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# Assessment of Genetic Diversity in Iranian Apple Genotypes Using SSR Markers

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**ABSTRACT**

Various markers can be used for accurate identifications of plant genotypes and cultivars. Since microsatellite markers of simple sequence repeats (SSRs) are abundant and reliably reproducible, 14 pairs were used for evaluating polymorphic levels among 33 apple genotypes. All 14 pairs of primers had high degrees of polymorphism, ranging from three alleles (in the case of primers CH01h01 and CH02d12) to 12 alleles in primer CH05d04. In total, 83 polymorphic alleles appeared in these 14 SSR loci (with an average of 5.92 alleles per gene locus), and the polymorphic information content averaged 0.71. Dendrograms for molecular data were drawn based on the UPGMA method, and genotypes were divided into six main groups. The genotypes of Shahrood 20 and Shahrood 21 (95%) had the highest similarity with each other, while Shahrood 3 and Palestinian Malayer (14%) had the lowest. Principal component analysis confirmed the results of cluster analysis to determine relationships between the genotypes.

## Introduction

Apple (*Malus domestica Borkh*.) is a complex species of genetic diversity that belongs to the Rosaceae family. Iran's proximity to the origin of apples in Central Asia and Kyrgyzstan makes the country a large secondary source of origin regarding apple diversity (Ariel et al., 2020). The global spread of this fruit tree species has mostly resulted from the extraordinary diversity of different cultivars and varieties, as well as the implementation of extensive breeding projects in important apple research centers (Ahmadi et al., 2015). The study and determination of genetic diversity in plant materials have great importance as they foreground fundamental steps in identifying and preserving hereditary resources, essential for genetic research and breeding programs. Therefore, accurate collection, identification of the country's genetic resources, and evaluation of these genotypes can be regarded as the first step in genetic and breeding research. Contrary to their similar appearance, some apple cultivars are given different local names and, unfortunately, their genetic relationships with each other are not very clear at this stage of scientific progress (Gharghani et al., 2016).

Previously, the identification and evaluation of genetic diversity and classification of cultivars were based on morphological and physiological traits, as well as agronomic characteristics and responses (Schmidt et al., 1985). These characteristics sometimes generate limitations as they are affected by environmental factors and, thus, a large population of mature plants is required before aiming to achieve accurate results. Today, DNA-based markers are used for evaluating genetic diversity and molecular fingerprints. With respect to research on fruits, we can refer to SSR and AFLP as the most famous molecular markers (Salehi

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Sardoei and Noorbakhash, 2020; Gharghani et al., 2009). Microsatellite markers, like SSRs, are second-generation DNA markers that have been used commonly in research, probably because of their high diversity, frequency, reproducibility, Mendelian inheritance, and dominant nature. Microsatellites are simple-repetitive or highly-variable sequences, with arrays of 1-6 nucleotide short motifs. Applications of microsatellite markers include genetic structure analysis of populations, cluster analysis, kinship determination, and gene loci that control quantitative traits (Li et al., 2000). To date, research has evaluated molecular markers to study the genetic diversity of different apple cultivars. An inquiry into 44 Iranian apple cultivars showed that the average number of effective alleles was 2.2. The content of polymorphic information ranged from 0.18 ($Hi0_3ao_3$) to 0.76 ($CH0_3c0_2$), and the average was 0.49 (Farrokhi et al., 2011). Another research on 66 commercial apple cultivars, using six SSR primers, showed 55 polymorphic alleles with a polymorphic information content (PIC) value of 0.72 and an average of 9.2 alleles per gene locus (Hokanson et al., 2001). Pereira et al. (2007) studied 114 local apple cultivars and 26 non-local commercial cultivars to assess the extent of genetic differences among them using 10 pairs of SSR markers. In all cultivars, 119 alleles were found, of which 10 alleles were less abundant than the other alleles. Naghshin et al. (2008) evaluated genetic relationships between Iranian Gollab apples and foreign cultivars, as well as native ones, using 28 pairs of SSR primers. In their study, each gene locus had a number of alleles that varied from 2 to 11, averaging 5.4. Moreover, the polymorphic information content ranged from 0.37 to 0.77 with an average of 0.68. The overall result was a common origin of rose apples in Iran. The heterozygosity values, which were expected and observed in different gene loci, showed differences although the means of these two indices for different loci were close to each other. Apple microsatellite markers are highly polymorphic and can be useful in studying apple genetic diversity because of their high heterozygosity and the high number of alleles observed in them, with an average of 6.22 alleles that described all gene loci in a general manner (Boteh Khak et al., 2018). In identifying 24 genotypes, among which were 5 native apple genotypes, as well as foreign ones, the results showed the high efficiency of SSRs in the molecular identification of genetic diversity (Gahromee et al., 2009).

Today, the available information on the genetic diversity of Iranian native apples is insufficient. There is no specific identity for many genotypes that deserve to become cultivars. Many apple genotypes can enter horticultural recognition if they are identified scientifically and in accurate molecular terms. Since Iran is one of the largest apple producers in the world, this study evaluated the possibility of identifying several native apples in Iran, along with their genetic relationships, using microsatellite markers. This research aims to identify the genetic diversity of Iranian native apples as a pioneering research in the Iranian context. Here, it is hypothesized that the genetic diversity of native Iranian apples is not entirely a function of geographical diversity. Meanwhile, it is highlighted that SSR markers are apt for relevant studies on genetic diversity and for the identification of different apple genotypes.

## Materials and Methods

### *Plant materials*

This research considered 33 distinctive local apple genotypes, which had been indexed within the apple cultivars collection of the Seed and Plant Breeding Institute in Kamalabad, Karaj, Iran (Table 1). The tested plant samples were collected from offshoots. The young branches were promptly frozen in liquid nitrogen and then exposed to a temperature of -80 °C. SSR markers were selected based on the degree of polymorphism and locus-specific genetics so as to ensure uniform genomic coverage (Silfverberg-Dilworth et al., 2006).

### *DNA Extraction and Polymerase Chain Reaction (PCR)*

DNA extraction was conducted according to the Doyle and Doyle method (Doyle and Doyle, 1987) with slight modification. The purification procedure was distinct in that it was repeated for each sample, using a chloroform/isoamyl alcohol mixture (ratio: 1:24). Then, fourteen pairs of microsatellite primers were selected according to previous indications in the available literature (Celton et al., 2009; Goulao and Cristina, 2001; Gianfranceschi et al., 1998; Guilford et al., 1997; Kenis and Keulemans, 2005; Liebhard et al., 2002; Nicole and Russo, 2008; Sawamura et al., 2000; Celton Yamamoto et al., 2004) (Table 2). The polymerase chain reaction operated using PCR. The mixture of each PCR reaction contained 10 µl of the materials purchased from CinnaGen and comprised 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 µM of each primer, 1U Taq DNA polymerase, 5 ng DNA, 10 mM Tris-HCL pH 9.0, and 0.2 mM of each dNTPs. The PCR program ran with an initial denaturation at 94 ºC for 10 min, followed by 25 denaturation cycles for 30 seconds at 94 ºC. Then, it involved 30 seconds at 50-55 ºC, followed by an expansion for 10 min at 72 ºC, which was terminated with a 10-min expansion at 72 ºC.

**Table 1.** Thirty three genotypes were used in the current study

| No | Genotype name | No | Genotype name | No | Genotype name |
|---|---|---|---|---|---|
| 1 | Shahrood 1 | 12 | Shahrood 17 | 23 | Malayer 6 |
| 2 | Shahrood 2 | 13 | Shahrood 18 | 24 | Malayer 8 |
| 3 | Shahrood 3 | 14 | Shahrood 19 | 25 | Malayer 9 |
| 4 | Shahrood 4 | 15 | Shahrood 20 | 26 | Palestinian Malayer |
| 5 | Shahrood 5 | 16 | Shahrood 21 | 27 | Rootstock chenger |
| 6 | Shahrood 8 | 17 | Shahrood 22 | 28 | Rubina |
| 7 | Shahrood 10 | 18 | Shahrood 23 | 29 | Yellow Autumn Gollab |
| 8 | Shahrood 12 | 19 | Shahrood 24 | 30 | Zeynal Autumn Gollab |
| 9 | Shahrood 14 | 20 | Shahrood 31 | 31 | Gami Almasi |
| 10 | Shahrood 15 | 21 | Malayer 1 | 32 | Manuchehri |
| 11 | Shahrood 16 | 22 | Malayer 5 | 33 | Yasuj Autumn Gollab |

**Table 2.** Characteristics of SSR primers for studying genetic diversity in the native Iranian apple genotypes

| Primer Name | Forward primer sequence (5'_3'_)<br>Reverse primer sequence (5'_3'_) | Annealing Temperature (AT) | References |
|---|---|---|---|
| CH05g08 | CCA AGA CCA AGG CAA CAT TT<br>CCC TTC ACC TCA TTC TCA CC | 50 | Smolik et al. 2004 |
| 02b1 | CCG TGA TGA CAA AGT GCA TGA<br>ATG AGT TTG ATG CCC TTG GA | 50 | Smolik et al. 2004 |
| CHO2b10 | CAA GGA AAT CAT CAA AGA TTC AAG<br>CAA TG GCT TCG GAT AGT TG | 53 | Smolik et al. 2004 |
| CHO2d12 | AAC CAG ATT TGC TTG CCA TC<br>GCT GGT GGT AAA CGT GGT G | 50 | Smolik et al. 2004 |
| CHO1hO1 | GAA AGA CTT GCA GTG GGA GC<br>GGA GTG GGT TTG AGA AGG TT | 50 | Smolik et al. 2004 |
| MS14h03 | CGC TCA CCT CGT AGA CGT<br>ATG CAA TGG CTA AGC ATA | 50 | Smolik et al. 2004 |
| CHO2h11a | CGT GGC ATG CCT ATC ATT TG<br>CTG TTT GAA CCG CTT CCT TC | 51 | Smolik et al. 2004 |
| CH03a04 | GAC GCA TAA CTT CTC TTC CAC C<br>TCA AGG TGT GCT AGA CAA GGA G | 51 | Smolik et al. 2004 |
| CH02g09 | TCA GAC AGA AGA GGA ACT GTA TTT G<br>CAA ACA AAC CAG TAC CGC AA | 53 | Smolik et al. 2004 |
| CH01f03b | GAG AAG CAA ATG CAA AAC CC<br>CTC CCC GGC TCC TAT TCT AC | 51 | Smolik et al. 2004 |
| CH01f07a | CCC TAC ACA GTT TCT CAA CCC<br>CGT TTT TGG AGC GTA GGA AC | 53 | Smolik et al. 2004 |
| CH05d04 | ACT TGT GAG CCG TGA GAG GT<br>TCC GAA GGT ATG CTT CGA TT | 51 | Smolik et al. 2004 |
| CH04c07 | GGC CTT CCA TGT CTC AGA AG<br>CCTCAT GCC CTC CAC TAA CA | 53 | Smolik et al. 2004 |
| CH02d11 | AGC GTC CAG AGC AAC AGC<br>AAC AAA AGC AGA TCC GTT GC | 53 | Smolik et al. 2004 |

Subsequently, all samples were stored at 4 ºC, and PCR products were electrophoresed on polyacrylic gel (6%) before being stained with silver nitrate.

### *Data analysis*

The scorable DNA parts were scored as 0 and 1 for the nonexistence and nearness of DNA groups, respectively, to analyze the DNA information. The

NTSYS V2.02 computer program was used for measuring population parameters, the number of observed successful alleles, homozygosity, as well as anticipated and vital components. Moreover, an information cluster examination was performed based on the Dice closeness coefficient and UPGMA strategy.

## Results

To evaluate the kinship rate of Iranian apple genotypes, 33 genotypes were selected and evaluated by applying 14 pairs of SSR primers which were able to amplify aptly in all genotypes herein. A total of 83 alleles were identified as polymorphisms. Table 3 provides information about the polymorphism of alleles. The highest number of observed alleles was 12 and related to the CH05d04 primer, whereas the lowest number of observed alleles was 3 and related to the $CH0_2d_{12}$ and $CH0_1h0_1$ primers. The highest degree of heterozygosity was equal to 0.89 in the $CH0_5d0_4$ primer, the lowest was equal to 0.61 in the $CH0_1h0_1$ primer, and the average was 0.74 (Table 3). The highest expected heterozygosity was 0.89 in $CH0_3a0_4$, and the relevant average was 0.79. The highest PIC (0.88) was related to gene locus CH05d04 with 12 alleles. At the same time, the lowest PIC (0.55) was observed in $CH0_1h0_1$.

The diversity among genotypes was relatively low because the average heterozygosity was 0.74, which was not much different from the expected average (0.79). (Table 2). On the other hand, CH01F03b was found to have the lowest PIC with four alleles. Twenty red- and white-fleshed genotypes had a total of 56 alleles, and the number of alleles in each locus ranged from 3 to 11, with 8 as average. The average number of effective alleles was estimated to be 5.74.

**Table 3.** Polymorphic parameters for each of the microsatellite primers used in the current research

| Primers | Major Allelic frequency | Number of alleles | Expected heterozygosity | Observed heterozygosity | PIC |
|---|---|---|---|---|---|
| CH05g08 | 0.52 | 5 | 0.70 | 0.64 | 0.60 |
| 02b1 | 0.40 | 6 | 0.75 | 0.72 | 0.67 |
| CH02b10 | 0.44 | 6 | 0.86 | 0.70 | 0.66 |
| MS14h03 | 0.42 | 5 | 0.8 | 0.78 | 0.74 |
| CH02d12 | 0.51 | 3 | 0.71 | 0.65 | 0.58 |
| CH01h01 | 0.26 | 3 | 0.8 | 0.61 | 0.55 |
| CH02h11a | 0.44 | 5 | 0.83 | 0.71 | 0.66 |
| CH03a04 | 0.37 | 7 | 0.89 | 0.76 | 0.73 |
| CH02g09 | 0.46 | 7 | 0.78 | 0.73 | 0.70 |
| CH01f03b | 0.24 | 7 | 0.80 | 0.83 | 0.81 |
| CH01f07a | 0.35 | 4 | 0.82 | 0.70 | 0.64 |
| CH05d04 | 0.16 | 12 | 0.82 | 0.89 | 0.88 |
| CH04c07 | 0.20 | 8 | 0.82 | 0.85 | 0.83 |
| CH02d11 | 0.43 | 5 | 0.73 | 0.71 | 0.67 |
| Mean | 0.37 | 5.92 | 0.79 | 0.74 | 0.70 |

Principal component analysis (PCA) was performed for two-dimensional (Fig. 2) and three-dimensional representations (Fig. 3). The accumulation of individuals in an area of a plot indicates the genetic similarity of those individuals. In this analysis, the three main components could explain 56.34% of the total variance (Table 4). The PCA and the Biplot results are significantly consistent with the results of cluster analysis and the corresponding dendrogram (Fig. 3). Six groups were created according to the incision line in an area of similarity coefficient (0.44) within the relevant dendrogram. Yasuj Autumn Gollab and Manuchehri genotypes had a similarity coefficient of 0.69, while Rubina had a similarity coefficient of 0.59, thereby being placed in the first subgroup. Gami Almasi and Yellow (Golden Delicious) genotypes were in the second subgroup with a similarity coefficient of 0.54. In the second group, the first subgroup comprised Shahrood 4 and 5 (with a similarity coefficient of 0.69 in relation to each other) and were separated from the second subgroup which had Shahrood 16 and 17. These two genotypes had a similarity coefficient of 0.52 in relation to each other, within the similarity coefficient area of 0.45. In the third group, the first subgroup, including the Palestinian Malayer genotype occurred in the similarity coefficient area of 0.55, and was separated from the second subgroup, including Malayer 1 and 9 genotypes (with a similarity coefficient of 0.78). In the fourth group which contained most of the genotypes, i.e. many of the Shahrood genotypes, the first subgroup was separated from the second subgroup in the similarity coefficient area of 0.5. In the first subgroup, however, some genotypes such as Shahrood 22, 20 21, 19, and 19 were separated from Shahrood 23, 24 and 18 in the 0.55 coefficient area. Also, Shahrood 22 was separated from Shahrood 20, 21, and 19 in the 0.76 coefficient area. Shahrood 20 and 21 were separated from Shahrood 19 in the 0.89 coefficient area as well. In the upper part of this subgroup, Shahrood 23 was separated from Shahrood 18 and 14, while having a similarity coefficient of 0.65 in relation to each other in the coefficient area of 0.61. In the second subgroup of the fourth group, Shahrood 8 was separated from Shahrood 3, 14, and 15 in the coefficient area of 0.56, while Shahrood 3 was subsequently separated

from Shahrood 14 and 15 in the area of 0.58, while having a similarity coefficient of 0.69 in relation to each other. In the fifth group, Shahrood 10, which appeared as the first subgroup, was separated from Shahrood 12 and Zeynal Autumn Gollab which appeared as the second subgroup (with a similarity coefficient of 0.58 in relation to each other). In the sixth group, the first subgroup included Malayer 6 and Rootstock changer genotypes (with a similarity coefficient of 0.54 in relation to each other), and was separated from the second subgroup which included Shahrood 2, Malayer 5 and 8, Shahrood 31, and Shahrood 1 genotypes in the coefficient area of 0.45. Shahrood 2 was separated from other genotypes of the first subgroup in the coefficient area of 0.47, in which Shahrood 1 was separated from Shahrood 31, Malayer 5 and 8 in the coefficient area of 0.51. Shahrood 31 was separated from Malayer 5 and 8 (with a similarity coefficient of 0.78) in an area of 0.53. In comparing PCA results with those of cluster analysis, it was found that Shahrood 3 was completely separated from other PCA samples (Fig. 2) and occurred in the second subgroup of the fourth group by cluster analysis (Fig. 4). However, based on Fig. 3, it is clear that this genotype was in the same row of samples of the same group, according to the second component. Such a situation can be imagined for the Yasuj Autumn Gollab genotype. The cophenetic correlation coefficient was obtained as r= 0.67.

## Discussion

Studying genetic diversity is an initial step toward identifying plant germplasm sources, especially when preserving and using the germplasm in breeding programs (Salehi Sardoei and Noorbakhash, 2020).

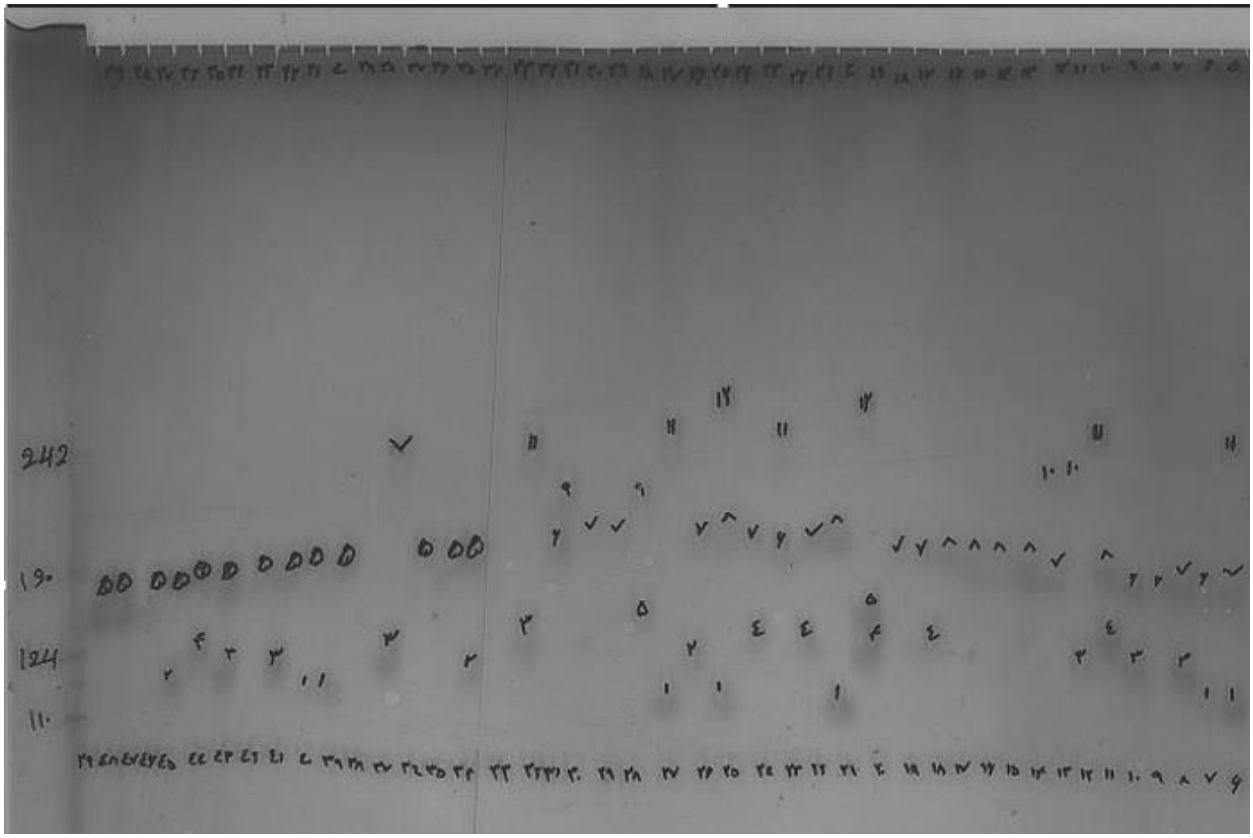

**Fig. 1.** The polymorphism of the primer CH02B10

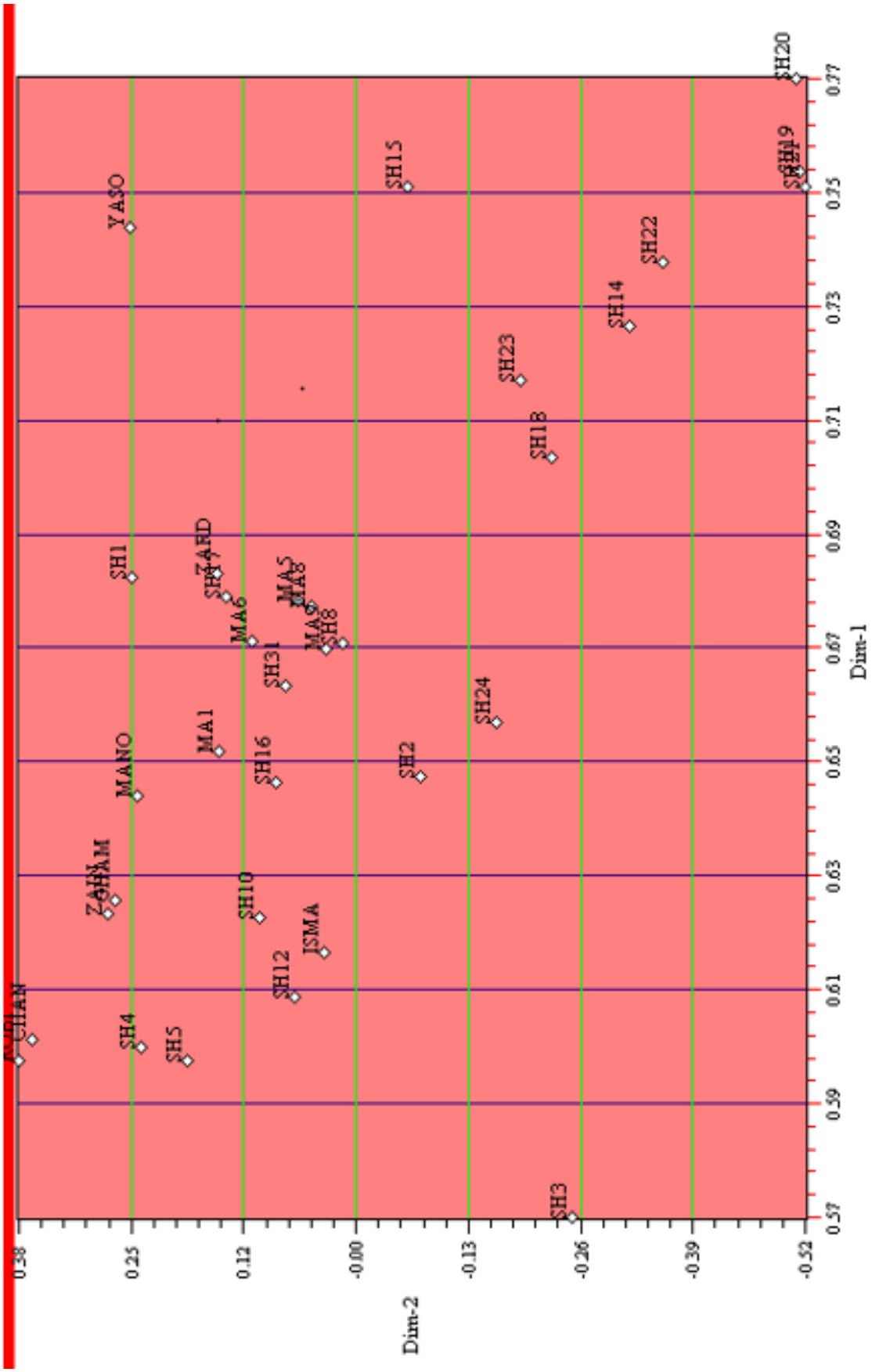


**Fig. 2.** Variability pattern of the 33 apple genotypes based on the first and second main components

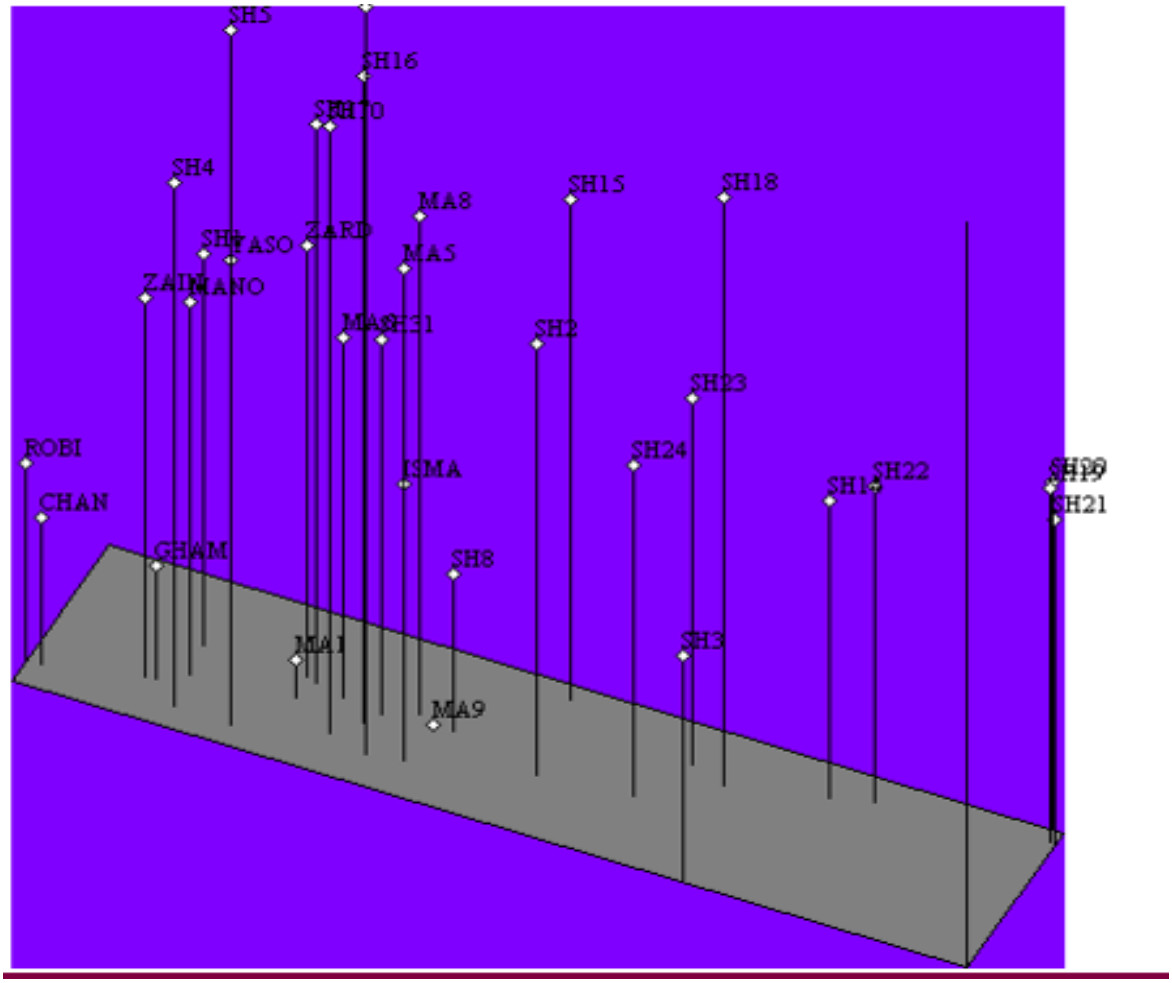


**Fig. 3.** Principal component analysis (PCA) was performed for three-dimensional representations

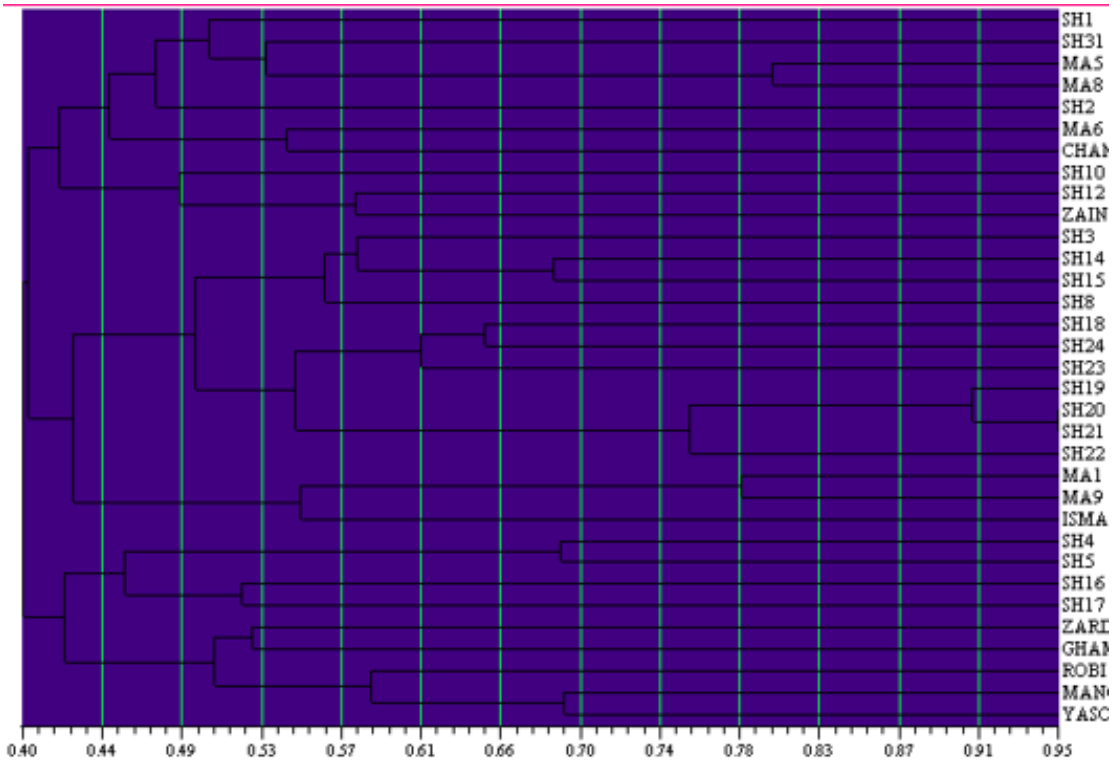


**Fig. 4.** Dendrograms of 33 genotypes of apple based on the data of 14 microsatellite markers using Dice similarity coefficient and UPGMA method

The present study identified genetic differences between apple genotypes by SSR markers. Indigenous genotypes of Iranian apple were divided into several groups, which indicates the high genetic diversity of apple trees in the country and confirms previous indications that Iran is a center of apple diversity. Overall, the SSR technique has advantages such as simplicity, cost-effectiveness and speed in identifying or differentiating the genetic diversity of apples. Therefore, the results of the present work can be used for selecting cultivars for breeding programs. The mean number of alleles observed was 5.92 in each gene locus, which was consistent with previous research on apples (Harris, 2002; Cavanna et al., 2008; Richards, 2009). A previous experiment on apples showed that 45 alleles were produced in the 16 SSR locus and that the polymorphic information content (PIC) varied from 0.18 to 0.76 (Farrokhi et al., 2011). The results also confirmed SSRs as reliable markers of DNA. Therefore, they can be used for studying genetic diversity in apples (Farrokhi et al., 2011). Since a more significant PIC number indicates a higher number of alleles and a higher level of polymorphism (Powell et al., 1996), an increase in heterozygosity was associated with an increase in PIC. Wichmann et al. (2007) identified 40 Hungarian apple varieties using six pairs of SSR primers, of which 71 were obtained with an average of 11.8 for each locus. In their study, the average heterozygosity for markers was very high (0.8). In a genetic analysis of 23 apple cultivars using two pairs of markers (05g8 and 02b1), Ershadi (2003) found that the said markers were effective in differentiating all Iranian apple cultivars except two, Zanuz Marand and Ahar. The above primer pairs amplified 5 and 6 alleles in the current work, respectively, while there were seven amplified alleles in Ershadi's study (2003). The dissimilarity in the proliferation of markers can be due to differences in the apple cultivars, because most of the genotypes herein were from Semnan province (Shahrood genotypes). A similar distinction was observed in a study by Jahromi Shirazi (2008). Other markers have also been valuable resources for addressing careful selection. In a study of 273 apple genotypes from plant genetic resources using 10 microsatellite markers (SSR), a total of 113 polymorphic alleles with 5-18 alleles were obtained in each locus (Patzak et al., 2012). In another study on 24 genotypes, including 5 native and numerous foreign apple genotypes, the average PIC index was 0.8 (Jahromi Shirazi, 2008). Research by Boteh Khak et al. (2018) showed that the mean PIC was 0.7, and the highest PIC with nine alleles was 0.82, which belonged to the CH01D09 locus. Also, the observable heterozygosity and expected heterozygosity were calculated 0.6 and 0.77, respectively (Mahmoudi et al., 2014). It was reported that in the apple tree, a total of 218 polymorphic fragments were obtained, and a high level of genetic diversity was observed in 19 cultivars. About 3 to 14 alleles per primer pair and an average of 7.51 alleles were observed in SSR markers (Ahmad Dar et al., 2019). The conservation of any species and its genetic resources, which includes the characteristics of local cultivars, requires knowledge of genetic diversity and the relationship between species and genotypes (Gharghani et al., 2009). An accurate assessment of the extent and pattern of genetic diversity is also effective in plant breeding programs. This study was carried out to determine the relationship and genetic diversity of several apple genotypes collected from different parts of Iran, using SSR molecular markers. Genetic differences between genotypes from the same region were not significant and were grouped together in cluster analysis. In most cases, however, the grouping was independent of the geographical origin of the genotypes.

## Conclusion

For the first time, a portion of apple cultivars in the Seed and Plant Breeding Research Institute of Kamalabad Garden, Karaj, Iran, was evaluated in terms of genetic diversity. According to the research results, 83 polymorphic alleles appeared in 14 gene loci (on average, 5.92 alleles per gene locus), and the polymorphic information content was 0.71 on average, which was higher than that reported in many previous cases of research.

According to the present results, it seems that the genetic diversity of native Iranian apples is not entirely a function of geographical diversity. Accordingly, apples with different local distributions were placed next to each other in some cases. Furthermore, the results confirmed that SSR markers proved effective for genetic diversity studies in the identification of different apple genotypes. Thus, it is expected that a significant step could be taken in the future to select and breed native apple genotypes for introduction purposes and production.

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**Conflict of Interest**

The authors declare no competing interests.

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