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A panel of cultivate specific marker based on polymorphisms at microsatellite markers for Iranian cultivated Almonds (*Prunus dulcis*)

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Abstract

Molecular markers developed for *Prunus* also offer a powerful tool to study the evolution of the genome, and for understanding of genome structure and determinants of genetic diversity. Two hundred eighty almond genotypes/cultivars from different origins distributed throughout Iran besides some foreign cultivars and their hybrids with Iranian ones were collected. Microsatellite analysis was carried out using 9 pair flanking SSR sequences previously cloned and sequenced specifically in almond. The total number of detected alleles was 152 (9 to 20 alleles per locus with an average of 16.87). The mean PIC value of the polymorphic loci was relatively high (0.81) and the mean value for H_e was 0.83, so that we were able to distinguish 98% of the genotypes using 5 loci. In cluster analysis, the genotypes were divided into 2 major groups, foreign cultivars and Iranian almond genotypes. Principal coordinate analysis based on Shared Allele method indicated proper distribution of the studied markers through the genome. Some specific markers were recorded among the germplasm which can be used efficiently in rapid and precise identification of the related genotypes and also in breeding programs through MAS. Genotypes were coded using our suggested coding method for genotype molecular identification.

Keywords: Almond, DNA barcoding, genetic identification, molecular marker, microsatellite, *Prunus dulcis*.
Abbreviations: Polymorphism information index (PIC), Simple sequence repeat (SSR), Expected heterozygosis (H_e), Marker assisted selection (MAS), Polymerase chain reaction (PCR), Neighbor joining (NJ), Shanon information index (I), Polymorphism information content (PIC), Base pair (bp), Dinucleotide triphosphate (dNTP)

Introduction

The genus *Prunus* in Prunoideae, a subfamily of Rosaceae, includes several economically important fruit tree species such as apricot (*P. armeniaca*), cherry (*P. avium*), peach (*P. persica*), plum (*P. mume*), and common almond (*P. dulcis* Mill. syn. *P. communis* Fritsch.). Twenty-six almond species form a distinct and easily identified taxonomic group in the world (Browicz and Zohary, 1996). In Iran, almond after pistachio is the most important nut fruit and 21 almond species and 6 natural hybrids grown in different climatic regions have been described (Khatamsaz, 1992). Iran is amongst the most important countries in the world producing *Prunus* fruits, as its ranking regarding to production of cherries, apricot and almond is the first, second and third, respectively. Traditionally, the identification and characterization of cultivars and species has been based on morphological and physiological traits which are sometimes difficult. Since morphological markers are prone to equivocal interpretations and time consuming, not always available for analysis and are affected by changing environmental conditions, molecular marker technology offers several advantages over the sole use of conventional markers in cultivar identification and breeding programmes. Molecular tools developed for the characterization for biodiversity may allow classification of synonyms and detection of the origin of species and cultivars (Rahman *et al*., 2009). Molecular markers developed for *Prunus* also offer a powerful tool to study the evolution of the genome, and for understanding of genome structure and determinants of genetic diversity (Martínez-Gómez *et al*., 2005). Microsatellites or simple sequence repeats (SSR) are sequences of a few repeated and adjacent base pairs, well distributed over the eukaryote genome (Powell *et al*., 1996). PCR-based, SSR markers are becoming the marker of choice for fingerprinting and genetic diversity studies for a wide range of plants. Because of their high polymorphism, abundance, and codominant inheritance, they are well suited for the assessment of genetic variability within plant species, and of the genetic relationships among species. Since there was not any comprehensive molecular database about Iranian almonds, in this work microsatellite markers (SSR) besides morphological traits were used in the

Table 1. List of the SSRs used in the study, locus code, primers sequence, allele size range, number of alleles, effective number of alleles, Shanon information index, heterozygosity, gene diversity and PIC.

SSR locus/ GeneBank no.	Sequence (5'-3')	Genotype No[1]	Allele size range (bp)	Allele No.	N_e[2]	I[3]	Gene Diversity[4]	Heterozygosity[5]	PIC[6]
UDA-002 BV102479	5'AAACGTGAGGTCTCACTCTCTC 5'-GCCATTTAAGGGTCTGGTCA	53	110-200	18	6.75	2.25	0.85	0.43	0.84
UDA-005 BV102480	5'CATCACACACAAACACAAATGC 5'-GCATTGTGCTCTTCATGGAC	70	146-212	18	7.64	2.35	0.87	0.7	0.86
UDA-006 BV102471	5'-ATTCTCCAAGGCGATAAGCA 5'-TTAGGCACCTGTCCCCTACA	19	149-177	9	3.43	1.51	0.71	0.16	0.67
UDA-008 BV102481	5'AGACGCTTTGCATACATACAAG 5'-TGCAGGAACTGGGATTAGAGA	88	111-189	20	12.06	2.62	0.92	0.78	0.91
UDA-009 BV102482	5'-AAAACATCTCTCTCCTCCATGC 5'-AGTTCTCTGGCAGCACAAGC	74	168-236	19	10.43	2.53	0.9	0.47	0.9
UDA-015 BV102474	5'-ACTCCATCGCTTGCATTTTC 5'-GCTCCGTGTGTGTTTGTGTG	23	101-192	12	4.44	1.71	0.77	0.37	0.74
UDA-022 BV102476	5'-GCCGTCTCATTTTCCCATTA 5'-GTGCGATGGAGGAGCACT	36	145-279	19	4.94	1.96	0.8	0.34	0.77
UDA-023 BV102477	5'-TTGCCGTGATACACTAACAACT 5'-ACCTGCCAAGTAAGTGCCTA	49	110-200	20	6.55	2.21	0.85	0.61	0.83
Mean		52	101-279	16.87	7.03	2.14	0.83	0.48	0.81
St. Dev		24.78		4.09	2.97	0.39	0.07	0.20	0.08

[1] Genotype No: number of the obtained allelic genotypes, [2] Ne: effective number of alleles (Kimura and Crow, 1964), [3] I: Shanon information index (Lewontin, 1972), [4] Gene Diversity: expected heterozygosity, [5] Heterozygosity: observed heterozygosity, [6] PIC: Polymorphism Information Content.

characterization of 280 almond genotypes/cultivars which most of them were Iranian along with some foreign cultivars. The objectives of the present study were search for a panel of cultivate specific marker based on polymorphisms at microsatellite markers for Iranian cultivated Almonds *(P. dulcis).*

Materials and methods

Plant material

Two hundred and eighty almond genotypes/cultivars from different origins distributed throughout Iran besides some foreign cultivars and their hybrids with Iranian ones were used in this study (Fig. 1). The genotypes maintained at two experimental orchards located at the Seed and Plant Improvement Research Institute in Karaj and Isfahan, Iran (supplementary table 1).

Molecular characterization

Genomic DNA extractions were performed as previously described (Kadkhodaei *et al*. 2005). The purified total DNA was quantified by gel electrophoresis, and its quality verified by spectrophotometry. DNA samples were stored at 4°C. Extracted almond genomic DNA was PCR-amplified using 9 pair flanking SSR sequences previously cloned and sequenced specifically in almond (Testolin et.al 2004). Details of the microsatellites analyzed (Fig. 2) and their origin are given in Table 1. As a parameter for the selection of some of these microsatellites we have used the genetic informativeness and the technical aspects of the analysis. None of the microsatellites used have not been studied extensively for almonds earlier, and almost all of the previous studies on almonds have been carried out using peach isolated SSRs. Amplification was conducted in a total volume of 15 µL with 10 ng DNA, 0.05 µM of both specific primers, 0.2 mM of 10 mM dNTP mix, 0.1 U Taq polymerase (Roche Applied Science), 1.5 mM $MgCl_2$, and 1X PCR buffer (100 mM Tris HCl, pH 8.3, 500 mM KCl). The amplification program consisted of 3 min at 94 °C, 34 cycles of 45 s at 94 °C, 45 s at the appropriate annealing temperature and 45 s at 72 °C, followed by a 5-min extension at 72 °C. Five micro-liters of the PCR product were separated on a 1% agarose gel to check the PCR amplification and determine approximated size of the amplified fragments. Then electrophoresis of PCR products were carried out on a 6-10 % polyacrylamid gel (depended on allele ranges in each locus) in 0.5 X TBE. The gels were run for 3 h at constant power (120 W) in ATTO Electrophoresis system. A 50-bp DNA Ladder (Fermentas) was used as a molecular size standard in each gel.

Statistical analysis

Allele size was measured with UVDoc 99.02 analysis software (UVI Tech, Cambridge, UK) by manual editing to increase accuracy. This procedure carried out two times to exclude wrong scorings. We developed software (ABRIISTAT30) for manipulating the large amount of data (Kadkhodaei, 2008). Using the ABRIISTAT software, input data (allele sizes) were processed to produce data format for different analysis softwares such as PowerMarker, PopGene, NTSys, and also different statistical parameters related to genetic diversity. We used the PowerMarker3.25 software package (Liu, 2005) to generate a similarity matrix by calculating the proportion of bands shared by each pair of accessions (Shared Allele coefficient) and to produce a dendrogram using Neighbor Joining (NJ) method due to high evolutionary rate of the germplasm. Different genetic diversity parameters were calculated as follows: Heterozygosity (the proportion of heterozygous individuals in the population) at a single locus, Gene diversity (often referred to as expected heterozygosity, is defined as the probability that two randomly chosen alleles from the population are different), PIC (Polymorphism information content- Botstein, 1980) and the shared allele distance (D_{SA}) (a commonly used distance- Chakraborty and Jin, 1993).

Fig 1. Iran map; the colored provinces are those from where genotypes were collected.

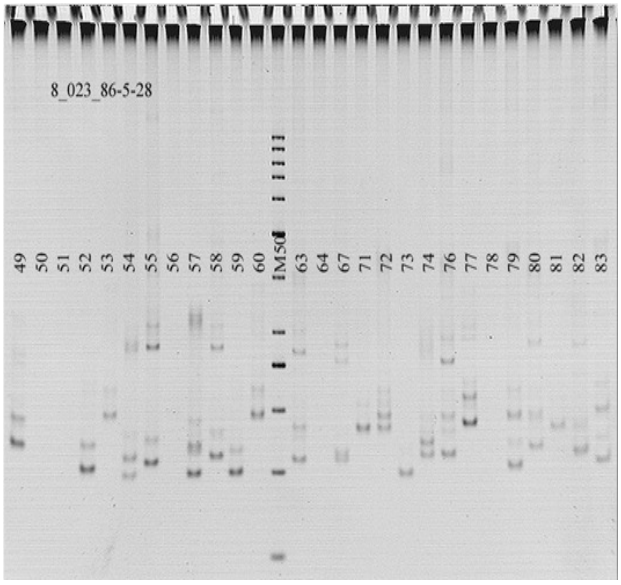

Fig 2. Alleles amplified using SSR primer set UDA-023 (GenBank accession number BV102477) for a group of the studied almond genotypes and cultivars.

Results and discussion

SSR polymorphism and distribution of alleles among the germplasm.

All of the SSR primers amplified polymorphic products except one (UDA010) which excluded from the analyses. Eight of the 9 genomic SSRs showed polymorphism in the 280 cultivated almond accessions and the total number of bands and alleles detected among them was 2879 and 152, respectively. The number of alleles per locus varied from 9 (UDA006) to 20 (UDA008, UDA023) with an average of 16.87 alleles per locus (table 1). The separable allelic genotypes detected for each locus ranged from 88 to 19 for UDA008, UDA009, UDA005, UDA002, UDA023, UDA022, UDA015 and UDA006, respectively. Other parameters including N_e (number of effective alleles), Shanon information index (I), gene diversity (H_e) and polymorphism information content (PIC) showed the same sequence for loci, so that increasing the amount of N_e (and not necessarily the total number of allele per locus) leaded to an increase in H_e and also indirectly in ability of the loci for separation of genotypes through increasing the number of allelic genotypes (Table 1). Frequency of the loci alleles except UDA015 and UDA022 showed a relatively normal distribution, so that alleles with lower and higher sizes had less frequency.

In the almond germplasm examined, the mean PIC value of the polymorphic loci was 0.81, ranging from 0.67 to 0.91, and the mean value for He was 0.83 (from 0.71 to 0.92). Six SSR primer pairs had a PIC value higher than 0.75. Because of the high polymorphic value of the studied loci, we were able to distinguish 98% of the genotypes using 5 loci (UDA002, UDA005, UDA006, UDA008 and UDA009). The mean value of observed heterozygosity (H_o) was lower than H_e, which represent the presence of null alleles among the germplasm. Considering the range of allelic size in 009, 015 and 005 loci, they may become the choice loci for using in multiplex PCR for genotype identification; this also can be applied slightly for the locus 023.

Genetic similarity and phylogenetic relationship

The maximum genetic similarity based on the shared allele index showed 6 groups included group 1 (No.8 and Hamedan21, Butte, Arak56, 1-a13 and 10-a16), group 2 (Sahand and Nonpareil3, group 3 (ShirazPN1-1.2 and ShirazPN1-1.6), group 4 (Tajeri12-3 and Tajeri12-5), group 5 (Butte and 1-a13), group 6 (Arak52.5 and Arak51). Several genotypes showed the minimum value of similarity. The average for similarity among the germplasm was as low as 0.2. The least genetic similarity among populations belonged to Italian almond cultivars (0.11), and the most one was 0.22 for hybrid genotypes. Regarding to homozygosity for the studied loci, genotypes MamaeiZefreh, Yazd103, Karaj15, 16-a30 and *A.lycioides*2 were heterozygote for all loci except one. Contrarily, genotypes GX, Kerman20, ShahrekordA1, Tafresh, Esfahan-Yarallah, 101-2-19.3, 16-a8 and 3-a11 represented the most homozygosity for all of the loci.

Cluster analysis

In the dendrogram (Fig. 3), the 280 genotypes were divided into 2 major groups: group 1 included mainly foreign cultivars besides their hybrids with Iranian cultivars, and also there were a few of Iranian genotypes; group 2 included nearly most of the Iranian almond genotypes which comprised subgroups according to their geographical origin. Group 1 further divided into two subgroups, the American cultivars and their offspring's with Iranian ones; Subgroup 2 included Italian cultivars. There were some Iranian genotypes distributed in the subgroups. In group 2, all of the genotypes were Iranian except Padre, Price and two Spanish cultivars. Distribution of Iranian cultivars in secondary subgroups was mainly due to geographic origin, so that genotypes of Isfahan, Shahrekord, Hamedan, Yazd and Shiraz clustered together separately.

Table 2. Independent axial coordinates (the first ten), with the corresponding Eigen value, percentage of explained variation and cumulative value.

PCoA	Eign value	percent of variation	cumulative value
1	23.167	5.8163	5.8163
2	16.905	4.2442	10.0605
3	15.605	3.9179	13.9784
4	13.15	3.3016	17.28
5	11.229	2.8368	20.1168
6	10.584	2.6574	22.7742
7	10.523	2.6419	25.4161
8	9.4574	2.3744	27.7905
9	8.3565	2.098	29.8885
10	7.8375	1.9677	31.8562

ShirazPoostnazok1 and ShirazPoostnazok2 and also Tajeri12-3 and Tajeri12-5 were duplicated so they have been tested for possible differences. About two genotypes, Sahand and Nonpareil3, it assumed that may be mislabeling occurred, because another accession of this genotype (Nonpareil) have grouped with American genotypes. In this study Azarbayjan genotypes and cultivars and American ones were grouped together which is interesting. Iranian breeding programs were started by importing foreign cultivars, specifically American cultivars and using them as Genitor in hybridizing with Iranian genotypes to release high yield and late flowering cultivars. Azar and Shekofe were obtained from a cross between Cristomorto×Ai and Nonpariel×Ai respectively. Both mentioned cultivar besides Sahand (which is known as a native cultivar in Azarbayejan) identified and released as late flowering cultivars. Yalda is the same American Neplus ultra. A few genotypes including Tajeri12.3 and -12.5, soft shell ShirazPoostnazok1 and -2, Arak No.1 and No.2 and also two Spanish genotypes assumed to be mislabeled, but the study indicated that this hypothesis was not true for them.

While three Nonpariel genotypes, two Shekofeh genotypes and two Mamaee genotypes, showed differences in their molecular profiles in spite of same labeling and the hypothesis was true for them. Considering their different sampling origins, it may be occurred some mistakes in naming or numbering during transplanting and cultivation. About Shekofeh genotypes, only the most similar one to Nonpareil can be considered as Shekofeh cultivar, because according to its pedigree information, Nonpareil is one of the parents of Shekofeh. Frangness and shahrod12 showed a high genetic similarity, indicating that maybe these two genotypes are the same and/or have very similar genetic background. Genotypes 101 and 103 which were found accidentally in an orchard located in Najaf Abad (Esfahan –central region of Iran), showed high genetic similarity with Rabee cultivar (a native cultivar in this region), indicating that may be these two genotypes are a possible Bud Sport of Rabee cultivar. Interestingly, among the studied genotypes these two genotypes had the highest percentage of (double flower in buds) and consequently high flower density which is important in almond breeding programs for increasing of the yield. Iranian and foreign genotypes were segregated separately, indicating their separate origin and development.

PCO analysis

A possible aggregation among some almond cultivars was suggested by the principal coordinate analysis. Principal coordinated analysis (PCO) based on Shared Allele method and calculated Eigenvalues have been presented in Table 2. The first ten principal coordinates accounted for 32 percent of the total variability. Unlike quantitative traits, which the first two or three coordinates account for nearly 90 percent of the total variability (particularly when there is a high correlation between traits), in molecular markers data especially when the markers distributed through the genome equally, some first coordinates may not account for a large amount of the total variability. In genetic diversity studies using molecular markers, whereas covering the genome with markers thoroughly is desired for sampling the whole genome and consequently because of the low correlation among the markers, so more principal coordinates are needed to account for the total variability. In this study, as we expected, some first coordinates accounted for a small percentage of the total variability, indicating proper distribution of the studied markers through the genome. As it has been shown in table 2, the first and second coordinates accounted for nearly 5.8 and 4.2 % of the total variability, respectively. Accordingly, until the thirtieth coordinate, 57% of the total variability will be accounted. On the other hand, the indication of such aggregations has a potential value to optimize parental choice in breeding crosses and for germplasm conservation programs (Resta *et al*., 1998).

Specific markers

Some alleles were shown to be unequally distributed in almond cultivars from different origins. The specific alleles, rare alleles and allelic genotypes (alleles and genotypes with frequency lower than 1%) were recorded among the germplasm. These specific markers can be used efficiently in rapid and precise identification of the related genotypes and also in breeding programs through marker assisted selection (MAS).

Genotype identification

Using the ABRIISTAT software, input data (allele sizes) were processed to produce data format for different analysis softwares such as PowerMarker3.25 (Liu and Muse, 2005), PopGene (Yeh and Boyle, 1997), NTSys (Rohlf, 2000), and also different statistical parameters related to genetic diversity.Another feature of ABRIISTAT software was development of a molecular identification key for the studied loci. The method includes two parts, the first for locus characteristics or Gene Bank No. and second for allele(s) size, for example UDA002-125/115. In the case of *Prunus* species, primer pairs flanking SSRs have been cloned and sequenced in peach (Dirlewanger *et al*., 2002; Georgi *et al*., 2002), apricot (Decroocq *et al*., 2003), cherry (Schueler *et al*., 2003) and almond (Testolin *et al*., 2004). These SSR markers have been used primarily for the molecular characterization and identification of cultivars in the related *Prunus* species. Also SSRs developed from different *Prunus* species have high potential for transportability across each other (Fathi *et al*., 2008; Shiran *et al*., 2007; Mnejja *et al*., 2005).

There are some other studies related to molecular identification of Iranian almond germplasm, in which all of them are restricted to limited number of the almond cultivars. Among the molecular markers used in this regard, RAPD (Shiran *et al*., 2007; Kadkhodaei *et al*., 2006), SSR (Shiran *et al*., 2007; Fathi *et al*., 2008; Kadkhodaei *et al*., 2009) and AFLP (Sorkhe *et al*., 2007) are most popular. Shiran *et al*. (2007) used SSRs (developed in peach) and RAPDs to study

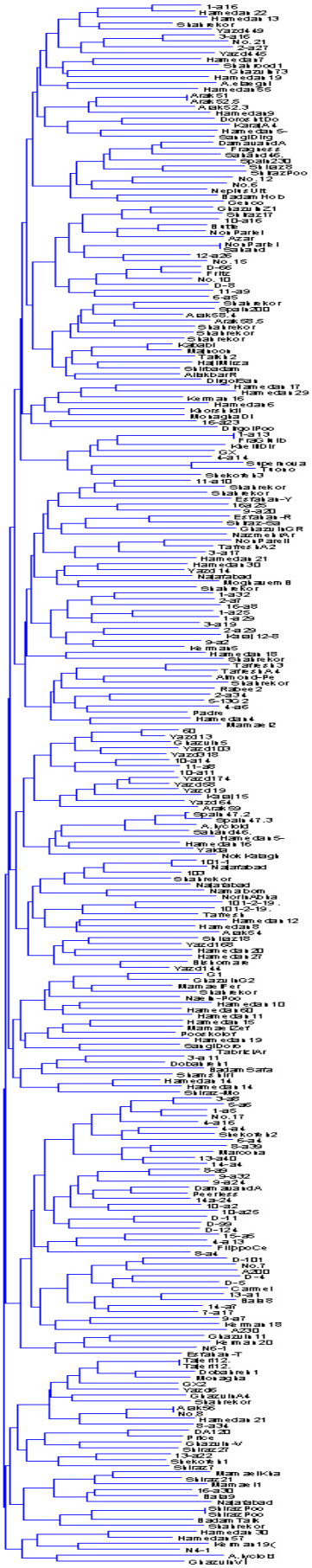

Fig 3. Dendrogram based Shared Allele genetic distance using SSR data of nine primer combinations and Neighbor Joining (NJ) method among the 280 almond germplasm.

the genetic diversity of eight Iranian almond cultivars and their relationship to important foreign cultivars and three wild almonds (*P. communis, P. orientalis* and *P. scoparia*). Results demonstrated an extensive genetic variability within the tested cultivars. Fathi *et al*. (2008) analysed 35 microsatellite markers markers among 56 almond (*Prunus dulcis*) genotypes which out of them 25 simple sequence repeats (SSRs) markers, were polymorphic, producing 215 alleles that varied from 2 to 16 with an average of 8.76 alleles per locus. Amplified fragment length polymorphism (AFLP) analysis was used by Sorkheh *et al*. (2007) for producing DNA fingerprints and molecular characterization of 45 almond cultivars from Iran, Europe, and America, assaying 19 primer combinations. In two studies Kadkhodaei *et al*. (2006 and 2009) evaluated almond germplasm in Iran (the most important 10 cultivated almonds and 5 wild almod species including *Prunus communis, P. hausknechtii, P. lycioides, P. elaegnifolia and P. scoparia*) using RAPD and SSR marker, which were considered as the preliminary studies to design thereof the more comprehensive present project. Comparing with the similar studies on almond cultivars in European and other Asian countries as well as in USA (Resta *et al*., 1998; Testolin *et al*., 2004), overall results show more polymorphism and heterozygosity among the Iranian almonds, indicating the presence of a rich almond germplasm and gene pool and confirming Iran as a center of origin for almond. In conclusion, the results of this study on almond cultivars and genotypes grown in Iran were: (i) the detection of considerable variation at the DNA level; (ii) Suggestion of a molecular code for identification of the genotypes; (iii) the selection of primers highly polymorphic; (iv) the revelation of high similarity between some pairs of cultivars; (v) identification of specific markers; and (vi) the consistency of SSR data with independent observations in estimating genetic relationships.

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