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Looking for Genetic Diversity in Iranian Apple Cultivars (*Malus* × *domestica* Borkh.)

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Abstract

The cultivated apple (*Malus domestica*) is one of the most important fruit crops cultivated in different regions of the world including Iran. For production and breeding of high quality apple, the knowledge of genetic diversity of the cultivated apples is necessary. Therefore we studied genetic diversity of 25 genotypes by using ISSR molecular markers. In present study, ten ISSR primers produced 85% polymorphism. (AGC)5GG and (GA)9C primers produced the highest number of bands, while primers (GA)9A, (GA)9A and UBC 807 produced the lowest number. Some of the genotypes showed presence of specific bands. PCoA (principal co-ordinate analysis) plot of genotypes based on geographical origin (Netherland, France, Lebonan and Iran) grouped cultivars from different geographical regions together and intermixed. UPGMA (unweighted paired group method with arithmetic average) and NJ (Neighbor Joining) dendrograms produced 4 major clusters with few subclusters in each of them indicating intra-group genetic diversity. AMOVA (Analysis of molecular variance) test performed on molecular features of the genotypes present in the 4 clusters revealed significant difference among the clusters. This high value of within clusters genetic variation is in agreement with subclusters formed in each major cluster due to intra-population group variation. STRUCTURE plots of k = 2-7 were obtained and the best groupings was done by k =7. It indicated differences in allelic composition and frequency of the subclusters. The results showed presence of high level of genetic diversity in apple samples studied which can be used in planning further selection and hybridization of apple trees.

Keywords: Genetic variation, ISSR, *Malus domestica*, polymorphism.

Introduction

Apple (genus *Malus* Mill., family Rosaceae) is one of the most important fruit crops cultivated in temperate and subtropical climate. The cultivated apple is *Malus* × *domestica* Borkh., while its wild relatives are *Malus sieversii* and *Malus sylvestris* [3]. With the ever-growing requirements for environmental protection and

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food safety in the production of high quality apples, the modern apple breeding becomes more and more dependent on resistant gene resources from the cultivated and wild genetic resources [5].

The apple (*Malus* × *domestica* Borkh.) is believed to have originated from central Asia and from there spread to the rest of the world [16, 17]. Apple trees are mostly diploid (2n=2x=34), but some varieties are haploid, with 17 single chromosomes. Others, especially among crab apples, are polyploid, which means that their chromosomes are not paired but tripled, quadrupled, quintupled, or even wadded up into bundles of six [2**,** 10]. In fact, some apples have as many as eighty-five chromosomes. And each of the genes on each of the chromosomes can have different alleles (alternative forms). A single seed may thus contain a lot of genetic variation that has accumulated down through the ancestral lines. For example, 'Gravenstein' apples are triploid with a chromosome number of 51 (3n=51). They are produced by the union of a diploid egg (2n=34) and a haploid sperm (n=17). This is accomplished by crossing a tetraploid plant (4n=68) with an ordinary diploid plant (2n=34). Because the triploid (3n) varieties are sterile, they must be propagated by grafting, where the scions of choice cultivars are grafted to hardy, pest-resistant root stalks [2].

Breeding and selection of high-quality, disease resistant apple cultivars and cultivar identification, require knowledge and understanding of the genetic diversity and the availability of genetic linkage maps which have been investigated by using different molecular markers [1, 9, 13, 14, 15, 27, 30, 35, 36]. Different molecular markers have been used to study genetic diversity and cultivar discrimination in apple. RFLP [36] and RAPD [18, 34] markers have been employed to identify cultivars and group them according to their genetic similarity. For example, the existence and usefulness of restriction fragment length polymorphisms (RFLPs) among apple clones were investigated by Watillon et al. [35]. Similarly, Tancred et al. [34] used RAPD markers to differentiate early maturing Queensland apple cultivar (GB 63-43) from 3 cultivars of similar appearance, namely Summerdel, Jonathan and Royal Gala. SSRs markers, due to their high polymorphism, random distribution and co-dominant Mendelian inheritance, have been used for cultivars identification and genetic diversity studies in apple germplasm [1, 11, 12, 17]. Hokanson et al. [17] used eight microsatellite markers developed in the cultivar "Golden Delicious" to characterize 66 apple accessions. Gharghani et al. [11] investigated the relationships of 159 accessions of wild and domesticated apples including some Iranian indigenous apple cultivars and landraces, selected wild species, and old apple scion and rootstock cultivars with nine simple sequence repeat (SSR) markers.

Apple is a favorite fruit in Iran and several apple cultivars (genotypes) are cultivated in the country. we have no knowledge about the genetic structure of these genotypes for future breeding program. Therefore, the present study considers the study of genetic variation of 25 apple cultivars based on ISSR analysis. We also studied inter-cultivar genetic relationship.

Materials and Methods

Tweny-five apple genotypes (including 23 cultivars and 2 rootstocks of Malling 7 and Malling 9) were obtained from Research Center of Seeds and Seedlings, Karaj, Iran. Details of localities and origin of these genotypes are provided in Table 1.

Three to five fresh leaves were randomly collected from at least 5 trees of each genotype and used for DNA extraction. The total genomic DNA was extracted using the CTAB method by De la Rosa *et al*. [6]. Quality and quantity of extracted DNA were examined by 0.8% agarose gel electrophoresis and UV-illuminator respectively.

ISSR assay

Ten ISSR primers; (AGC)5GT, (CA)7GT, (AGC)5GG, UBC 810, (CA)7AT, (GA)9C, UBC 807, UBC 811, (GA)9A and (GT)7CA commercialized by UBC (University of British Columbia) were used. PCR reactions were performed in 25 µL of 10 mM Tris- HCl buffer at pH 8; containing 50 mM KCl; 1.5 mM $MgCl_2$; 0.2 mM of each dNTP (Bioron, Germany); 0.2 µM of a single primer; 20 ng genomic DNA and 3 U of *Taq* DNA polymerase (Bioron, Germany). Amplifications reactions were performed in Techne thermocycler (Germany) with the following program: 5 min initial denaturation step 94°C, 30 s at 94°C; 1 min at 50°C and 1 min at 72°C. The reaction was completed by final extension step of 7 min at 72°C. Amplification products were visualized by running on 2% agarose gel, following ethidium bromide staining. Fragment size was estimated by using a 100 bp molecular size ladder (Fermentas, Germany). The experiment was replicated 3 times and constant ISSR bands were used for further analyses.

Data analyses

ISSR obtained bands were treated as binary characters and coded accordingly (presence=1, absence=0). Jaccard's similarity as well as Nei's genetic

Table 1. Apple cultivars and their geographical features.

Cultivar	Origin	Locality of cultivation	Altitute (M)	Coordinates
Delbarstival	Holand	Shahriar	1161.0	35.7117°N 51.4070°E
Redstarking	Holand	Shahriar	1161.0	35.7117°N 51.4070°E
Idared	France	Shahriar	1161.0	35.7117°N 51.4070°E
Royal-gala	Holand	Shahriar	1161.0	35.7117°N 51.4070°E
Red-delicious	Lebenan	Karaj	1312.5	35° 50′ 8″ N, 51° 0′ 37″ E
Gala-beauty	Holand	Shahriar	1161.0	35.7117°N 51.4070°E
Arbabi Bojnoord	Iran-Khorasan	Karaj	1312.5	35° 50′ 8″ N, 51° 0′ 37″ E
Alimoori-dirras	Iran-Khorasan	Karaj	1312.5	35° 50′ 8″ N, 51° 0′ 37″ E
Shasti	Iran-Khorasan	Karaj	1312.5	35° 50′ 8″ N, 51° 0′ 37″ E
Golshahi-ghermez	Iran-Khorasan	Karaj	1312.5	35° 50′ 8″ N, 51° 0′ 37″ E
Roobin Esfarayen	Iran-Khorasan	Karaj	1312.5	35° 50′ 8″ N, 51° 0′ 37″ E
Kadoo-sib	Iran-Khorasan	Karaj	1312.5	35° 50′ 8″ N, 51° 0′ 37″ E
Zeynab-banoo	Iran-Khorasan	Karaj	1312.5	35° 50′ 8″ N, 51° 0′ 37″ E
Golshahi	Iran-Khorasan	Karaj	1312.5	35° 50′ 8″ N, 51° 0′ 37″ E
Akharres	Iran-Khorasan	Karaj	1312.5	35° 50′ 8″ N, 51° 0′ 37″ E
Alimoori	Iran-Khorasan	Karaj	1312.5	35° 50′ 8″ N, 51° 0′ 37″ E
Mohammadabadi	Iran-Khorasan	Karaj	1312.5	35° 50′ 8″ N, 51° 0′ 37″ E
Abbasi-gerd	Iran-Khorasan	Karaj	1312.5	35° 50′ 8″ N, 51° 0′ 37″ E
Ghasemshahi	Iran-Khorasan	Karaj	1312.5	35° 50′ 8″ N, 51° 0′ 37″ E
Vasatres	Iran-Khorasan	Karaj	1312.5	35° 50′ 8″ N, 51° 0′ 37″ E
Atlasi	Iran-Khorasan	Karaj	1312.5	35° 50′ 8″ N, 51° 0′ 37″ E
Shafiabadi	Iran-Shahreyar	Shahriar	1161.0	35.7117°N 51.4070°E
Golab Kahnaz	Iran-Shahreyar	Shahriar	1161.0	35.7117°N 51.4070°E
M7	Holand	Shahriar	1161.0	35.7117°N 51.4070°E
M9	Holand	Shahriar	1161.0	35.7117°N 51.4070°E

distance [26], was determined among the cultivars studied and used for the grouping of the genotypes by unweighted paired group method with arithmetic average (UPGMA) and Neighbor Joining (NJ) clustering methods as well as ordination plot based on principal co-ordinate analysis (PCoA) [31]. Cophenetic correlation and bootstrapping (1000 replications) were performed to check the fit of dendrograms obtained. NTSYS Ver. 2.02 (1998) and DARwin ver 5 (2010) were used for clustering and PCoA analyses.

Genetic diversity parameters were determined in clusters obtained. These parameters were Nei's gene diversity (H), Shannon information index (I) [20, 25], number of effective alleles and percentage of polymorphism. AMOVA (Analysis of molecular variance) test (with 1000 permutations) as performed in GenAlex 6.4 [29], was used to show molecular difference among the groups obtained.

Moreover, model-based clustering was performed to elucidate the genetic structure of the cultivars by using STRUCTURE ver. 2.3 [32]. The program STRUCTURE uses genotype data consisting of unlinked markers and assumes the existence of *K* clusters [32]. We took advantage of an admixture ancestry model under the correlated allele frequency model. The Markov chain Monte Carlo simulation was run 20 times for each value of *K* (2-7) for 10^6 iterations after a burn-in period of 10^5. All other parameters were set at their default values. Data were scored as dominant markers and analysis followed the method suggested by Falush et al. [8]. STRUCTURE Harvester web site [7] was used to visualize the STRUCTURE results and also to perform Evanno method to identify proper number of K [7]. Since apple materials obtained are from different geographical populations and it is possible to consider them in different populations groups, we performed STRUCTURE analysis for k=2 to k=6. The choice of the most likely number of clusters (K) was carried out comparing log probabilities of data [Pr(X|K)] for each value of K [32], as well as by calculating an ad hoc statistic ΔK based on the rate of change in the log probability of data between successive K values.

Reticulation was performed by T-REX (Tree and Reticulogram Reconstruction) ver. 3. (2000), which infer reticulogram from distance matrix. For reticulation, we first built a supporting phylogenetic tree using Neighbor Joining (NJ), Followed by a reticulation branch that minimizes the least-squares at each step of

the algorithm [21]. Two statistical criteria, Q1 and Q2, were used to measure the gain in fit provided by each reticulation branch as follows:

$$Q_1 = \frac{\sqrt{\sum_{i \in X} \sum_{j \in X} (d(i,j) - \delta(i,j))^2}}{n(n-1)/2 - N}$$

and

$$Q_2 = \frac{\sum_{i \in X} \sum_{j \in X} (d(i,j) - \delta(i,j))^2}{n(n-1)/2 - N}$$

The numerator of these functions is the square root of the sum (or the sum itself) of the quadratic differences between the values of the given evolutionary distance δ and the corresponding reticulogram estimates d, n is the number of taxa in the considered set X and N is the number of branches in the reticulogram, i.e. total of the phylogenetic tree branches and reticulation branches. The minimum of Q1 and Q2 can define a stopping rule for the addition of reticulation branches [21, 23, 24].

Results

Ten ISSR primers used produced 123 bands (Figure 1) of which 105 bands were polymorphic (85% polymorphism) and 8 bands were monomorphic. (AGC)5GG and (GA)9C primers produced the highest number of bands (19 and 18 bands respectively), while primers (GA)9A, (GA)9A and UBC 807 produced the lowest number (7, 8 and 8 bands respectively).

In total 8 specific bands were produced by ISSR primers. Primer (CA)7GT and (AGC)5GT produced the highest number of specific bands (3). Some of the genotypes showed presence of specific bands for example, Abbasi-gerd genotype showed a single specific band of the primer (AGC)5GT (1000 bp), which was not observed in other genotypes studied. Similarly, Vasatres cultivar had 3 specific bands produced by primer (CA)7AT(850 bp), (AGC)5GT (830 bp) and (CA)7GT (2500 bp). The Red-Delicious cultivar had two specific bands (1300 and 1400 bp respectively) produced by the primer (AGC)5GG, while Redstarking cultivar had only one specific band (430 bp) which was produced by primer (AGC)5GG.

Jaccard similarity as well as Nei's genetic identity and genetic distance were determined among 25 genotypes studied. The values of jaccard similarity ranged from 0.31 to 0.70. High values of similarities occurred between the genotypes Delbarstival and redstarking and then between Golshahi-red and Alimoori as well as Atlasi and M7. The values of genetic identity ranged from 0.58 to 0.88. High values of genetic identity was observed between the genotypes Delbarstival and Redstarking, Idared and Royalgal, Arbabi Bojnoord and Alimoor-dirras, Zeynab-banoo and Alimoor-dirras, Shasti and Ghasemshahi, Alimoor-dirras and Vsatres, Alimoor-dirras and M7, Kadoosib and Zeynab-banoo, These genotypes also showed lower values of Nei's genetic distances (data not shown).

We tried to group the genotypes by PCoA based on

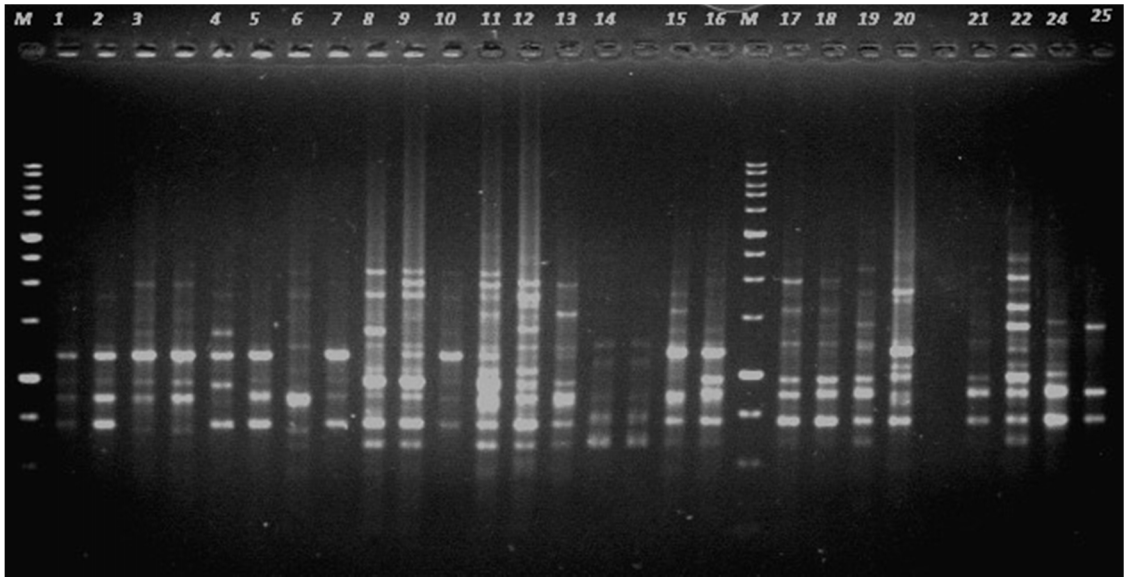

Figure 1. ISSR profile of the primer (CA)7GT. Genotypes 1-25 are based on Table 1

their geographical origin (Netherland, France, Lebonan, Karaj (Iran) and Shahreyar (Iran). The plot (data not shown) obtained showed that cultivars from different geographical regions were placed intermixed and are not placed in different groups. Therefore, we used UPGMA and NJ clustering methods to group the genotypes based on genetic distance. Both methods produced similar results but since NJ tree showed a higher cophenetic correlation value (r=0.98) it is discussed below.

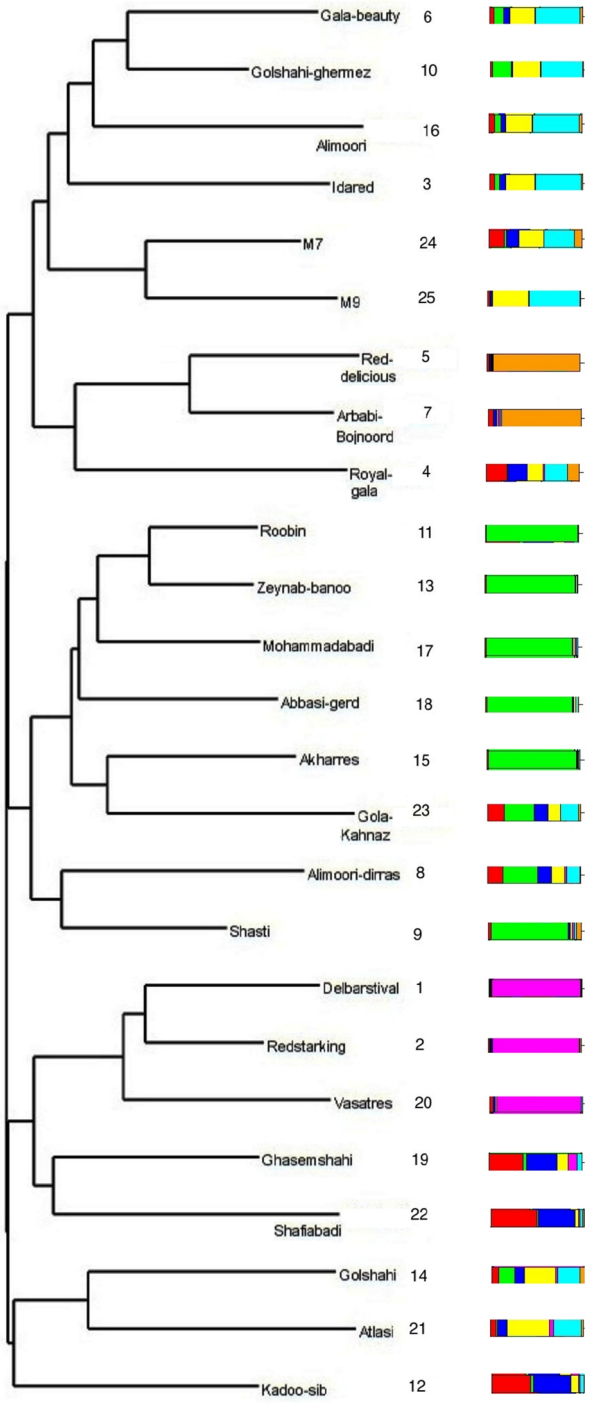

Figure 2. Neighbor Joining tree and STRUCTURE plot of ISSR data.

In general 4 major clusters are formed (Figure 2). The first major cluster is comprised of 9 genotypes distributed in 2 subclusters. The cultivars Gala-beauty, Golshahi ghermez, Alimoori, Idared, Malling 7 and Malling 9 comprised the first subcluster while, 3 cultivars namely Red-delicious, Arbabi-Bojnoord and Royal-gala formed the second subcluster. These 2 subclusters joined each other with some distance due to their genetic difference.

The second major cluster also showed presence of 2 subclusters. Cultivars Roobin, Zeynab-banoo, Mohammadabadi, Abbasi-gerd, Akharres and Gola-kahnaz formed the first subcluster while, 2 cultivars namely Alimoori-dirras and Shasti formed the second subcluster. These 2 cultivars joined the others with some distance.

Five cultivars comprised the third major cluster but were distributed in 2 subclusters. Cultivars Delbarstival, Redstarking and Vasatres formed the first subcluster, while Ghasemshahi and Shafiabadi cultivars formed the second subcluster. The forth major cluster contained only 3 genotypes namely Golshahi, Atlasi and Kadoo-sib of which the first 2 cultivars showed more genetic affinity and joined each other with less distance.

The presence of different number of subclusters in each major cluster formed, indicated intragroup genetic diversity which was latter on taken in to consideration while performing STRUCTURE analysis and assuming different population groups (k=2-7).

The mean number of variable alleles obtained for all cultivars studied was 1.364. The value of this parameter (Table 2) ranged from 1.057 (in forth major cluster/population group) to 1.553 (in first major cluster), among the four groups recognized by NJ tree. Similarly the mean number of effective alleles obtained for all cultivars was 1.334, while it ranged from 1.252 (in forth major cluster) to 1.387 (in first major cluster). The lowest and the highest values for I and He which are other genetic diversity parameters studied also occurred in the first and forth major clusters respectively (Table 2). Similarly, the highest percentage of allelic polymorphism was observed in population group 1 (73.00%) while, the lowest percentage occurred in the forth population group (38.00%).

AMOVA test performed on molecular features of the genotypes present in the 4 clusters revealed significant difference among the clusters (Table 3). The analysis showed 10% of the total variation to occur among the clusters while, 90% of variation occurs within clusters. This high value of within clusters genetic variation is in agreement with subclusters formed in each major cluster due to intra-population group variation.

UPGMA dendrogram constructed based on genetic diversity values (Figure 3) showed that the cultivars of the forth major cluster differed the most and joins the others with some distance while, the cultivars of the

Table 2. Genetic parameters determined among major clusters obtained by clustering.

Population		N	Na	Ne	I	He	UHe
Pop1	Mean	9.000	1.553	1.387	0.350	0.231	0.244
	SE	0.000	0.068	0.032	0.024	0.017	0.018
Pop2	Mean	8.000	1.407	1.346	0.307	0.203	0.217
	SE	0.000	0.074	0.034	0.025	0.018	0.019
Pop3	Mean	5.000	1.439	1.353	0.319	0.210	0.234
	SE	0.000	0.070	0.033	0.025	0.017	0.019
Pop4	Mean	3.000	1.057	1.252	0.221	0.148	0.178
	SE	0.000	0.078	0.032	0.025	0.017	0.021

N= Number of cultivars in the cluster, Na= Number of Different Alleles, Ne= Number of Effective Alleles, I= Shannon's Information Index, He= Expected Heterozygosity, UHe= Unbiased Expected Heterozygosity. Populations abbreviations: Pop1= includes genotypes 1, 2, 4, 5, 7 and 20 of Table 1; Pop2= includes genotypes 3, 6, 10, 15, 23, 24 and 25; Pop3= 12, 14, 19, 21; Pop4= includes genotypes 8, 9, 11, 13, 17, 18 and 22.

Table 3. AMOVA test used among 4 clusters obtained by clustering. df= digree of freedom, SS= sum of square, MS=mean of square, Est.Var=estimated variance and % Var=percentage of Variance

Source	df	SS	MS	Est. Var.	%Var
Among Pops	3	85.079	28.360	1.883	10%
Within Pops	21	360.361	17.160	17.160	90%
Total	24	445.440		19.043	100%
Stat	Value	P(rand >= data)			
PhiPT	0.099	0.010			

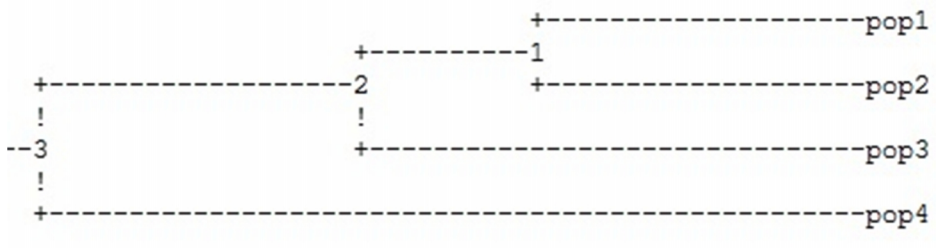

Figure 3. Grouping of the clusters obtained based on genetic diversity index. (Details of genotypes included in each cluster are given in the text).

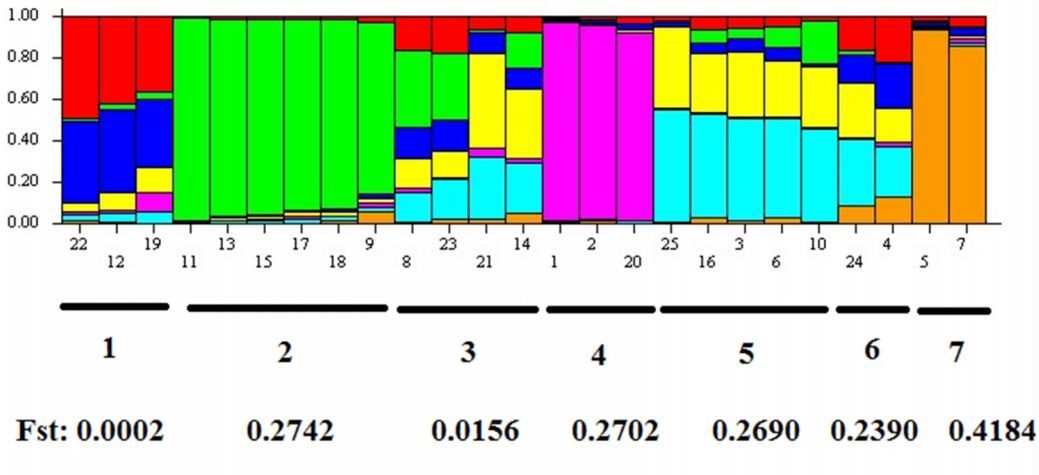

Figure 4. STRUCTURE plot k=7. The lines and numbers bellow lines indicate the cluster members and its number. (Fst values of each cluster is given in bellow of plot).

second and fourth major clusters are closer to each other.

STRUCTURE plots of k=2-7 were obtained and STRUCTURE Harvester analysis showed that the best groupings was done by k=6 or 7. Analysis for k=7 produced estimated Ln Prob of data = -2164.4, mean value of ln likelihood=-1605.6, variance of ln likelihood=1117.6 and mean value of alpha=0.0467.

The overall proportions of membership of the sample in each of the 7 clusters were 1=0.100, 2=0.280, 3=0.094, 4= 0.139, 5=0.125, 6=0.167 and 7=0.097. These values were almost the highest when compared with other k values assumed in the analysis and showed a better fit of data.

Allele-frequency divergence among pops (Net nucleotide distance), computed using point estimates of P, ranged from 0.002 between cluster No. 1 and 4 as well as between 1 and 3, to 0.3987 between 1 and 5. The average distances (expected heterozygosity) between individuals in same cluster ranged between -3.8361 in cluster 1 to -4.5034 in cluster 7. These data showed also good separation of clusters from each other and presence of genetic variation among the genotypes inside each cluster.

The mean value of Fixation index (Fst) in each cluster is provided in Figure 4. The lowest Fst value occurred in cluster 1 (0.0002) while, the highest value occurred in cluster 7 (0.4184). The highest Fst value of cluster 7, showed that 2 rootstock materials Mailing 7 and Mailing 9 are genetically differentiated from the others cultivars. Low Fst values in cluster 1 and 3 indicated genetic diversity among genotypes in these 2 clusters. Other clusters showed a medium Fst values and showed moderate degree of genetic variability compared to other clusters formed.

We also placed STRUCTURE plot results on NJ tree and could obtain more detailed information about allelic composition and frequency in each cluster/ subcluster formed by NJ tree (Figure 2). For example, in the first subcluster of major cluster No. 1, cultivars 6, 10, 16, 3, 24 and 25 showed almost similar allelic composition but differed in their frequency. This was also evident in both cultivars 6 and 10 which showed higher genetic affinity. Rootstockes Mailing 7 and 9 which were placed close to each other also differed in their allelic composition and frequency.

The same holds true for members of the second subcluster viz. cultivars 5, 7 and 4. They differed genetically from each other and also varied greatly from cultivars of the first subcluster. The great allelic difference was observed particularly between cultivars 5 and 7 and the others.

The cultivars grouped in the second major cluster (11, 13, 17, 18, 15, 23, 8 and 9) showed presence of alleles (green colored section) which were either absent in the other genotypes or present in very low frequency. The first 5 cultivars in this major cluster although showed high degree of genetic affinity but still differed in allele's frequency due to genetic rearrangements in their genome. Detailed comparison of the cultivars placed in the major cluster 3 and 4 also showed allelic difference. These genetic differences which are both quantitative (frequency of different alleles) and qualitative (allelic composition) in nature resulted in significant molecular difference among cluster groups obtained by AMOVA test discussed before.

Reticulated tree obtained by TIREX is presented in Figure 5. As it is evident in this figure, genetic

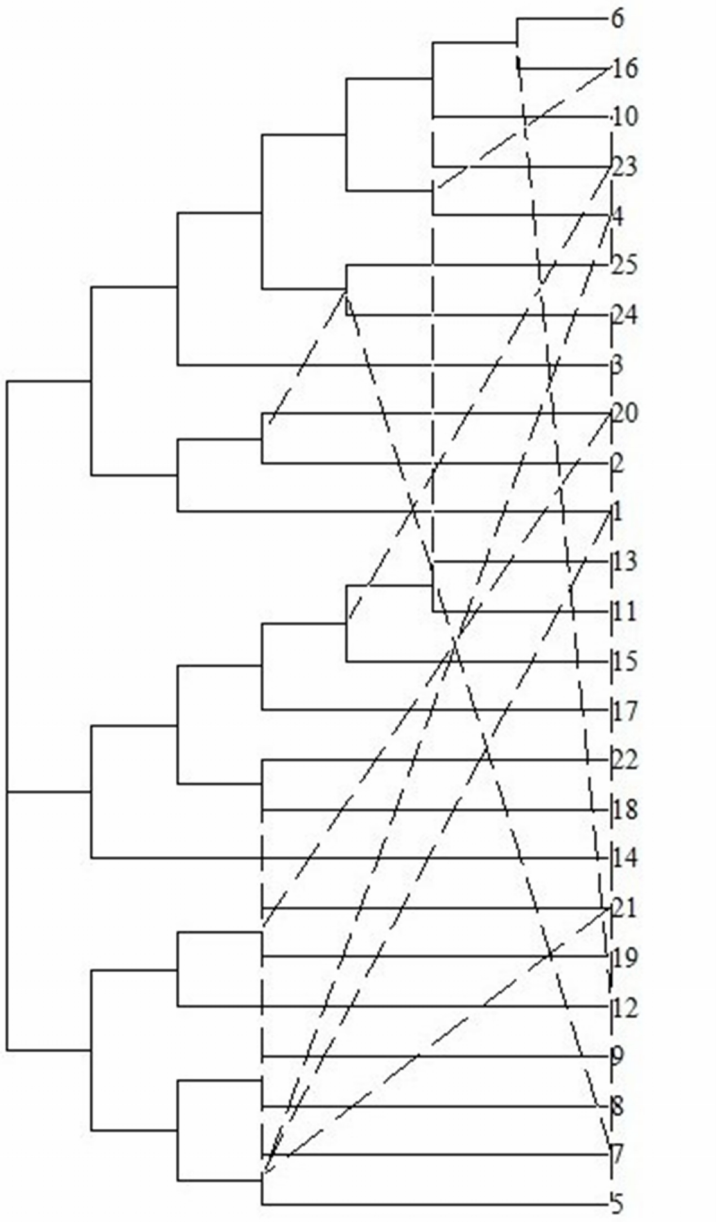

Figure 5. Reticlogram obtained based on ISSR data using T-REX ver. 3 . Genotypes 1-25 are based on Table 1

exchange/allelic similarities occurred both within members of each major cluster/subcluster and between different major clusters. For example, cultivars 6 and 16 of the first major cluster showed gene exchange with cultivar 12 from another cluster. Similarly, cultivar 16 showed gene exchange with cultivar 4 of the same cluster group. Cultivars 24 and 25 of the first subcluster showed gene exchange with cultivars 20 and 2 of another subcluster of the same major cluster group.

Discussion

The presence of ISSR polymorphic bands in the studied apple cultivars indicated the presence of genetic polymorphism in these genotypes. Moreover, the occurrence of specific bands/loci only in some of the cultivars illustrates the occurrence of unique insertion/deletion in DNA material of these genotypes which may be used in planning apple hybridization.

Smolik et al. [33] used ISSR markers to study genetic similarity of 8 apple cultivars. Out of 30 primers, 11 produced bands and were used for analysis. A total of 414 bands were obtained out of which 342 (83%) were polymorphic, which in close agreement with our findings. Moreover, they could detect specific ISSR products for each apple cultivar too, indicating the use of ISSR markers for apple cultivar finger printing and identification. According to Smolik et al. [33], the level of polymorphism of the ISSR markers is higher than AFLP markers reported by Goulão and Oliveira [13], and that of RAPD markers, described by Zhou and Li [36].

In another attempt, Goulão and Oliveira [13] used SSR and ISSR markers for potential use in fingerprinting and determination of the similarity degree between 41 commercial cultivars of apple previously characterized using RAPD and AFLP markers. A total of 13 SSR primer sets were used and 84 polymorphic alleles were amplified. Seven ISSR primers yielded a total of 252 bands, of which 176 (89.1%) were polymorphic. Except for cultivars obtained from somatic mutations, all cultivars were easily distinguishable employing both methods. The similarity coefficient between cultivars ranged from 0.20 to 0.87 for SSR analysis and from 0.71 to 0.92 using the ISSR methodology. Dendrograms constructed using UPGMA cluster analysis revealed a phenetic classification that emphasizes the existence of a narrow genetic base among the cultivars used, with the Portuguese cultivars revealing higher diversity. This study indicates that the results obtained based on the RAPD, AFLP, SSR and ISSR techniques are significantly correlated. The marker index, based on the effective multiplex ratio and expected heterozygosity, was calculated for both analyses (MI=1.7 for SSR and MI=8.4 for ISSR assays) and the results obtained were directly compared with previous RAPD and AFLP data from the same material. The SSR and ISSR markers were found to be useful for cultivar identification and assessment of phenetic relationships, revealing advantages, due to higher reproducibility, over other commonly employed PCR-based methods, namely RAPD and AFLP.

Pathak and Dhawan [28] used a total of 24 ISSR primers assess the genetic stability of micropropagated plants regenerated through axillary buds of clonal apple (*Malus* × *domestica* Borkh.) rootstock MM111 after twenty second passages and obtained 147 amplification products with an average of 10 bands per primer. A homogenous amplification profile was observed for all the micropropagated plants and no variation was observed.

Liebhard *et al*. [22] used a combination of AFLP, RAPD, SSR and SCAR molecular markers to investigate their potential for QTL (Quantitative Trait Loci) identification and found these markers useful and transferable to populations or cultivars other than the ones on which it has been constructed.

In the present study AMOVA test showed genetic differences among the cultivars studied and that much of variation exists within each group. This is also supported by NJ tree which showed presence of different subclusters in each major cluster group and also by STRUCTURE analysis as explained in detail before. All these data indicate presence high level of genetic diversity among apple germplasm available in the country which may be used in breeding and improvement of apple trees. Both STRUCTURE and Reticulation analyses showed high level of genetic admixture in apple samples studied. This indicates that we can mix the genetic materials among different apple cultivars and if this admixture is accompanied with new and desirable agronomic or disease resistance features we shall have the opportunity of improving apple trees. Faramarzi et al. [9] and Farrokhi et al. [10] reported intra genetic variations in Iranian apple cultivars. They also showed that distribution of the cultivars was independent from their geographical distribution which were in agreement with our finding.

Coart et al. [4], studied the genetic variation within and between wild apple samples (*Malus sylvestris*) and cultivated apple trees by using AFLP and SSR markers to develop a conservation genetics program for the endangered wild apple in Belgium and observed that although some geographical pattern of genetic differentiation among wild apple populations exists, most variation is concentrated within samples, which is

similar to what we obtained in ISSR analysis.

They studied 76 putative wild, six presumed hybrids and 39 cultivars. Both clustering and PCoA analyses classified the apples into three major gene pools: wild genotypes, edible cultivars and ornamental cultivars. All presumed hybrids were assigned to the edible cultivar gene pool. They found that the gene flow between wild and cultivated gene pools is shown to be almost absent.

In conclusion we suggest that ISSR and other molecular markers are of use in apple genotype discrimination and in further studies we should use these molecular markers to identify other variants in the apple germplasm of the country and try to correlate molecular data with useful agronomic traits of apple tree.

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