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

Dilek TOK¹ Funda SENTURK-AKFIRAT² Duygu SEVINC¹ Yildiz AYDIN¹ Ahu ALTINKUT-UNCUOGLU*¹

¹Marmara University, Faculty of Science and Letters, Department of Biology, Turkey
²Gebze Institute of Technology, Faculty of Science, Department of Molecular Biology and Genetics, Turkey
³Istanbul University, Faculty of Science, Department of Biology, Turkey
⁴Marmara University, Faculty of Engineering, Department of Bioengineering, Turkey
*Corresponding author’s e-mail: ahu.uncuoglu@marmara.edu.tr

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 grouped in one subcluster. Similar to this, in the dendrogram created based on epimarker data which differentiate between genetic and methylation polymorphism in the studied cultivars. Dendrogram created based on genetic polymorphism showed that PI178383 and Sonmee2001 (0.30) were genetically very different from each other, but in the dendrogram constructed by methylation polymorphism Sonmee2001 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 Sonmee2001 and ES14 (0.57) as in the second sub-cluster. 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 Quirós 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
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 Weinig 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 Lejak-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. Polymorphism information content (PIC) or average heterozygosity was calculated as per the formula of Roldan-Ruiz et al. (2000):

\[
PIC = 2f_i (1 - f_i), \quad f_i = \frac{f_i}{N}
\]

where \(f_i\) is the frequency of the amplified allele and \(1 - f_i\) is the frequency of null allele. Average heterozygosity (\(H_w\)) is obtained by taking the average of PIC values obtained for all the markers and it is calculated as \(H_w = \frac{\sum [2f_i (1-f_i)]}{N}\). \(N\) is sample size and \(m + p/n\) 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 (\(MI = H_w \times MR\)) was obtained by multiplying the average heterozygosity (\(H_w\)) 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 Aytin98 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 OPD04, OPD05, OPD06, OPD09, OPD14, OPD15, OPD16, OPD03, OPD06, OPD11, OPD13, OPD01, OPD05, OPD07, OPD10, OPD14, OPD07, OPD11, OPD05, OPD07, OPD14, OPD15, OPD04, OPD11, OPD12, OPD15, OPD17, OPD20, OPL03, OPM05, OPM09, OPM18, UBC212, UBC320, UBC537. The number of primers which gave distinct pattern for genotypes were 13, 7, 7, 12, 5 in PI178383, Izgi2001, Sonmez2001, Harmankaya99, ES14, and Aytin98 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**

<table>
<thead>
<tr>
<th>Cultivars</th>
<th>RAPD No.</th>
<th>RAPD %</th>
<th>SSR No.</th>
<th>SSR %</th>
<th>ISSR No.</th>
<th>ISSR %</th>
<th>SRAP No.</th>
<th>SRAP %</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI178383</td>
<td>111</td>
<td>111.1</td>
<td>239</td>
<td>17.59</td>
<td>235</td>
<td>16.6</td>
<td>241</td>
<td>17.2</td>
</tr>
<tr>
<td>Izgi2001</td>
<td>175</td>
<td>175</td>
<td>235</td>
<td>16.6</td>
<td>225</td>
<td>15.8</td>
<td>228</td>
<td>15.7</td>
</tr>
<tr>
<td>Sonmez2001</td>
<td>198</td>
<td>198</td>
<td>230</td>
<td>15.5</td>
<td>225</td>
<td>16.0</td>
<td>224</td>
<td>16.0</td>
</tr>
<tr>
<td>Harmankaya99</td>
<td>171</td>
<td>171</td>
<td>231</td>
<td>16.4</td>
<td>230</td>
<td>17.1</td>
<td>226</td>
<td>16.1</td>
</tr>
<tr>
<td>ES14</td>
<td>157</td>
<td>157</td>
<td>230</td>
<td>15.5</td>
<td>228</td>
<td>15.7</td>
<td>225</td>
<td>15.7</td>
</tr>
<tr>
<td>Aytin98</td>
<td>139</td>
<td>139</td>
<td>233</td>
<td>16.8</td>
<td>228</td>
<td>15.7</td>
<td>226</td>
<td>15.7</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Cultivars</th>
<th>No. of polymorphic loci</th>
<th>Percentage of polymorphic loci</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI178383</td>
<td>30</td>
<td>10.18</td>
</tr>
<tr>
<td>Izgi2001</td>
<td>26</td>
<td>10.18</td>
</tr>
<tr>
<td>Sonmez2001</td>
<td>21</td>
<td>8.06</td>
</tr>
<tr>
<td>Harmankaya99</td>
<td>22</td>
<td>5.47</td>
</tr>
<tr>
<td>ES14</td>
<td>19</td>
<td>4.01</td>
</tr>
<tr>
<td>Aytin98</td>
<td>43</td>
<td>17.39</td>
</tr>
</tbody>
</table>

**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 was 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 Aytin98 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

<table>
<thead>
<tr>
<th>Marker index (MI)</th>
<th>RAPD</th>
<th>SSR</th>
<th>ISSR</th>
<th>SRAP</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of primers used</td>
<td>106</td>
<td>65</td>
<td>7</td>
<td>18</td>
<td>196</td>
</tr>
<tr>
<td>Number of primers amplified</td>
<td>98</td>
<td>37</td>
<td>6</td>
<td>15</td>
<td>156</td>
</tr>
<tr>
<td>Total number of polymorphic bands</td>
<td>678</td>
<td>232</td>
<td>46</td>
<td>149</td>
<td>1105</td>
</tr>
<tr>
<td>Total number of monomorphic bands</td>
<td>598</td>
<td>218</td>
<td>57</td>
<td>140</td>
<td>1013</td>
</tr>
<tr>
<td>Total number of bands</td>
<td>1276</td>
<td>450</td>
<td>103</td>
<td>289</td>
<td>2118</td>
</tr>
<tr>
<td>Average percentage of polymorphism (%)</td>
<td>48.66</td>
<td>36.27</td>
<td>37.4</td>
<td>41.1</td>
<td>40.86</td>
</tr>
<tr>
<td>Average number of polymorphic bands/primer</td>
<td>6.4</td>
<td>3.6</td>
<td>6.6</td>
<td>8.3</td>
<td>6.23</td>
</tr>
<tr>
<td>Multiplex ratio (MR) - (n/T, total number of bands/total number of bands)</td>
<td>12.03</td>
<td>6.92</td>
<td>14.71</td>
<td>16.05</td>
<td>12.43</td>
</tr>
<tr>
<td>Average heterozygosity (Hav)</td>
<td>0.7976</td>
<td>-</td>
<td>0.9369</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>[(mR): multiple ratio, MR was estimated by dividing the total number of bands; (m): monomorphic and (p): polymorphic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Marker index (MI) = Hav × MR</td>
<td>0.8761</td>
<td>0.7727</td>
<td>0.8998</td>
<td>0.9330</td>
<td>0.8704</td>
</tr>
</tbody>
</table>

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 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.

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

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI178383</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Izgi2001</td>
<td>0.34</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ES14</td>
<td>0.36</td>
<td>0.39</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sonmez2001</td>
<td>0.30</td>
<td>0.36</td>
<td>0.39</td>
<td>0.42</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>Aytin98</td>
<td>0.32</td>
<td>0.30</td>
<td>0.40</td>
<td>0.45</td>
<td>0.41</td>
<td>1.00</td>
</tr>
</tbody>
</table>

**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
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

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI178383</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Harmankaya99</td>
<td>0.28</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Izgi2001</td>
<td>0.30</td>
<td>0.39</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ES14</td>
<td>0.29</td>
<td>0.39</td>
<td>0.41</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sonmez2001</td>
<td>0.29</td>
<td>0.37</td>
<td>0.38</td>
<td>0.44</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>Aytin98</td>
<td>0.29</td>
<td>0.33</td>
<td>0.35</td>
<td>0.39</td>
<td>0.39</td>
<td>1.00</td>
</tr>
</tbody>
</table>

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 drawed 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...
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

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

<table>
<thead>
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<td>0.48</td>
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</tr>
</tbody>
</table>

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


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