. Camadro, R.W. Masuelli, 2009. Phenotypic instability epigenetic variability in a diploid potato of hybrid origin, *Solanum ruiz-leali*. *BMC Plant Biol.* 9, 21.
- Martienssen, R.A., V. Colot, 2001. DNA methylation epigenetic inheritance in plants lamentous fungi. *Science* 293, 1070-1074.
- Matzke, M.A., M.F. Mette, W. Aufsatz, J. Jakowitsch, A.J.M. Matzke, 1999. Host defenses to parasitic sequences the evolution of epigenetic control mechanisms. *Genetica* 107, 271-287.
- Mukhtar, M.S., M.U. Rehman, Y. Zafar, 2002. Assessment of genetic diversity among wheat (*T. aestivum* L.) cultivars from a range of localities across Pakistan using random amplified polymorphic DNA (RAPD) analysis. *Euphytica* 28, 417-425.
- Nagaoka, T., Y. Ogihara, 1997. Applicability of inter-simple sequence repeat polymorphisms in wheat for use as DNA markers in comparison to RFLP RAPD markers. *Theor. Appl. Genet.* 94, 597-602.
- Nei, M. 1978. Estimation of average heterozygosity genetic distance from a small number of individuals. *Genetics* 583-590.
- Nei, M., W.H. Li, 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proc. Natl. Acad. Sci. USA* 76, 5269-5273.
- Peng, J.H., N.L.V. Lapitan, 2005. Characterization of EST-derived microsatellites in the wheat genome development of eSSR markers. *Funct. Inter. Genom.* 5, 80-96.
- Pestsova, E., M.W. Ganal, M.S. Röder, 2000. Isolation mapping of microsatellite markers specific for the D genome of bread wheat. *Genome* 43, 689-697.
- Portis, E., A. Acquadro, C. Comino, A. Lanteri, 2004. Analysis of DNA methylation during germination of pepper (*Capsicum annum* L.) seeds using methylation-sensitive amplification polymorphism (MSAP). *Plant Sci.* 166, 169-178.
- Powell, W., Morgante, M., Andre, C., Hanafey, M., Vogel, J., Tingey, S., Rafalski, A. (1996) The comparison of RFLP, RAPD, AFLP SSR (microsatellite) marker for germplasm analysis. *Mol. Breed.* 2, 225-238.
- Rangwala, S.H., E.J. Richards, 2004. The value-added genome: building maintaining genomic cytosine methylation landscapes. *Cur. Opin. Genet. Dev.* 14, 686-691.
- Rapp, R.A., J.F. Wendel, 2005. Epigenetics plant evolution. *New Phytol.* 168, 81-91.
- Repkova, J., A. Dreiseitl, 2009. Candidate markers for powdery mildew resistance genes from wild barley PI284752. *Euphytica* DOI 10.1007/s10681-009-0096-0.
- Röder, M.S., J. Plaschke, S.U. König, A. Börner, M.E. Sorrells, 1995. Abundance, variability chromosomal location of microsatellites in wheat. *Mol. Gen. Genet.* 246, 327-333.
- Roldan-Ruiz, I., J. Dendauw, E. Van Bockstaele, A. Depicker, M. De Loose, 2000. AFLP markers reveal high polymorphic rates in rye grasses (*Lolium spp.*). *Mol. Breed.* 6, 125-134.
- Selker, E.U. 1997. Epigenetic phenomena in filamentous fungi: useful paradigms or repeat-induced confusion? *Trends Genet.* 13, 296-301.
- Shaked, H.K., K. Kashkush, H. Ozkan, M. Feldman, A.A. Levy, 2001. Sequence elimination cytosine methylation are rapid reproducible responses of the genome to wide hybridization allopolyploidy in wheat. *Plant Cell* 13, 1749-1759.
- Takata, M., Y. Kishima, Y. Sano, 2005. DNA methylation polymorphisms in rice wild rice strains: detection of epigenetic markers. *Breed. Sci.* 55, 57-63.
- Temel, A., G. Kartal, N. Gozukirmizi, 2008. Genetic and epigenetic variations in barley calli cultures. *Biotechnol. & Biotechnol. Eq.* 22(4), 911-914.
- Teshale, E.T., S. Bansal, A. Mishra, Vijaipal, V.K. Khanna, 2003. DNA fingerprinting of wheat genotypes by RAM markers. *Wheat Infor. Serv.* 96, 23-27.

- Tsaftaris, A.S., A.N. Polidoros, 2000. DNA methylation plant breeding. *Plant Breed. Rev.* 18, 87-176.
- Xiao, W., K.D. Custard, R.C. Brown, B.E. Lemmon, J.J. Harada, R.B. Goldberg, R.L. Fischer, 2006. DNA methylation is critical for *Arabidopsis* embryogenesis seed viability. *Plant Cell* 18, 805-814.
- Xiong, L.Z., C.G. Xu, M.A. Saghai Maroof, Q.F. Zhang, 1999. Patterns of cytosine methylation in an elite rice hybrid its parental lines, detected by a methylation-sensitive amplification polymorphism technique. *Mol. Genet. Genom.* 261, 439-446.
- Wang, G., J.S. Pan, X.Z. Li, H.L. He, A.Z. Wu, R. Cai, 2005. Construction of a cucumber genetic linkage map with SRAP markers location of the genes for lateral branch traits. *Sci. China Ser. C: Life Sciences* 48, 213-220.
- Wang, Y., X. Lin, B. Dong, Y. Wang, B. Liu, 2004. DNA methylation polymorphism in a set of elite rice cultivars and its possible contribution to intercultivar differential gene expression. *Cellular and Molecular Biology Letters* 9, 543-556.
- Weining, S., P. Langridge, 1991. Identification mapping of polymorphisms in cereals based on the polymerase chain reaction. *Theor. Appl. Genet.* 82, 209-216.
- Wenguang, C., P. Hucl, G. Scoles, R.N. Chibbar, P.N. Fox, B. Skovm, 2002. Cultivar identification pedigree assessment of common wheat based on RAPD analysis. *Wheat Infor. Serv.* 95, 29-35.
- Zietkiewicz, E., A. Rafalski, D. Labuda, 1994. Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. *Genomics* 20, 176-183.