Assessment of Lime Genetic Diversity in Three Regions of Iran Using Morphological and ISSR Markers

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ABSTRACT
Identification of genetic diversity level is important in characterizing germplasm, clarifying genetic relationships and introducing new cultivars. In the present investigation, morphological and ISSR markers were used to identify genetic diversity among 29 citrus samples including 21 genotypes and eight cultivars. A significant variability was observed in the selected Citrus genotypes at morphological and molecular levels. Thirty-two qualitative characteristics revealed similarity ranged from 0.19 to 0.88, then the samples based on these similarities were divided into two main groups. One of these has two individual members were pummelo (Citrus maxima) and N44 (Citrus sp.). The other main group at similarity coefficient 0.38 and 0.41 were divided into three subgroups. In subgroup 3, Mexican lime (Citrus aurantifolia) was placed (with other samples that might be lime) in one group. Eight ISSR primers produced 102 fragments with an average of 66.67% polymorphism. The PIC analysis showed ranging from 0.42 to 0.49. From these results it could be understood that these set of primers can distinguish genetic differences very well. ISSR cluster divided samples into two main groups and each group was divided into two subgroups. Similarity range which was calculated based on ISSR, was recorded between 0.42-0.96. Cumulative analyses of morphological traits and ISSR data also divided genotypes in to four clusters such as morphological and ISSR. In all D-plots, the same classification was found with clustering. Both morphological and molecular analyses showed a high degree of variation among the genotypes. The present study revealed that morphological and molecular markers could be successfully used to identify genetic diversity and relationship of lime group for lime breeding programs.

Keywords: Citrus, cluster analysis, ISSR marker, morphological marker, PIC analysis.

Abbreviations:
ISSR: Inter Simple Sequence Repeats; PCA: Principal Components Analysis; PIC: Polymorphic Information content; SMC: Simple Matching Coefficient.

INTRODUCTION
The genus Citrus (family Rutaceae; subfamily Aurantioideae) includes some of the principal fruit crops of the world, such as the citrons [C. medica L.], lemons [C. × limon (L.) Osbeck], limes [C. × aurantium (L.) Blanco], mandarins [C. reticulata Blanco], sour oranges [C. × aurantium L.], sweet oranges [C. × aurantium L.; C. sinensis (L.) Osbeck], grapefruits [C. × aurantium L.; C. paradisi Macf.] and pummelos [C. maxima (Burm.) Merr.]. Based on phylogenetic relationships, different theories about the origin of citrus have been noted which most report that citrus is distributed in a wide area from Indian to North of Africa (Soost and Roose, 1996). The origin of limes ‘according to Swingle reports’ is an island in east India (Davies and Albrigo, 1994).

Although nearly all cultivated Citrus are diploids, several factors made the taxonomy of Citrus, including Lime, intricate. Hybridization of Citrus with other genera that belong to this citrus group is also possible (Iwamasa et al., 1988) and so many natural hybrids could be seen. Correct classification and identification of them, especially in breeding program, is important and helpful. Moreover, this can solve management problems such as avoiding duplication in the exchange and in
the conservation of the germplasm, certifying propagated material and inferring the genetic variability that the collection represents in order to increase or maintain an appropriate range of genetic diversity (Campos et al., 2005).

Using Molecular markers along with morphological or biochemical descriptions, provides more accurate and detailed information than classical phenotypic data. Some of these methods are used routinely in the characterization of germplasm collections of horticultural species (Karp et al., 1997). Investigating genetic diversity by using only morphological markers has serious limitations. Especially in species of a complex genus like *Citrus*, whose taxonomy is in an amorphous state due to frequent occurrence of hybridization, apomixis, polyploidy and bud mutations (Kumar et al., 2010).

Morphological traits are highly influenced by the environment. Therefore, sometimes they cannot be distinguished between closely related cultivars. On the other hand, in two different areas with different environmental conditions, two phylogenetic closely related plants may be considered different (Fang et al., 1998). However, morphological traits have been affected by the environment, they generally could be used in genetic investigations. Especially, when morphological characters were combined with molecular markers, it can be more helpful in identification and characterization of most closely related cultivars at intra-specific level.

Today, assessment of genetic diversity in plants has become far more simple, cost-effective, reliable and reproducible. They are indebted to the PCR-based DNA marker techniques, such as random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), inter simple sequence repeats (ISSR), simple sequence repeats (SSR), directed amplification of mini-satellite DNA (DAMD) and etc. (Weising et al., 2005).

Inter simple sequence repeats (ISSR) for its effective potential in disclosing polymorphisms was investigated. It can be used as markers, co-dominant advantage, extending genetic linkage map in citrus, (Cai et al., 1994). ISSR markers have been used in several earlier molecular studies in citrus for genetic diversity (Nicosi et al., 2000; Shahsavar et al. 2007; Biswas et al. 2010; Kumar et al. 2010; Marak and Laskar, 2010; Uzun et al. 2010; Uzun et al. 2011; Pal et al., 2013; Tripolitsiotis et al. 2013)

Nowadays, molecular marker techniques are routinely used for worthy characterization, management and conservation of germplasm collections of horticultural species (Karp et al., 1997). The purposes of the present study were: 1) evaluate the genetic diversity and relationships among Iranian lime genotypes and compare them by commercial cultivars, 2) compare the capability and effectiveness of morphologic traits in combination by ISSR markers on *Citrus* genotypes especially limes and 3) facilitate the use of related cultivars and hybrids in future breeding programs exploiting the Iran citrus research Institute and other researchers.

**MATERIALS AND METHODS**

**Plant Materials:**

Twenty nine citrus samples (Table 1), including 21 undefined local and native genotypes and eight known cultivars were collected from four citrus production areas in different regions in Iran (south “Minab”, central “Darab and Manojan” and north “Ramsar and Kotra”) in order to conduct morphological and ISSR analyses.

**Morphological Analysis:**

Thirty-two qualitative characteristics were evaluated by using leaves, fruits and seeds. The selection of morphological characters was made based on the descriptors developed by International Plant Genetic Resources Institute (IPGRI, 2000). The YBAR option of the Stand program from the NTSYS-pc 2.1 software was used for morphological character data standardization (Rohlf, 2000). For each sample, duplicate measurements were averaged, and data matrix of pairwise similarities between genotypes was designed. Similarity was measured by simple matching coefficient (SMC), as it was the coefficient with the best results following a cophenetic test (Mantel, 1967). Principal components analysis (PCA) was used to depict non-hierarchical relationships among the samples. Eigenvalues and eigenvectors were calculated by Minitab 16.

**Genomic DNA Extraction:**

Total DNA was isolated using the procedure described by the Diversity Arrays Technology Pty Ltd (DArT P/L) company (Diversity Arrays Technology Pty Ltd (DArT P/L), 2003).

The required amount (0.2 g approximately) of plant materials was grinded by mortar and pestle under liquid nitrogen to fine powder, then transferred to a 2 ml tube and 1 ml of fresh buffer added and incubated at 65 °C for 1 h. Then cooled down for 5 min on ice and 1 ml of chloroform: isoamyl alcohol (24:1) mixture was added; then the suspension was mixed well for 30 min. After centrifugation at 10000 g for 20 min, the water phase transferred to fresh tube, and the same volume of ice cold isopropanol added, later the tubes inverted until nucleic acids become visible. Following centrifugation at 10000 g for 30 min, the supernatant discarded and the pellet of DNA washed with 2 ml 70 % ethanol. Ethanol discarded, pellet dried and dissolved in 250 μl of 1 X TE buffer.
Table 1. Citrus samples used in morphological and ISSR analysis.

<table>
<thead>
<tr>
<th>No.</th>
<th>Scientific name</th>
<th>Common name</th>
<th>Location</th>
<th>Plant code</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Citrus sp.</td>
<td>Feri lime</td>
<td>Darab</td>
<td>D1</td>
</tr>
<tr>
<td>2</td>
<td>Citrus sp.</td>
<td>Bakra lime</td>
<td>Darab</td>
<td>D2</td>
</tr>
<tr>
<td>3</td>
<td>Citrus sp.</td>
<td>Limequat</td>
<td>Darab</td>
<td>D5</td>
</tr>
<tr>
<td>4</td>
<td>Citrus sp.</td>
<td>Cucumber lime</td>
<td>Darab</td>
<td>D8</td>
</tr>
<tr>
<td>5</td>
<td>Citrus sp.</td>
<td>Mayer lime</td>
<td>Darab</td>
<td>D9</td>
</tr>
<tr>
<td>6</td>
<td>Citrus sp.</td>
<td>Unknown</td>
<td>Manojan</td>
<td>A5</td>
</tr>
<tr>
<td>7</td>
<td>Citrus sp.</td>
<td>Unknown</td>
<td>Manojan</td>
<td>A6</td>
</tr>
<tr>
<td>8</td>
<td>Citrus sp.</td>
<td>Unknown</td>
<td>Manojan</td>
<td>A7</td>
</tr>
<tr>
<td>9</td>
<td>Citrus sp.</td>
<td>Unknown</td>
<td>Manojan</td>
<td>A8</td>
</tr>
<tr>
<td>10</td>
<td>Citrus sp.</td>
<td>Rudan lime</td>
<td>Minab</td>
<td>M1-1</td>
</tr>
<tr>
<td>11</td>
<td>Citrus sp.</td>
<td>Unknown</td>
<td>Minab</td>
<td>M1-3</td>
</tr>
<tr>
<td>12</td>
<td>Citrus sp.</td>
<td>Unknown</td>
<td>Minab</td>
<td>M1-5</td>
</tr>
<tr>
<td>13</td>
<td>Citrus sp.</td>
<td>Unknown</td>
<td>Minab</td>
<td>M1-7</td>
</tr>
<tr>
<td>14</td>
<td>Citrus sp.</td>
<td>Unknown</td>
<td>Minab</td>
<td>M1-10</td>
</tr>
<tr>
<td>15</td>
<td>Citrus sp.</td>
<td>Unknown</td>
<td>Minab</td>
<td>M1-13</td>
</tr>
<tr>
<td>16</td>
<td>Citrus sp.</td>
<td>Unknown</td>
<td>Minab</td>
<td>M1-14</td>
</tr>
<tr>
<td>17</td>
<td>Citrus sp.</td>
<td>Mazandaran lime</td>
<td>Kotra (Tonekabon)</td>
<td>N45</td>
</tr>
<tr>
<td>18</td>
<td>Citrus sp.</td>
<td>Khoramab hybrid</td>
<td>Kotra (Tonekabon)</td>
<td>N38</td>
</tr>
<tr>
<td>19</td>
<td>Citrus sp.</td>
<td>Limo gelab amol</td>
<td>Kotra (Tonekabon)</td>
<td>N44</td>
</tr>
<tr>
<td>20</td>
<td>Citrus sp.</td>
<td>Unknown</td>
<td>Kotra (Tonekabon)</td>
<td>N39</td>
</tr>
<tr>
<td>21</td>
<td>Citrus sp.</td>
<td>Khoeshe lime</td>
<td>Kotra (Tonekabon)</td>
<td>N42</td>
</tr>
<tr>
<td>22</td>
<td>Citrus auranti folia (chrism.) Swing.</td>
<td>Mexican lime</td>
<td>Ramsar</td>
<td>S37</td>
</tr>
<tr>
<td>23</td>
<td>Citrus limon (L) Burm. F.</td>
<td>Lisbon lemon</td>
<td>Ramsar</td>
<td>S46</td>
</tr>
<tr>
<td>24</td>
<td>Citrus sinensis (L) Osbeck</td>
<td>Siavaraz sweet orange</td>
<td>Ramsar</td>
<td>S47</td>
</tr>
<tr>
<td>25</td>
<td>Citrus reticulata Blanco</td>
<td>Dancy mandarin</td>
<td>Ramsar</td>
<td>S48</td>
</tr>
<tr>
<td>26</td>
<td>Citrus grandis (L) Osbeck.</td>
<td>Pummelo</td>
<td>Ramsar</td>
<td>S50</td>
</tr>
<tr>
<td>27</td>
<td>Citrus paradisi Macf.</td>
<td>grapefruit</td>
<td>Ramsar</td>
<td>S52</td>
</tr>
<tr>
<td>28</td>
<td>Citrus aurantium L</td>
<td>Sour orange</td>
<td>Ramsar</td>
<td>S53</td>
</tr>
<tr>
<td>29</td>
<td>Citrus medica L</td>
<td>Citron</td>
<td>Ramsar</td>
<td>S54</td>
</tr>
</tbody>
</table>

ISSR Analysis:

For DNA amplification, 15 ISSR primers were initially screened and finally eight primers that produced scorable polymorphic bands were used for further analyses. DNA amplification was carried out in 12.5 μl reactions containing 50 ng of template DNA, 0.2 mM dNTPs, 0.8 μM primers, 1.25 μl of 10X PCR buffer (buffer containing of magnesium chloride) and 1 unit of Taq DNA polymerase (EC 2.7.7.7) (recombinant, Fermentas, Canada). Cycling conditions consisted of: 94 °C for 3 min; 35 cycles of 94 °C for 1 min, 55 °C for 45 s, and 72 °C for 1 min; and one final cycle of 72 °C for 7 min. PCR products were loaded on 1.5% (w/v) agarose gel containing 1X TBE (45 mM Tris–borate 1 mM EDTA) and 0.5 μg ml⁻¹ aqueous solution of ethidium bromide. Samples were then separated through electrophoresis at constant voltage 90 V for 2 h. DNA fragments were visualized and documented with the help of UVdoc system (UVP, USA). Reproducibility of the patterns was confirmed by running all the reactions in duplicates. Information of ISSR primers are provided in Table 2.

Molecular Data Analysis:

Reproducible DNA fragments were scored as present (1)/absent (0) for each reaction and were assembled in a binary data matrix table. Genetic similarities were calculated using the Dice coefficient and tree method UPGMA, complete and single for clustering, and then dendrogram was constructed. Mantel test was used to compute the cophenetic correlation and finding best dendrogram. All analyses were performed using the NTSYS-PC 2.01 software (Rohlf, 2000). The level of Polymorphic Information Content was calculated as $PIC_i = 2fi(1 - fi)$, where $PIC_i$ is the PIC of marker i, $fi$ the frequency of i th marker fragment when present and 1 - $fi$ is the frequency of ith marker when absent (Roldain-Ruiz, 2000).

RESULTS AND DISCUSSION

Morphological Analysis:

Characterization was done based on IPGRI descriptor (IPGRI, 2000) list on leaf, stem, and fruit morphology in some limes, which were collected from different regions in Iran (south “Minab”, central “Darab and Manojan” and north “Ramsar and Kotra”). Comparative analysis on 32
morphological characteristics in *Citrus* genotypes and cultivars showed moderate variations. A pairwise similarity among the samples ranged from 0.19 to 0.88 with an average of 0.4 based on morpho-metric data. The highest similarity (0.88) was observed between "M1-7 and M1-10", two genotypes of south region (Minab), while the lowest (0.19) was found among three standards and genotypes "S50-S54", "S50-A8" and "S37-M1-13". In previous study, it was clarified that pummelo (*C. maxima*) is a true citrus (Barrett and Rhods, 1976), so this difference was predictable. On the basis of morphological parameter, our obtained similarity was supported by the result of Campos et al. (2005) and Pal et al. (2013), reported similarity coefficient between 0.10-0.90 and 0.15-0.75, respectively. Considering these result, it could be understood that morphological characters can distinguish samples.

Dendrogram, which was generated based on morphological parameters, grouped samples into two main clusters (Fig. 1). Cluster one which was separated from other group in 0.35 of coefficient similarity (approximately), just has two individual members, N44 (Limo Golab Amol) that is a natural hybrid without any knowledge about its region and parents, next member was pummelo (*C. maxima*) or S50, both of them have exclusive characters. Considering the simple morphological data of N44 (Limo Golab Amol), it can be found that fruit parameters are very different and specific from every common citrus and also in leaves, flowers, so it could be inferred that Limo Golab Amol grouped in a different cluster of other *Citrus* genus and related.

Cluster number two has 27 members and was divided into three sub-clusters. At 0.38 coefficient similarity (approximately), the first sub-cluster which consists of Minab samples, was separated from other samples and this result points out that Minab samples have separate evolutionary paths or the environment has affected them. So, in order to answer the question, it should use stronger markers that have not been affected by environment also cover large amount of genome. Second sub-cluster included five cultivars [Sweet orange, Citron (*C. medica*), Sour orange (*C. aurantium*), Dancy mandarin (*C. reticulata*) and Grapefruit (*C. paradisi*)] and one genotype N38. Locating sample N38 beside other cultivars was not unpredictable, as it is manmade hybrid between sweet lime (*C. limettioides* Tan) and Valencia orange (*C. sinensis* Osbeck) in Iran Citrus research Institute. But, about this sample in its cluster, one thing that must be noted is that N38 separated from other cultivars that were studied in this investigation at 0.48 coefficient similarity. This was also predictable too, because its parents were sweet limes and it is different from other cultivars that have been studied. Same result, separation of sweet lime from other cultivars, was reported by shahsavar et al. (2007), when he used ISSR marker. So according to their report, our result "separation of N38 from other cultivars" is completely precise.

Third sub-cluster is the biggest group and has 14 members including two cultivars of Mexican lime (*C. auranifolia*) and Lisbon lemon (*C. limon*) and 12 genotypes (from north and central regions). Lisbon lemon (*C. limon*) is different from lime, as our selected samples were from north and central areas based on the limes, it was expected that Lisbon lemon (*C. limon*) should be separated from other samples and S37 or Mexican lime (*C. auranifolia*) should be placed between other samples and it can be done at the time when the cluster generated. Placement of Lisbon lemon (*C. limon*) in this cluster due to suggested parents for lemon (Barrett and Rhods, 1976) Lime and Citron was expected.

The copheneticalAnalyses comparing the UPGMA cluster analysis and the simple matching similarity matrix demonstrated that the correlation was 0.78, indicating that data in the matrix was good represented by the dendrogram. The greatest number comparing the coefficient matrix and cophenetic matrix indicating better fitting for the cluster and similarity matrix (Nei, 1972).

<table>
<thead>
<tr>
<th>No.</th>
<th>Primer</th>
<th>Sequences (50–30)</th>
<th>Total Number of Amplified Bands</th>
<th>Number of Polymorphic Bands</th>
<th>Percent of Polymorphism</th>
<th>PIC Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>P1</td>
<td>(AG)BG</td>
<td>11</td>
<td>7</td>
<td>63.64</td>
<td>0.48</td>
</tr>
<tr>
<td>2</td>
<td>P2</td>
<td>(GA)BC</td>
<td>12</td>
<td>8</td>
<td>66.67</td>
<td>0.48</td>
</tr>
<tr>
<td>3</td>
<td>P3</td>
<td>(AC)8C</td>
<td>14</td>
<td>9</td>
<td>64.29</td>
<td>0.42</td>
</tr>
<tr>
<td>4</td>
<td>P4</td>
<td>(ATC)6T</td>
<td>14</td>
<td>11</td>
<td>78.57</td>
<td>0.42</td>
</tr>
<tr>
<td>5</td>
<td>P5</td>
<td>(AC)8YC*</td>
<td>13</td>
<td>8</td>
<td>61.54</td>
<td>0.49</td>
</tr>
<tr>
<td>6</td>
<td>P6</td>
<td>(GA)RA</td>
<td>14</td>
<td>10</td>
<td>71.43</td>
<td>0.49</td>
</tr>
<tr>
<td>7</td>
<td>P7</td>
<td>(GAA)6</td>
<td>11</td>
<td>6</td>
<td>54.55</td>
<td>0.47</td>
</tr>
<tr>
<td>8</td>
<td>P8</td>
<td>(GA)8YG</td>
<td>13</td>
<td>9</td>
<td>69.23</td>
<td>0.48</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>102</td>
<td>68</td>
<td>66.67</td>
<td>0.48</td>
</tr>
</tbody>
</table>
A two-dimensional plot (2D plot) generated from PCA showed four groups. It was found almost similar to the clustering pattern of UPGMA dendrogram. In 2D plot, genotype M1-13 was placed with distance by other Minab genotypes. It placed by Dancy mandarin (C. reticulata) and pummelo (C. maxima) in one group which was a new group. In the dendrogram, N44 was grouped along with S50 but in 2D plot, it clustered with D8, S46, S52 and S53. This result is believable due to all samples in this new group which have similar fruits in size (bigger than lime especially). Interestingly, grapefruit (S52) (C. paradisi) and sour orange (S53) (C. aurantium) in this group were neighbors. This finding is supported by previous investigation which suggested sour orange (C. aurantium) as one of parents of grapefruit (Li et al., 2010). All Minab samples generated the third group except M1-13. In this group, all Minab genotypes were closely related except M1-3, at similarity matrix based on morphology data. These samples were represented similarity between 0.47-0.88, it could be understood that they have genetic relationships. Other samples were placed on the fourth group, most samples in this group belong to lime but it should be noted that, three members (S47, S53 and N38) were not lime. Fig. 2 shows D-plot that generated based on morphological characters.

![Dendrogram generated based on morphological traits of 29 of citrus samples and cultivars collected from different region of Iran based on SM coefficient using UPGMA method.](image)

The analysis oriented the first five principal components, which contributed 66.40 % of the total variability of collected genotypes. Maximum variability was contributed by the first component (45.27 %) followed by the second component (7.82 %), and the third component (5.10 %).

**ISSR Analysis:**

Eight primers were selected for the ISSR analysis based on the reproducibility and banding patterns from 15 ISSR primers which were used for ISSR amplification of 29 DNA samples extracted from citrus samples. The amplified fragments size ranged from 100 to 2000 bp with the scorable region being from 150 to 1500 bp. The number of fragments per primer ranged from 7 [(AG)8G] to 12 [(GA)8C and (GAA)6]. The number of polymorphic fragments scored range from 5 with (AG) 8G and (GA) 8A to 9 with (AC)8C with an average of 6.6 per primer. Between the total 78 scorable fragments, 67.94 percent of them were polymorphic among the samples (Table 2). At first, this percentage of polymorphism was sound low, while in previous investigation that samples with close relation were studied, this kind of percentage was found (Lombardo et al., 2011).

The correlation coefficient (r), based on Mantel Z-statistics (Mantel, 1967), was calculated for all Dice’s coefficient in three method for clustering (UPGMA, complete and single). UPGMA was shown upper correlation coefficient (0.831). The amount of similarity between similarity matrix and the cluster was shown with this coefficient. A better method for design cluster and calculate similarity
matrix could be found by the greatest number in comparison between the coefficient matrix and cophenetic matrix. To find the potential of ISSR markers, which used in our investigation to distinguish our samples in correct way, polymorphic information content (PIC) was calculated by using formula that was introduced for dominant marker by Roldain-Ruiz et al. (2000). Regarding the data presented in table 2, all the PICs were high (minimum was 0.42 and maximum was 0.49). This range of PIC showed the efficiency of the applied molecular marker used to detect polymorphism within the citrus and especially in lime group.

Pairwise DICE coefficient among 29 samples was calculated and ranged from 0.41 to 0.97. The highest and lowest similarities were observed between A7-A8 and S50-S53, with an average of 0.65 based on ISSR data. With comparison of similarities in our study and other studies, same results was found. Pal et al. (2013) reported max, min and average similarities values of 0.42, 0.96 and 0.69, respectively. Shahsavari et al. (2007) showed similarities between 58% and 92%. The lowest and the highest similarities which was calculated by Gulsen and Rose (2001) were 47% and 99%, respectively. These similar results confirm that, ISSR marker could be applied with high supremacy for distinguishing genetic relationships in Citrus and related genera.

Based on these polymorphic bands, similarity coefficient was calculated and similarity dendrogram (Fig. 2) was constructed using UPGMA cluster analysis. On the basis of clustering analysis, the 29 samples could be classified into two major groups at 58% similarity. Both groups were divided into two sub-groups at approximately 63±0.5% similarity.

Sub-group 1-1 consists of only Pummelo (C. maxima) which is separated from other groups. Our results were similar with previous studies such as: Shahsavari et al. (2007), Nematollahi et al. (2013), and Abedinpour et al. (2014). This result supported the previous theory suggested pummelo (C. maxima) as one of the true citrus (Barrett and Rhods, 1976). In other sub-group (1-2) in this cluster, all members were from Minab region. Maximum similarity in Minab samples found between M1-7 and M1-10 (92% approximately) as in morphological analysis. Therefore, Minab samples might have special evolution way, so placing them in a separate group was not strange. In selecting samples from Minab region, we tried to find some genotypes which have history behind themselves, not derived from central or north region of Iran. Approximately all of them may be derived from sexual propagation (in the past, lime was propagated by seed in Iran) or bud mutation.

Group two, also, divided into two sub-groups, including all standard samples (cultivars) which not known as lime or lemon plus N38 (manmade hybrid) were placed in sub-group 2-1. As described in morphological analysis, this classification (classification of N38 with standards) was predictable because parents of this hybrid were sweet lime and sweet orange (Valencia). So, its placement in a group with sweet orange (S47), Dancy mandarin (S48) (C. reticulata), grapefruit (S52) (C. paradisi), and sour orange (S53) (C. aurantium) could be predicted. In studies that
sweet lime or Valencia orange were investigated, it could be found that all sweet oranges generated one group with high similarity (Golein et al., 2012) or all samples that show orange type in morphological characteristics, have high similarity (Nematollahi et al., 2013). One thing about this sample that should not be forgotten that one of the parents was sweet lime. When back to calculated similarity matrix, it was found that N38 similarity with other standards was between 0.63-0.78. It confirmed from this matrix the similarities were not so high that placed N38 in orange group and not enough low that could differ between them. Separation of sweet orange (S47) from grapefruit and pummelo (C. maxima) were reported by Barrett and Rhodes (1976) previously. Genetic relationship that found between grapefruit (C. paradisi) and pummelo (C. maxima) was supported by result of using RAPD and SCAR marker (Gmitter, 1995; Nicolosi et al., 2000). As mentioned before, pummelo (C. maxima) is one of the three citrus types that Barrett and Rhodes (1976) proposed as a true species.

Considering the cluster, none of unknown samples (that classified as lime) were not placed with standards. Comparing this result with other studies on lime (Shahsavar et al., 2007; Lombardo et al., 2011) or lemon (Gulsen and Roose, 2001) it seems logic.

Sub-group 2-2 was the largest sub-group in four sub-clusters by covering of 17 samples in this study [Mexican lime (C. auranifolia) and Lisbon lemon (C. limon) as cultivars and 15 genotypes that are selected from three regions of Iran in this study]. Placement of Lisbon lemon (C. limon) in sub-cluster 2-2 is not amazing due to lemon was known as hybrid of citron and lime (Barrect and Rhods, 1976). The similarity of lemon with citron and lime were calculated 0.68 and 0.73, respectively, which support the opinion of origin of lemons. In Shahsavar et al.’s (2007) study on limes of central Iran, similar result and high similarity, was reported. Moreover, lime and lemon were placed into one group and separated at 64% similarity (approximately). However, despite differences in morphological characters, genetic variation which could be found in Citrus and related genera was low (Fang and Roose, 1997). According to recent studies using a large number of citrus, high level of genetic similarity were obtained among oranges (Shahsavar et al., 2007; Uzun, 2009; Lombardo et al., 2011; Golein et al., 2012; Nematollahi et al., 2013). Furthermore, genotypes arising from spontaneous mutations are also often difficult to be distinguished (Barkley et al. 2006).

High percent of heterozygosity was reported by Herrero et al. (1996) in limes and lemons. Therefore the major source of variation in these varieties is thought to be hybridization or bud mutation. Without much difficulty, Citrus species and related genera could fertilize each other and produce new hybrid (Iwamasa et al., 1988), so numerous instances of naturally hybrids have been reported (Swingle, 1943). Clustering of investigated samples are shown in Fig. 3.

**Fig. 3.** Dendrogram generated based on ISSR data of 29 of citrus samples and cultivars collected from different region of Iran based on DICE’s coefficient using UPGMA method.
For better visualization of relations among studied genotypes, the principal components analysis (PCA) was performed. The principal components analysis is probably an example of dimensionality reduction. The resulted D-plot according to ISSR data are demonstrated in Fig. 4.

A two-dimensional plot generated from PCA based ISSR data, showed four groups too, which were found to be almost similar to the clustering pattern of sub-groups in UPGMA dendrogram with some differences. In a 2D plot, citron (S54) (C. medica) standard sample was placed with limes and lemon in on group (A), this supported the theory of lemon was generated from citron and lime (Barrect and Rhods, 1976). In this group, it should be noted that Mexican lime (C. aurantifolia) and neighbors of it, have small fruits (which usually known as character of lime) and Lisbon lemon (C. limon) and its neighbors have larger fruits than them (which usually known as character of lemon). In group B, N38 (hand hybrid) revealed high similarity with S47 (C. sinensis). This is in agreement with the information of parents of this hybrid (one parents of this hybrid was Valencia (sweet orange). In D-plot analysis based ISSR data, S53 (sour orange) (C. aurantium) and S52 (grape fruit) were found in one group, interestingly these sample were tended to group A. these both were supported the genetic relation between sour orange, lime and grape fruit (grape fruit was derived from hybridization between lime and sour orange (C. aurantium) (Li et al., 2010). Minab samples were generated in a especial group (group C) that all members collected from south of Iran but two samples of this area were went and set in group A, which may be derived from another place or from bud mutation. Separation of pummelo (S50) (C. maxima) also happened in D-plot analysis and it clustered alone in a further group (D).

The analysis demonstrated that, the first five principal components have contributed 87.00% of total variability of collected genotypes. Maximum variability was created by the first component (67.29 %) followed by the second (6.97 %), and the third components (6.11 %).

Cumulative Data Analysis of Morphology and ISSR:
Cumulative data analysis of morphology and ISSR showed a well correlation between two dendrograms and similarity matrix ($r= 0.84$, $P=1.00$). A pairwise similarity among the samples in our study was ranged from 0.31 to 0.89 with an average of 0.54 based on combined morphological data and ISSR data derived from simple matching method (SM). Maximum similarity (89 %) was observed between two genotypes from Minab (M1-7 and M1-10) and the same result was observed in morphological analysis. This was while minimum similarity was observed between N38 and pummelo (S50) (C. maxima) with the similarity value of 0.31.
A dendrogram generated based on combined morphometric data and ISSR data grouped 29 samples into four major clusters (Fig. 5). Group one just had one member and it was pummelo (S50) (C. maxima) which was separated from other samples at 42% similarity. This result supported the theory
that said, it is one of true citrus (Barrect and Rhods, 1976). Group two consisted of 28 other samples that were divided to three sub-groups. Sub-group 2-1 covered Minab samples. In this sub-group, two genotypes (M1-13 and M1-14) that were separated from others in ISSR analysis, come back and placed with others samples but, they were separated at 0.55 similarity coefficient (approximately). Other five genotypes were shown high similarity (0.69-0.89). From these results it could be understood that these two genotypes (M1-13 and M1-14), may have had different ways of evolution.

Sub-group 2-2 had the same morphological and ISSR analyses and consisted of standard samples and one genotype from north (N38) except Mexican lime (S37) (C. aurantiifolia). Regarding the cluster of combined morphometric and ISSR data, it was found that Lisbon lemon (S46) (C. limon) was grouped with other standard samples in this sub-group. Genotype N38, as previously mentioned, had the parents of Valencia orange (C. sinensis) and sweet lime (C. limettioides). Classification of this group was in agreement with this result.

Sub-group 2-3 covered all samples that are known as lime and lemon. Four genotypes (D1, D5, A6 and N45) that in D-plot of ISSR data were placed near N46, in cumulative data analysis of morphology and ISSR, were classified with Mexican lime (C. aurantiifolia) into sub-group 2-3. Similarity coefficient of these four genotypes with Mexican lime (C. aurantiifolia) was higher than lemon (C. limon). So, it might be true that, all studied genotypes, belong to the lime group in citrus. Another thing that should be noted from cluster of these analyses is this fact that all genotypes which were collected from north of Iran, generated a new sub-group in sub-cluster 2-3 with Mexican lime (C. aurantiifolia). This kind of classification according to propagation method of citrus become true because in in south of Iran, citrus especially lime, is usually propagated by seed and in north by grafting. So genotypes from north might be derived from bud mutation from lime.

D-plot generated from PCA of combined morphometric and ISSR data (Fig. 6) also supported the clustering pattern of UPGMA dendrogram approximately with some differences. In D-plot, the samples were classified in three groups while in dendrogram samples were placed in four groups. Two groups of D-plot were the same as cluster group. By D-plot analysis, pummelo (C. maxima) was generated one group alone and Minab samples also formed another group but, M1-13 and M1-14 were placed on the third group with standard samples, north and central samples look alike ISSR D-plot. Generation of this group was not
against cluster category due to in similarity matrix data showed high similarity almost. In north and central parts of Iran all kinds of citrus were cultivated but in south mainly lime was cultivated so lime fertilized pistil of flowers in most cases, but in north and central part of Iran other cultivars could fertilized flowers. Another reason to confirm this classification is that in north and central Iran, propagation was done by grafting and seed. In grafting propagation usually mutant that showed especial character were grafted and propagated therefore integration of Citrus and related genera may have happened. On the other hand, in the south especially limes were propagated by sexual method (seed), as the main citrus were lime. So integration of Citrus and related genera is lower and in this case separation of them could be from other citrus.

First ten PCs contributed 86.18% of the total variability of the analyzed samples. The first five PCs accounted for 76.15% of the total variability and the first three accounted for 69.10% of the variance, in which maximum variability was contributed by first component (55.85%) followed by second component (7.07%), and third component (6.17%).

**CONCLUSION**

Morphological and ISSR analyses among some genotypes and cultivars from three regions of Iran were successfully employed to compute genetic variability and to calculate genetic relationships. According to our data, it was confirmed that morphological and ISSR markers analyses in limes and other Citrus species were successfully utilized for estimating genetic diversity and relationship which were in agreement with result of Malik et al. (2012). Characterization by using morphological parameters based on 32 characters, exposed significant diversity (32-88%) in traits of leaf, fruit and seed.

ISSR data generated from 21 genotypes and eight citrus cultivars with eight primers, respectively, were sufficient to provide inferences on genetic differentiation and relationships among them. PIC values were also recorded high in ISSR (0.48) markers, showing the efficiency of molecular marker application in order to detect polymorphism within the lime group. In both ISSR and morphological analyses, 29 genotypes were classified into four groups by some differences. These differences were previously reported by Koehler-Santos et al. (2003) in mandarins.

This study represented the first attempt to use morphological traits with ISSR markers to study genetic diversity of Iranian lime genotypes from three different regions that their relationships were somewhat clarified. The results of this study also open a door to tackle the long standing problem of citrus classification and identification in Iran. But, we suppose that this kind of study needs to be continued since Iran has a very large and numerous citrus germplasm. In south and central regions of Iran, it is being propagated by seed which gives researchers a chance to find new genotypes that need to be classified, investigated and introduced as a new cultivar.
References


Kumar, S., S.N. Jena and N.K. Nair. 2010. ISSR polymorphism in Indian wild orange (Citrus indica) Tanaka, Rutaceae) and related wild species in North-east India. Scientia Horticulturae. 123: 350–359.


