Genetic Diversity among Iranian Local and Commercial Apple Rootstocks by Using Simple Sequence Repeat Markers

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Summary

The objective of this research was to evaluate the genetic diversity among twelve apple rootstocks by using 14 simple sequence repeat primer pairs. Generally, 44 alleles were scored at SSR loci. The number of alleles (n_a) per locus ranged from two to five alleles with an average of 3.14. The maximum effective number of alleles (n_e), expected heterozygosity (H_e), and observed heterozygosity (H_{obs}) were 2.3, 0.56 and 0.36, respectively. Results showed that the studied genotypes were not in Hardy-Weinberg equilibrium in most of studied loci. The polymorphism information content (PIC) varied from 0.5 to 0.86 which confirmed the effectiveness of SSR markers in determination of molecular polymorphism and characterization of the rootstocks genotypes. Cluster analysis using UPGMA algorithm on the basis of Jaccard's similarity coefficient classified the studied genotypes into three distinct groups. In this study, three Iranian local rootstocks were located in separate groups.

Key words

apple rootstock; genetic diversity; simple sequence repeat marker; polymorphism information content; cluster analysis

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Introduction

The cultivated apple is supposed to be the result of inter-specific hybridization. The denomination *Malus* × *domestica* has been generally accepted as the appropriate scientific name fpr the cultivated apple (Korban and Skirvin, 1984). According to historically documents, Iranian plateau had an important role in domestication and transmission of apple from the East Asia to the ancient Roman and Greek civilizations (Gharghani et al., 2009). Due to specific geographic and climatic conditions of Iran, it is the fourth apple producer after China, USA and Poland (FAOSTAT, 2008). Generally, the roots and lower part of the stem of the commercial apple trees, below the graft union, are called the rootstock. It is well known that rootstock influences several aspects of fruit tree growth and development, including yield and fruit quality (Webster, 1995), resistance to drought stress, root pests, and disease (Beckman and Pusey, 2001; Sharma and Sharma, 2008). By using apple dwarfing rootstocks one can establish intensive orchards (Oliveira et al., 1999). In contrast, the phenological stage of trees such as flowering time isn't influenced by rootstocks (Loreti et al., 2001). The apple rootstocks constitute the main part of *Malus* germplasm. Evaluation of genetic diversity and the protection of local apple rootstocks are essential elements in the fruit industry (Koc et al., 2009). To reach these aims, plant breeders use different types of marker systems, while the most substantial of them are DNA markers. Among DNA markers, microsatellite or simple sequence repeats (SSRs) markers, due to their high polymorphism, random distribution and co-dominant Mendelian inheritance, are the most reliable marker system for QTLs mapping, cultivars identification, and genetic diversity studies (Hokanson et al., 1998). Microsatellites constitute the current marker system of choice for characterizing apple germplasm (Gianfranceschi et al., 1998; Gabor et al., 2005; Gharghani et al., 2009; Naseri et al., 2011). In taxonomic viewpoint, this marker system can be used in some botanical classes that are similar. For instance apple SSRs have been used for identifying several pear cultivars (Yamamoto et al., 2001). Recently by using SSR markers, it was suggested that Iranian native apple genotypes are likely a medium between cultivated apples and wild species (Gharghani et al., 2009). In order to reveal the genetic diversity among Iranian apple cultivars and genotypes several research works have been conducted in the recent years (Nagshin et al., 2008; Gharghani et al., 2009; Jahromi Shirazi et al., 2009; Naseri et al., 2011). In present work, genetic diversity among foreign and Iranian apple rootstocks was surveyed by SSR markers.

Materials and methods

Plant material and SSR primers

Twelve apple rootstocks from the Kahriz station germplasm bank were investigated in present work. The majority of studied rootstocks belonged to (MM104, MM109, MM106, M7, M4, M2, M26, and M9). The 'M' prefix refers to East Malling research station in England, in the 1900s. The 'MM' prefix, Malling Merton, refers to hybrid of the Malling series with 'Northern Spy' in Merton, England in the 1920s (Rom and Carlson, 1987). Moreover three famous Iranian rootstocks including 'Azayesh', 'Khan Almasi' and 'Gami Almasi' were used in this study. Out of the 60 SSR primers tested, 14 were found to be polymorphic and produced clear bands (Table 1).

DNA extraction

Total genomic DNA was extracted from the powdered (ground in liquid nitrogen) young leaves by using the method described by Dellaporta et al. (1983) with some modifications. Approximately 10-15 mg tissue samples from each genotype were snap-frozen in liquid nitrogen. A volume of 600 µl DNA extraction buffer [100 mM Tris–HCl, pH 7.0; 50 mM EDTA, pH 8.0; SDS 2% (w/v); 500 mM NaCl] and 3.5 µl RNAse were added and mixed well. The mixture was incubated at 65°C in a water bath for 30 minutes with intermittent shaking at 5 min intervals, and then 300 µl potassium acetate was added to it. After 10 min, chlorophorm: isoamyl alcohol solution (24:1) was added and the mixture was centrifuged at 10000 g for 10 min at 4°C. The supernatant was transferred to a sterile 1.5 ml tube, mixed with an equal volume of isopropyl alcohol (-20°C) and was centrifuged. After centrifugation the supernatant was collected and washed with Ethanol three times. The genomic DNA was re-suspended in 100 µl TE (10 mM Tris, 1 mM EDTA). The concentration of

Table 1. The name of used primer, its sequence and annealing temperature

Primer	Sequence of primer F (5′→3′)	Sequence of primer R (5′→3′)	Tm*	
Hi02b10	TGTCTCAAGAACACAGCTATCACC	GTTTCTTGGAGGCAGTAGTGCAG	60	Silfverberg-Dilworth et al., 2006
Hi02d04	TGCTGAGTTGGCTAGAAGAGC	GTTTAAGTTCGCCAACATCGTCTC	60	Silfverberg-Dilworth et al., 2006
CH05c07	TGATGCATTAGGGCTTGTACTT	GGGATGCATTGCTAAATAGGAT	59	Liebhard et al., 2002
CH03a02	TTGTGGACGTTCTGTGTTGG	CAAGTTCAACAGCTCAAGATGA	60.6	Liebhard et al., 2002
CH02b12	GGCAGGCTTTACGATTATGC	CCCACTAAAAGTTCACAGGC	59.7	Liebhard et al., 2002
Hi03e03	ACGGGTGAGACTCCTTGTTG	GTTTAACAGCGGGAGATCAAGAAC	60	Silfverberg-Dilworth et al., 2006
CN444636	CACCACTTGAGTAATCGTAAGAGC	GTTTGCCAGTTAAGGACCACAAGG	60	Silfverberg-Dilworth et al., 2006
CH05g07	CCCAAGCAATATAGTGAATCTCAA	TTCATCTCCTGCTGCAAATAAC	60	Liebhard et al., 2002
CH02c02	CTTCAAGTTCAGCATCAAGACAA	TAGGGCACACTTGCTGGTC	60	Liebhard et al., 2002
Hi03a03	ACACTTCCGGATTTCTGCTC	GTTTGTTGCTGTTGGATTATGCC	60	Silfverberg-Dilworth et al., 2006
CN444542	ATAAGCCAGGCCACCAAATC	GTTTGCAGTGGATTGATGTTCC	60	Silfverberg-Dilworth et al., 2006
GD96	CGGCGGAAAGCAATCACCT	GCCAGCCCTCTATGGTTCCAGA	55	Hokanson et al., 1998
CH05b06	ACAAGCAAACCTAATACCACCG	GAGACTGGAAGAGTTGCAGAGG	60	Liebhard et al., 2002
CN493139	CACGACCTCCAAACCTATGC	GTTTATGAAAGTACGGCACCCATC	60	Silfverberg-Dilworth et al., 2006
CH05g08	CCAAGACCAAGGCAACATTT	CCCTTCACCTCATTCTCACC	60	Liebhard et al., 2002

Note: *Tm: Temperature melting [Tm=4°C (G+C) + 2°C (A+T)].

each DNA sample was determined spectrophotometrically at 260 nm (Biophotometer 6131; Ependorf, Hamburg, Germany). The quality of DNA was checked by running 1µl of DNA in 0.8% (w/v) agarose gel in 0.5X TBE buffer (45 mM Tris-base, 45 mM Boric acid, 1mM EDTA, pH 8.0). DNA samples that gave a smear in the gel were rejected.

PCR amplification

PCR amplification was carried out in a final volume of 25 µl containing 1X reaction buffer [100 mM Tris–HCl, pH 7.0; 160 mM $(NH_4)_2SO_4$ 0.1% (v/v)], 2.0 mM $MgCl_2$, 0.4 mM of dNTPs (dATP, dCTP, dGTP, and dTTP) (CinnaGen Inc., Tehran, Iran), 0.16 µM of each SSR primer, 1.0 Unit Taq DNA polymerase (CinnaGen Inc., Tehran, Iran), and 25 ng of genomic DNA as template. Amplification was performed by using a 96-well Eppendorf Mastercycler Gradient (Type 5331, Eppendorf AG, Hamburg, Germany) programmed for an initial step of 95°C for 2 min, followed by 35 cycles of 93°C for 45 s, 50-55°C for 60 s and 72°C for 2 min, and 10 min at 72°C as a final step. The reaction products were mixed with an equal volume of formamide dyes (98% formamide, 10 Mm EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol) and resolved in a 3% (w/v) agarose gel in 0.5X TBE buffer, stained with ethidium bromide (1.0 µg ml^{-1}) and photographed under UV light using a Gel-Doc image analysis system (Gel Logic 212 PRO, USA).

Data analysis

Amplified bands were scored as present (1) or absent (0) across all studied individuals to construct a binary data matrix. Mean number of allele per locus (n_a), effective allele number (n_e), allele frequency, F statistics (F_{IS}) (Wright, 1978), observed and expected heterozygosities (H_o and H_e respectively) were estimated using the GenAlEx software version 6.41 (Peakall and Smouse, 2006). $n_a = \sum_{i=1}^{n} n_{ai} / n$ where n_{ai} is the number of alleles at i^{th} locus. $n_e = \sum_{i=1}^{n} n_{ei} / n = \sum_{i=1}^{n} (1 - \sum_{j=1}^{m_i} q^2_{ij}) / n$, where n_{ei} is the effective allelic number at i^{th} locus, and q_j the frequency of the j^{th} allele. Allele frequency = $\frac{2N_{XX} + N_{XY}}{2N}$, calculated locus by locus (Hartl and Clark, 1997); where N_{XX} is the number of homozygotes for allele $X(XX)$, N_{XY} is the number of heterozygotes containing the allele X (Y can be any other allele), N = the number of samples. $H_o = \sum_{i=1}^{n} H_{oi} / n = \sum_{i=1}^{n} (1 - \sum_{j=1}^{m_i} q^2_{ij}) / n$ (Hartl and Clark, 1997), where H_{oi} represents the observed heterozygosity of the i^{th} locus, and q_{ij} is the frequency of the j^{th} allele at i^{th} locus. $H_e = \sum_{i=1}^{n} H_i / n = \sum_{i=1}^{n} (1 - \sum_{j=1}^{m_i} q^2_{ij}) / n$ (Lynch and Milligan, 1994), where H_i is the expected heterozygosity of the i^{th} locus, and q_{ij} is the frequency of the j^{th} allele at i^{th} locus. Allelic polymorphism information content (PIC) was calculated as described by Anderson et al. (1992):

$$PIC = 1 - \sum_{i=1}^{n} (Pi)^2$$

where Pi is the proportion of the population carrying the i^{th} allele, calculated for each micro satellite locus. Hardy-Weinberg equilibrium test was done using PopGene32 program (Yeh et al., 1997). Different methods were used for calculating similarity matrices and constructing dendrograms. The efficiency-of-clustering algorithms and their goodness-of-fit were determined based on co-phenetic correlation coefficients. The significance of co-phenetic correlation observed was tested using the Mantel matrix correspondence test (Mantel, 1967). Data analyses were performed using the NTSYS-pc (Numerical Taxonomy and Multivariate Analysis for personal computers) software (Rohlf, 1998). Dendrograms were constructed by using the SAHN subprogram (Sequential, Agglomerative, and Hierarchical and Nested clustering) in NTSYS-pc software. AMOVA (Analysis of Molecular Variance) test was used to determine the slice point of the dendrogram.

Results and discussion

In this research, 14 SSR loci out of 60 SSRs were used for apple rootstocks fingerprinting. Generally, 44 polymorphic alleles were identified using 14 SSR loci in studied rootstock genotypes. The number of alleles per locus (n_a) varied from 2 to 5 with an average of 3.14 alleles (Table 2). This was similar to findings of Naseri et al. (2011) and Nagshin et al. (2008) in Iranian apple cultivars and genotypes. In controversy, Pereira-Lorenzo et al. (2007) and Gharghani et al. (2009) reported high value of the average number of alleles per locus. The number of effective alleles per locus (n_e) varied from 1.52 to 3.56 with an average of 2.29 alleles per locus. Whereas, using the SSR markers the n_a was 8.7 in Liebhard et al. (2002), 12.3 in Pereira-Lorenzo et al. (2007), and 17 in Gharghani et al. (2009) reports. Different values of n_a in several studies may be the consequence of different number of studied genotypes, their genetic background, number of used markers and polymorphism detection technique.

The maximum and minimum of effective alleles (n_e) were observed in "Hi02d04" and "CH05g08" loci, respectively (Table 2). Herein, the mean number of effective allele (n_e) (2.29) was lower than the previous report (Gharghani et al., 2009). The low n_e could reflect the low heterozygosity (Nei, 1978). The maximum and minimum of observed heterozygosity (H_{obs}) belonged to "CN444636-SSR" and "CN444542-SSR" loci, the maximum of expected heterozygosity (H_e) was detected in "Hi02d04" and "CH05c07" loci and the minimum one was detected in "CH05g07" locus (Table 2). In the current study, expected heterozygosity (H_e) was greater than H_{obs} values. The mean of H_{obs} and H_e were 0.36, and 0.56, respectively.

The Fixation index (F_{IS}) can vary from -1 (all individuals are heterozygous) to +1 (all individuals are hemozygous) (Wright, 1978). According to Table 2, the F_{IS} values ranged from -0.33 in locus "Hi02d04" to 1 in loci: "Hi03e03", "Hi02d04", "CH05c07", "CN444542-SSR", and "GD96". The extent of F_{IS} values in the previous studies varied from -0.0002 to -0.088 (Larsen et al., 2006; Pereira-Lorenzo et al., 2007). Opposite of the previous report using Iranian apple cultivars (Gharghani et al., 2009), the average value of F_{IS} was high (0.29) in this research. Regarding to positive F_{IS} values and significant chi-square test ($P < 0.05$) in most of studied loci, it is inferable that studied genotypes are not in Hardy-Weinberg (H-W) equilibrium. Generally, disequilibrium could be observed in open-pollinated species with possibility of random mating system. The apple rootstocks propagate by some

Table 2. A summary of genetic parameters across single sequence repeat loci and apple rootstocks

Primer	n_a	n_e	Prob	Allele frequency				
				a	b	c	d	e
Hi02b10	3	2.72	0.16	0.44	0.38	0.19	-	-
Hi02d04	4	3.56	0.01	0.13	0.25	0.38	-	0.25
CH05c07	5	3.5	0.1	0.14	0.36	0.36	0.07	0.07
CH05g08	3	1.52	0.09	0.8	0.1	0.1	-	-
CH02b12	3	2.72	0.03	0.38	0.44	0.19	–	–
Hi03e03	5	2.41	0.00	0.05	0.1	0.05	0.6	0.2
CN444636-SSR	2	2.00	0.16	0.5	0.5	-	-	-
CH05g07	2	1.8	0.3	0.33	0.67	–	–	–
CH02c02	2	1.85	0.04	0.36	0.64	–	–	–
Hi03a03	4	1.71	0.32	0.13	0.75	0.06	-	0.06
CN444542-SSR	2	1.6	0.00	0.25	0.75	-	-	-
GD96	3	1.68	0.00	0.15	0.75	0.1	–	–
CH05b06	2	1.75	0.02	0.31	0.69	–	–	–
CN493139-SSR	4	3.27	0.53	0.14	0.43	0.29	–	0.14
Mean	3.14	2.29						

Note: n_a= number of alleles, n_e= number of effective alleles, Prob= Probability level of Chi-Square test for Hardy – Weinberg equilibrium

Table 2. continued

Primer	H_e	H_{obs}	PIC	F_{IS}				
				a	b	c	d	e
Hi02b10	0.68	0.88	0.68	0.78	-	-	0.23	0.07
Hi02d04	0.77	0.63	0.8	1.00	-	-0.33	-0.07	0.33
CH05c07	0.77	0.57	0.82	1.00	-0.08	-0.08	0.07	0.07
CH05g08	0.38	0.4	0.73	-0.25	-	-0.11	-	-0.11
CH02b12	0.68	0.25	0.86	-	-	0.59	0.75	0.47
Hi03e03	0.62	0.25	0.75	-0.05	1.00	1.00	-0.05	-0.11
CN444636-SSR	0.6	1.00	0.5	-1.00	-	-	-	-1.00
CH05g07	0.46	0.33	0.78	0.25	-	-	-	0.25
CH02c02	0.49	0.14	0.83	0.69	-	0.69	-	-
Hi03a03	0.44	0.38	0.79	-0.14	-0.07	-	-0.07	0.33
CN444542-SSR	0.4	0.00	0.79	-	-	1.00	-	1.00
GD96	0.43	0.1	0.79	0.61	1	-	-	0.73
CH05b06	0.46	0.13	0.77	0.71	-	-	-	0.71
CN493139-SSR	0.75	0.57	0.83	-0.17	-	-0.17	0.30	0.42
Mean	0.56	0.36	0.76					

Note: PIC=Polymorphism Information Content, H_{obs}= Observed heterozygosity, H_e = Expected heterozygosity, F_{IS} = Fixation index

asexual ways such as layering and this would cause to fix the alleles during the time (Gregorian, 2002). Hence, the absence of random mating system can be considered the main reason of low allelic frequency and missing of H-W equilibrium in clonally varieties and rootstocks. Polymorphism information content (PIC) varied from 0.5 to 0.86, implying the high potential of allelic segregation and detection ability of used SSR primers. The high value of PIC could be caused by the high number of polymorphic bands or existence of specific alleles per locus. The average of polymorphism information content (PIC) was 0.76 and it was in agreement with Koc et al. (2009) (PIC=0.72) and Gabor et al. (2005) (0.78) in commercial apple cultivars.

In this study, different methods were used to construct the similarity matrices and dendrograms (Table 3). The co-phenetic correlation coefficients as a measure of the correlation between the similarities is presented on the dendrograms and the actual degree of similarity was calculated for each dendrogram (Table 3). Among the different methods, the highest value (r=0.7) was observed for the UPGMA method based on Jaccard's similarity coefficients (Jaccard, 1908) (Table 3). Therefore, Jaccard's similarity coefficient was used to depict the genetic diversity of the rootstock individuals (Table 4). The highest similarity coefficient was GS_j= 0.47 and the lowest one was GS_j= 0.19 (Table 4). So, there was moderate level of genetic variation among studied Iranian local and commercial apple rootstocks.

Table 3. Comparison of different methods for constructing similarity matrices and dendrograms

Similarity coefficient	UPGMA	Complete linkage
Jaccard (J)	r=0.70	r=0.49
Dice (D)	r=0.67	r=0.44
Simple Matching	r=0.57	r=0.45
Phi	r=0.52	r=0.42

Note: J: (Jaccard, 1908); D: Dic (Nei; Li, 1979); Simple Matching: (Sneath; Sokal, 1973); Phi: Pearson's Phi coefficient (Sokal; Sneath, 1963); UPGMA: Un –weighted Pair Grouped Method with Arithmetic average

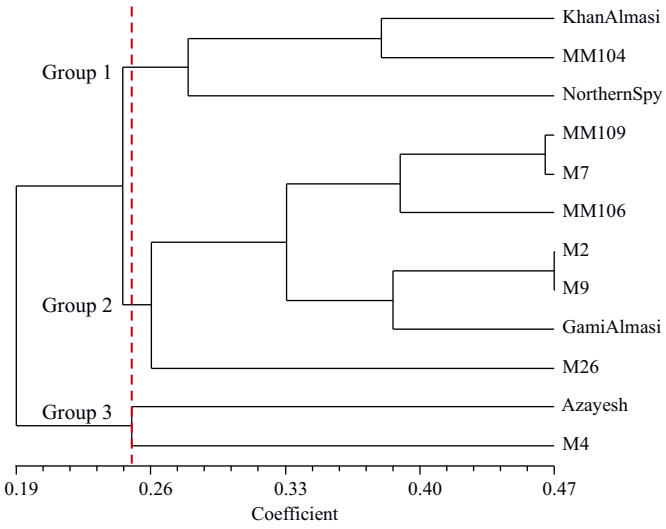

Figure 1. The dendrogram of 12 studied apple rootstocks using SSR markers on the basis of Jaccard similarity matrix

(*Agrobacterium tumefacience*), and deficiency of Mg^{+2} levels in calcareous soils. Iranian rootstock 'Gami Almasi', which is well-adapted for the north west of Iran, showed the highest similarity to 'M9' (r= 0.39). It has breakable roots and shoots, and produces more suckers; however, when it is used as a rootstock for a vigorous variety, the ability for sucker production decreases. Its flower buds are formed on spurs and branches and it grows well in calcareous soils. This rootstock is resistant to temperatures lower than –17°C and it is tolerant to *Podosphaera leucotricha* (Naseri et al., 2006). Similar to finding of Naseri et al. (2011), genotypes 'Azayesh' and 'M4' were located together in Group III. In this study, three Iranian native rootstocks: 'Khan Almasi', 'Gami Almasi', and 'Azayesh' were located in three distinguished groups.

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Table 4. The Jaccard similarity matrix of studied apple rootstocks using SSR markers

	Khan Almasi	Azayesh	Northern Spy	MM104	MM109	MM106	M7	M4	M2	M26	M9
Azayesh	0.19										
Northern Spy	0.28	0.19									
MM104	0.38	0.19	0.28								
MM109	0.25	0.19	0.25	0.25							
MM106	0.25	0.19	0.25	0.25	0.39						
M7	0.25	0.19	0.25	0.25	0.46	0.39					
M4	0.19	0.25	0.19	0.19	0.19	0.19	0.19				
M2	0.25	0.19	0.25	0.25	0.33	0.33	0.33	0.19			
M26	0.25	0.19	0.25	0.25	0.26	0.26	0.26	0.19	0.26		
M9	0.25	0.19	0.25	0.25	0.33	0.33	0.33	0.19	0.47	0.26	
Gami Almasi	0.25	0.19	0.25	0.25	0.33	0.33	0.33	0.19	0.39	0.26	0.39

Based on UPGMA clustering algorithm and Jaccard's similarity coefficients the studied local and commercial apple rootstock genotypes were divided into three main groups (Fig. 1). The majority of genotypes are located in the first and second groups. Group I included 'Khan Almasi', 'MM104', and 'Northern Spy' genotypes. 'MM104' is produced from cross between 'Northern Spy' and 'M2' (Gregorian, 2002). The highest genetic similarity in the group I was observed between 'Khan Aalmasi' and 'MM104' (GS_j= 0.38). Group II included 'MM109', 'M7', 'MM106', 'M2', 'M9', 'Gami Almasi', and 'M26'' genotypes and it is divided into two sub-groups (Fig. 1). The highest genetic similarity (GS_j = 0.47) in the group II was observed between 'M2' and 'M9' genotypes (Table 4).

'M2' rootstock previously denominated as 'Paradise English' can be easily propagated by trench layering. Its yield performance is lower than 'M9' but it has higher growth power. (Loreti et al., 2001). 'M9' has fragile roots and it is sensitive to drought stress condition, wooly apple aphid (*Eriosoma lanigerum*), crown gall

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