A comparative analysis of ISSR and RAPD markers for studying genetic diversity in Iranian pistachio cultivars

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Abstract

Pistachio is one of the most important horticultural products of Iran. The best way to get the maximum yield is to have genetically pure and monotonous gardens. Thereby, study of genetic variation and providing genetic identifications, make this possible to have homogenous gardens with high performance genotypes. In the present study, genetic diversity of 19 Iranian pistachio cultivars was assessed using ISSR and RAPD primers. Among these markers, RAPDs generated 127 amplification products, out of which 88 were polymorphic and ISSRs produced 114 amplification products, out of which 73 were polymorphic. RAPD fingerprinting detected more polymorphic loci (67%) than the ISSR (63%). Mean of polymorphism information content (PIC) for each of the marker systems (0.39 for RAPD and 0.39 for ISSR) suggested that both the marker systems were equally effective in determining polymorphisms. The dendrograms constructed using RAPD and ISSR marker systems were highly correlated with each other as revealed by high Mantel correlation (r= 0.83). These two marker systems were found to be useful for genetic diversity studies in pistachio and identify variation within Pistacia vera cultivars.

Key words: Pistachia, fingerprinting, horticultural product, molecular maker.

Introduction

Pistachio (Pistacia vera L.) is one of the most important horticultural products of Iran. It is a diploid (2n = 30) member of the Anacardiaceae family. The genus Pistacia consists of eleven species that P. vera is the only cultivated and commercially grown species in this genus (Zohary 1996). The other species grow as wild and their seeds are used mainly as a rootstock seed source. Due to its drought and salinity resistance and ability to growth in weak soils, this plant can be cultivated in desert-like and dry lands. It is cultivated in many places around the world, Asia, Middle East, southern Europe, South Africa, Australia and America. The main world producers of pistachio nuts are Iran, USA, Turkey and Syria (FAO, 2006). The best way to get the maximum yields is related to have genetically pure and monotonous gardens. Thereby, the study of genetic diversity and providing genetic identification, make this possible to have homogenous gardens with high performing genotypes. Initial classification of Pistacia species was carried out morphologically based on tree, leaf, flower and nut characteristics. Isozyme markers have also been used to investigate the genetic diversity of pistachios (Rovira et al., 1995). DNA markers have proved to be an efficient tool for the molecular characterization of the plant species. The classification of Pistacia species at molecular level was first done based on chloroplast DNA profiles by Parfitt and Badenes (1997). RAPD has been the
most used method in pistachio to study genetic diversity and relationship among Pistacia species and cultivars (Hormaza et al., 1994, 1998; Kafkas et al., 2002; Katsiotis et al., 2003; Golan-Goldhirsh et al., 2004; Mirzaei et al., 2005; al-saghir et al., 2006). Also AFLP, SSR, ISSR and comparisons of these markers were the other used markers in pistachio cultivars characterization (Katsiotis et al., 2003; Ahmad et al., 2003; Golan-Goldhirsh et al., 2004; Ahmad et al., 2005; Kafkas et al., 2006; Ahmadi-Afzadi et al., 2007; Ibrahim-Basha et al., 2007; Salehi Shanjani et al., 2009; fares et al., 2009).

RAPD markers are DNA fragments from PCR amplification of random segments of genomic DNA with single primer of arbitrary nucleotide sequences, which are able to differentiate between genetically distinct individuals. Inter simple sequence repeat (ISSR) is a RAPD like marker system. It can reveal variation in the numerous microsatellite regions by using primers that may be anchored with one or two nucleotides on either the 5’ or 3’ end of a repeat region and extend into the flanking region. These methods are widely applicable because they are rapid, inexpensive, and simple to perform, do not require prior knowledge of DNA sequence, and require very little starting DNA template (Esselman et al., 1999).

In this study two markers, the random amplified polymorphic DNA (RAPD) (Williams et al., 1990) and inter simple sequence repeat (ISSR) (Vos et al., 1995) were used to explore the genetic diversity among 19 Iranian genotypes of pistacia vera and were compared for their relative efficiency.

**Materials and Methods**

**Plant Material and DNA Extraction**

In the present study, genetic relationships and diversity among nineteen cultivars were assessed using RAPD and ISSR primers. Plant material for this study was collected from Iranian Pistachio Resources Institute (IPRI) (Table 1).

Total genomic DNA was extracted from freeze-dried leaf tissue using (CTAB) mini-extraction protocol based on the method by Hormaza et al., (1998), with minor modifications. Quality and quantity of DNA were estimated by spectrophotometer and electrophoresis on 0.8% agarose gel. DNA samples were diluted to 25 ng/µl for polymerase chain reaction (PCR) amplification.

**RAPD Amplification**

Twenty RAPD primers obtained from Metabion Company were tested and 10 were used (Table 2). Amplification reactions were performed in 25 µl volume containing 13.5 µl PCR master kit, 8.5 µl dH₂O, 1 µl template DNA and 2 µl primer. PCR reactions were performed with thermal cycler (model TC-512). Amplification condition was an initial denaturing at 94°C for 5 min followed by 45 cycles of 1 min denaturing at 94°C, 1 min annealing at 31°C -34 °C, and 2 min extension at 72 °C plus 7 min final extension at 72°C. PCR products were analyzed by gel electrophoresis in 1.5% (w/v) agarose gel in 1x TBE buffer, gels were stained with ethidium bromide and digitally photographed under ultraviolet light in a transilluminator documentation system (UVP, USA).

**ISSR Amplification**

PCR amplification was performed with primers obtained from Metabion Company. The experiment was carried out by 20 ISSR primers, of which 10 were used (Table 2). Amplification reactions were done in a 25 µl volume containing 12.5 µl PCR master kit, 10 µl dH₂O, 1µL template DNA and 1.5 µl primer. The mixture was overlaid with mineral oil and subjected to PCR on a thermal cycler (model TC-512), programmed for an initial denaturing of 5 min at 94°C, followed by 35 cycles of 30 sec denaturing at 94°C, 1 min annealing at 51°C -59°C, 2 min extension at 72°C, plus a 5 min final extension at 72°C. PCR products were analyzed by gel electrophoresis in 1.5% agarose in 1x TBE buffer, gels were stained with ethidium bromide and digitally photographed under ultraviolet light in a transilluminator documentation system (UVP, USA).
Table 1. Names and codes of nineteen Iranian pistachio cultivars, used in this study.

<table>
<thead>
<tr>
<th>Cultivar code</th>
<th>Cultivar name</th>
<th>Cultivar code</th>
<th>Cultivar name</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>Akbari</td>
<td>P11</td>
<td>Gholamrezai</td>
</tr>
<tr>
<td>P2</td>
<td>Ahmad-aghai</td>
<td>P12</td>
<td>Khanjari-damghan</td>
</tr>
<tr>
<td>P3</td>
<td>Kale-ghochi</td>
<td>P13</td>
<td>Sefid-pest</td>
</tr>
<tr>
<td>P4</td>
<td>Ohadi</td>
<td>P14</td>
<td>Lavare 2</td>
</tr>
<tr>
<td>P5</td>
<td>Ghazvini</td>
<td>P15</td>
<td>Fandoghi-riz</td>
</tr>
<tr>
<td>P6</td>
<td>Mohseni</td>
<td>P16</td>
<td>Ebrahim-abadi</td>
</tr>
<tr>
<td>P7</td>
<td>Javad-aghai</td>
<td>P17</td>
<td>Seifedin</td>
</tr>
<tr>
<td>P8</td>
<td>Amiri</td>
<td>P18</td>
<td>Rezai-zodras</td>
</tr>
<tr>
<td>P9</td>
<td>Sheni</td>
<td>P19</td>
<td>Momtaz</td>
</tr>
<tr>
<td>P10</td>
<td>Hosein-khani</td>
<td></td>
<td></td>
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</tbody>
</table>

Table 2. ISSR and RAPD primer sequences and annealing temperatures (Ta).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'–3')</th>
<th>Ta °C</th>
<th>Primer</th>
<th>Sequence (5'–3')</th>
<th>Ta °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>K 10</td>
<td>(AC)₈YG</td>
<td>54</td>
<td>Z 16</td>
<td>TCCCCATCAC</td>
<td>31</td>
</tr>
<tr>
<td>K11</td>
<td>(CA)₉AG</td>
<td>59</td>
<td>29</td>
<td>CCGGCCCTAC</td>
<td>34</td>
</tr>
<tr>
<td>K13</td>
<td>(AG)₈YT</td>
<td>54</td>
<td>28</td>
<td>CCGGCCTTAA</td>
<td>34</td>
</tr>
<tr>
<td>K15</td>
<td>(GT)₈YC</td>
<td>57</td>
<td>1</td>
<td>CCTGGGCTTC</td>
<td>34</td>
</tr>
<tr>
<td>K16</td>
<td>(CA)₉RC</td>
<td>53</td>
<td>51</td>
<td>CTACCCGTC</td>
<td>34</td>
</tr>
<tr>
<td>K25</td>
<td>(AG)₉G</td>
<td>54</td>
<td>OPAC-06</td>
<td>CAGAAACGGA</td>
<td>32</td>
</tr>
<tr>
<td>K26</td>
<td>(AG)₈T</td>
<td>53</td>
<td>OPA₁₁</td>
<td>CAAATCGCCGT</td>
<td>32</td>
</tr>
<tr>
<td>K24A</td>
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<td>53</td>
<td>OPA-04</td>
<td>CACAGAGGGA</td>
<td>32</td>
</tr>
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<td>K24B</td>
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<td>51</td>
<td>Z 06</td>
<td>GTCCCCGTTC</td>
<td>33</td>
</tr>
<tr>
<td>UBC 840</td>
<td>(GA)₉TT</td>
<td>53</td>
<td>P 6</td>
<td>TCGGCCTT</td>
<td>34</td>
</tr>
</tbody>
</table>

Photographed under ultraviolet light in a transilluminator documentation system (UVP, USA).

**Data Analysis**

The amplifications were independently repeated three times using the same procedure, in order to ensure that the amplifications obtained with the primers were reproducible and consistent. The amplified bands were scored manually as 1 (present) and 0 (absent). A binary matrix was obtained by visual scoring of the bands in the cases of both RAPD and ISSR. Data analyses were performed using NTSYS-pc version 2.0 program package. Mantel (1967) test was used to measure the degree of relationship between similarity index matrices produced by any two-marker systems. Clustering dendrograms were constructed by un-weighted pair group method using arithmetic average (UPGMA) method. The polymorphism information content (PIC) of each marker that provides an estimate of the discriminatory power of a locus or loci was calculated by taking into account not only the number of alleles that are expressed but also relative frequencies of those alleles (Kumar *et al.*, 2003). Also marker index (MI), which is the product of polymorphic information content (PIC) and effective multiplex ratio (EMR), was used to evaluate the overall utility of each marker system. EMR (E) is defined as the product of the fraction of polymorphic loci and the number of polymorphic loci for an individual assay.
**Fig 1.** Genomic DNA amplification pattern in 19 pistachio cultivars, with ISSR primer (primer K26). (M molecular weight marker).

**Table 3.** The total number of loci, polymorphic loci, polymorphism percentage, PIC and MI, revealed by ISSR and RAPD primers.

<table>
<thead>
<tr>
<th>Primer code</th>
<th>NO. locus</th>
<th>polymorph Locus</th>
<th>Polymorphism percentage</th>
<th>PIC</th>
<th>Emr</th>
<th>MI=PIC*E</th>
</tr>
</thead>
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<tr>
<td><strong>ISSR</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>k10</td>
<td>12</td>
<td>11</td>
<td>0.92</td>
<td>0.39</td>
<td>6.71</td>
<td>2.64</td>
</tr>
<tr>
<td>k16</td>
<td>8</td>
<td>3</td>
<td>0.37</td>
<td>0.39</td>
<td>2.37</td>
<td>0.94</td>
</tr>
<tr>
<td>k11</td>
<td>12</td>
<td>9</td>
<td>0.75</td>
<td>0.45</td>
<td>5.92</td>
<td>2.66</td>
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<tr>
<td>k15</td>
<td>9</td>
<td>7</td>
<td>0.78</td>
<td>0.46</td>
<td>4.34</td>
<td>1.98</td>
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<tr>
<td>k13</td>
<td>11</td>
<td>7</td>
<td>0.64</td>
<td>0.39</td>
<td>5.56</td>
<td>2.19</td>
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<tr>
<td>k26</td>
<td>12</td>
<td>7</td>
<td>0.58</td>
<td>0.40</td>
<td>5.10</td>
<td>2.02</td>
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<td>k25</td>
<td>15</td>
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<td>0.53</td>
<td>0.40</td>
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<td>2.70</td>
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<td>k24a</td>
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<td>0.27</td>
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<td>1.51</td>
</tr>
<tr>
<td>k24b</td>
<td>11</td>
<td>6</td>
<td>0.55</td>
<td>0.34</td>
<td>4.59</td>
<td>1.56</td>
</tr>
<tr>
<td>UBC840</td>
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<td>8</td>
<td>0.67</td>
<td>0.43</td>
<td>6.38</td>
<td>2.73</td>
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<tr>
<td>Z 16</td>
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<td>5</td>
<td>0.71</td>
<td>0.32</td>
<td>3.87</td>
<td>1.26</td>
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<tr>
<td>OPAC-06</td>
<td>11</td>
<td>9</td>
<td>0.82</td>
<td>0.43</td>
<td>6.20</td>
<td>2.68</td>
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<tr>
<td>28</td>
<td>22</td>
<td>19</td>
<td>0.86</td>
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<td>13.14</td>
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<tr>
<td>1</td>
<td>16</td>
<td>10</td>
<td>0.63</td>
<td>0.30</td>
<td>7</td>
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<tr>
<td><strong>RAPD</strong></td>
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<td></td>
<td></td>
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<td></td>
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<tr>
<td>OPA-11</td>
<td>9</td>
<td>6</td>
<td>0.67</td>
<td>0.39</td>
<td>4.67</td>
<td>1.80</td>
</tr>
<tr>
<td>OPA-04</td>
<td>8</td>
<td>4</td>
<td>0.5</td>
<td>0.28</td>
<td>3.63</td>
<td>1</td>
</tr>
<tr>
<td>Z 06</td>
<td>11</td>
<td>8</td>
<td>0.73</td>
<td>0.41</td>
<td>4.98</td>
<td>2.05</td>
</tr>
<tr>
<td>P 6</td>
<td>14</td>
<td>10</td>
<td>0.71</td>
<td>0.45</td>
<td>5.83</td>
<td>2.61</td>
</tr>
<tr>
<td>29</td>
<td>14</td>
<td>7</td>
<td>0.5</td>
<td>0.39</td>
<td>5.24</td>
<td>2.07</td>
</tr>
<tr>
<td>51</td>
<td>15</td>
<td>10</td>
<td>0.67</td>
<td>0.36</td>
<td>6.56</td>
<td>2.37</td>
</tr>
</tbody>
</table>

**PIC:** Polymorphic information content.

**MI:** Marker index.

**Results**

Out of 20 ISSR and 20 RAPD primers, only 10 ISSR and 13 RAPD primers produced DNA fragments which were scorable. All the 10 scorable ISSR primers were polymorphic and reproducible, while out of 13 RAPD primers, only 10 were polymorphic and reproducible.
ISSR banding pattern
The 10 chosen ISSR primers produced various numbers of DNA fragments, depending on their simple sequence repeat motifs. A total of 114 fragments were produced, out of which, 73 (64.03%) were polymorphic (Fig. 1). The size of the amplified products ranged from 250 bp to 2 kb. The range of polymorphism was between (37%) for K16 primer up to (91%) for K10 primer. The polymorphic information content (PIC) varied from 0.27 (K24a) to 0.45 (K15) with an average PIC of 0.39. The marker index (MI) varied from 0.93 (K16) to 2.72 (UBC 840) with an average of 2.09. Therefore the most informative primers were K11, K25 and UBC 840. The number of polymorphic loci varied from 3 (K16) to 11 (K10) with an average of 7.3 per primer (Table 3).

RAPD banding pattern
In total, 127 loci were produced out of which, 88 loci (69%) were polymorphic (Fig. 2). All the selected primers amplified DNA fragments in the 19 genotypes studied, with the number of polymorphic locus varying from 4 (OPA-04) to 19 (28) with an average of 8.8 per primer (Table 3). The size of amplified products ranged from 250 bp to 2.5 kb. The range of polymorphism was between 50% for primers 29 and OPA-04 up to 86% for primer 28. The polymorphic information content (PIC) varied from 0.28 (OPA-04) to 0.45 (P6) with an average PIC of 0.39. The marker index (MI) of primers varied from 1(OPA-04) to 5.26 (28) with an average of 2.32. The most informative primers were 28 and OPAC-06.

Grouping the cultivars
RAPD
Estimates of genetic relationships were obtained from the markers data using Jaccard’s similarity coefficient. According to the results, genetic similarity ranged from 0.56 (low similarity) between Amiri and Javad-aghai up to 0.79 (high similarity) between Gholamrezai, Khanjari-damghan and Ebrahim-abadi. Cluster analysis revealed 4 main groups (Fig. 3a). Cluster I consisted of Gholamrezai, Ahmad-aghai, Kale-gochi, Ohadi, Hosein-khani, Rezaei-zodras, Seifoddin. Cluster II consisted of Mohseni, Fandoghi-riz and Cluster III was comprised of Ghazvini, Momtaz. The last group included Shen and Ebrahim-abadi. The result of principal component analysis (PCA) was comparable to the cluster analysis and the first three most informative PC components explained 76.1 % of the total variation (Fig. 3a).

ISSR
The genetic similarity matrices were constructed using Jaccard’s similarity coefficient. According to the results, genetic similarity ranged from 0.53 (low similarity) between Kale-gochi, Hosein-khani and Ebrahim-abadi up to 0.83 (high similarity) between Kale-gochi and Ahmad-aghai. Cluster analysis using UPGMA revealed three main groups with 13, 2 and 4 pistachio varieties in each group (Fig. 3b). Cluster I consisted of Akbari, Gholamrezai, Ahmad-aghai, Kale-gochi, Ohadi, Hosein-khani, Fandoghi-riz, Shen, Lavare 2, Rezaei-zodras, Seifoddin and Momtaz (Fig. 3b). Cluster II consisted of Ghazvini and Sefid-peste and Cluster III was comprised of Mohseni, Ebrahim-abadi, Amiri, Khanjari-damghan (Fig. 3b). The result of principal component analysis (PCA) was comparable to the cluster analysis. The first three most informative PC components explained 78.4 % of the total variation.

Combined (ISSR and RAPD) data
Combining both markers, a total of 241 DNA fragments were produced, of which 161 of them (85.8%) were polymorphic with an average of 5.45 polymorphic fragments per primer. By combining markers data (RAPD + ISSR), the similarity coefficient varied from a minimum of 0.56 (Hosein-khani and Ebrahim-abadi) to the maximum of 0.81 (Ohadi and Kale-gochi) with an average similarity value of 0.68. The Mantel test between the two Jaccard’s similarity matrices gave r = 0.83 that indicated very

1.
good agreement between RAPD and ISSR marker systems. The genetic relatedness of cultivars studied through RAPD and ISSR markers, showed similar dendrogram topologies with some exceptions. However, the general dendrogram constructed using Jaccard’s similarity coefficient values of combined molecular data (RAPD + ISSR) revealed a better representation of the relationship than individual markers. The cultivars grouped into 3 main clusters and some of the clusters divided further into sub-clusters (Fig. 3c). A single cultivar, Ebrahim-abadi formed a distinct cluster and showed a distant relationship with Akbari (Fig. 3c).

**Fig 2.** Genomic DNA amplification pattern in 19 pistachio cultivars, using 10-mer RAPD primer (primer 28) (M, molecular weight marker).

**Fig 3.** UPGMA dendrogram of the 19 pistachio cultivars, based on Jaccard’s similarity coefficient using (a) RAPD (b) ISSR and (c) ISSR + RAPD data.
Results indicated the presence of a wide genetic variability among different genotypes of Pistachio. The three-dimensional ordination confirmed the cluster analysis results and the first three most informative PC components explained 76.4% of the total variation (Fig. 4a-c). The

**Fig 4.** Three-dimensional plot of principle component analysis of 19 pistachio cultivars using (a) RAPD (b) ISSR (c) RAPD+ISSR data.
grouping of cultivars using three first PC components was in agreement with the results in clustering. It showed that Ebrahim-abadi is the most distant cultivar, Ghazvini and Momtaz clustered in the same group, Mohseni, Amiri and Khandjari-damgan in other group, lavare 2, Seifedin, Rezai-zodras, Gholamrezaei, Hosein-khani, Fandoghi-riz, Ohadi, Javad-aghai and Kale-ghochi as the close cultivars in the last group.

**Discussion**

Comparative studies in Pistacia species involving RAPD, AFLP and ISSR marker systems have been successfully used by very limited researchers (Kafkas et al., 2006). In this work we compared the applicability of ISSRs and RAPDs as genetic markers for the study of genetic diversity in Iranian pistachio cultivars. Between the two marker systems employed, 10 RAPD primers produced a total of 127 DNA fragments, whereas 10 ISSR primers produced only 114 DNA fragments. The level of polymorphism revealed by RAPD (67%) was higher than ISSR (63%). In agreement with our results, Archak et al., (2003) detected 87.9 % of polymorphism in 19 cashew accession with RAPDs and 73.7 % with ISSRs. Mohd-arif et al., (1997) also reported similar results for Shisham (*Dalbergia sissoo*). It has been reported that the ability to resolve genetic variation may be more directly related to the degree of polymorphism detected by the marker system (Sivaprakash et al., 2004). Comparison of PIC values for the two marker systems indicated that the range of PIC values for RAPD primers was from 0.27 (OPA-04) to 0.45 (P6). This is because of poly-allelic nature of RAPD markers. Also the comparison of the average PIC values of ISSR primers revealed that the lowest value was 0.27 with K24a and the highest value was 0.46 with k15. RAPD and ISSR markers grouped the 19 cultivars into four and three clusters. The clustering of genotypes within groups was similar with some exceptions. A possible explanation for the difference in the resolution of RAPD and ISSR is that the two marker techniques targeted different regions of the genome. These differences may also be attributed to marker sampling errors and/or the level of polymorphism detected, reinforcing the importance of the number of loci and their coverage of the whole genome for obtaining reliable estimates of genetic relationships among cultivars (Souframanien and Gopalakrishna 2004). The correlation between Jaccard’s similarity values obtained from two marker techniques was high (r> 0.83). This indicates very good correlation between ISSR and RAPD based similarities. ISSR method has been reported to be more reproducible (Goulao and Oliveira 2001) which produces more complex marker patterns than the RAPD approach, and is advantageous when differentiating closely related cultivars. ISSR has also been used for cultivar identification. Nevertheless, on the basis of PIC values (RAPD=0.39; ISSR=0.39), percent of polymorphism (RAPD=67%; ISSR=63%), and similarity matrix, the RAPD markers were marginally more informative than ISSR in the assessment of genetic diversity in Iranian pistachio cultivars. Similar results are reported in Caldesia grandis (Chen et al., 2006). This may be because of the fact that two marker techniques targeted different regions of the genome. Some researchers have considered RAPD markers to represent segments of DNA with non-coding regions and to be selectively neutral (Bachmann 1997; Landerdott et al., 2001). On the contrary, some other studies have shown that RAPD markers are distributed throughout the genome and may be associated with functionally important loci (Penner 1996). However, there is little information to indicate that ISSR markers are functionally important (Esselman et al., 1999).
The high similarity between Kale-gochi and Ohadi was noticed in both RAPD and combined analysis indicating that these genotypes are closely related. But high similarity of Kale-gochi and Ahmad-aghai was observed in ISSR marker system. Grouping by ISSR, showed the akbari and khanjari-damghan as most divergent ones while the Akbari and Ebrahim- abadi were most diverse using RAPD results. This revealed the existence of sufficient amount of genetic variability among the pistachio, which could be exploited further. A close genetic similarity was found in some of the cultivars analyzed as shown by high values of similarity index. Also, the similarities detected with ISSRs were greater than the similarities observed with RAPDs. Fernandez et al., (2002) studied 16 barley cultivars form different countries and found a higher similarity index by ISSRs than by RAPDs. It may be due to highly polymorphic, abundant nature of the microsatellites due to slippage in DNA replication. In summary, the markers employed either individually or in combination were effective in discriminating the different types and may be useful for better management of germplasm resources.

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References


