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The Study of Morphological Traits and Identification of Self-incompatibility Alleles in Almond Cultivars and Genotypes

Mousa Rasouli

Department of Horticulture and Landscape, Faculty of Agriculture, Malayer University, P.O. Box 65719-95863 Malayer, Iran

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Abstract

The evaluation of an almond collection using morphological variables and identification of self-incompatibility genotype is useful for selecting pollinizers and for the design of crossing in almond breeding programs**.** In this study, important morphological traits and self-incompatibilities in 71 almond cultivars and genotypes were studied. Simple and multiplex specific PCR analyses were used in order to identify self-incompatibility alleles. Based on the results, cultivars and genotypes including 'Dir Ras–e-Savojbolagh', 'D-124', 'D-99', 'Shahrood 12', 'Tuono', 'Nonpareil', 'Price', 'Mirpanj-e-Tehran', 'Pakotahe-e- Taleghan', 'V-13-34', 'V-16-8, 'V-11-10', 'Zarghan 10', 'Uromiyeh 68', 'Barg dorosht-e-Hamedan' and 'Yazd 60' were late flowering and had the highest quality of nut and kernel characters. The result of the PCR method using combined primers AS1II and AmyC5R showed amplification of ten self-incompatibility alleles (S_1, S_2, S_3, S_5, S_6, S_7, S_8, S_{10}, S_{12},and *S* unknown allele) and three S_f alleles. Moreover, S_1 had the highest frequencies in comparison with other known S-alleles. Also, unknown alleles with different sizes were detected and 58 new bands were found in some cultivars.

Keywords: AS1II, AmyC5R , Incompatibility, Kernel, *Prunus dulcis*, S-allele, Specific PCR.

Introduction

The cultivated almond [*Prunus dulcis* Miller (D.A. Webb)] belongs to the Rosaceae family, subfamily Prunoideae, and typified by a drupe fruit structure (Kester and Gradiziel, 1996). Almond originated in central and southwest Asia and represents divergent evolution under cold and xerophytic environments. Related *Prunus* species are found growing wild from eastern China to the mountainous areas and deserts of western China, Afghanistan and Iran (Kester and Gradiziel, 1996; Browicz and Zohary, 1996). Almond is one of the most important nut crops worldwide and produces fruits with high commercial value. Iran is the main producer of this crop (FAO, 2012; Sepahvand *et al.*, 2015).

The efficiency of cross-breeding programs mainly depends on the choice of the progenitors and knowledge on the transmission of traits that are to be improved. A high efficiency is especially important in fruit breeding, almond included, due to the high cost and time consuming of breeding programs of these species (Sanchez –Perez *et al.*, 2007). Late blooming, time of maturity and some of the fruit and kernel traits are the most important objectives of the almond breeding programs, and there are studies on transmission of these traits (Kester and Gradiziel, 1996; Sanchez –Perez *et al.*, 2007; Spiegel-Roy and Kochba 1974; Dicenta *et al.*, 1993).

*Corresponding author: Email: m.rasouli@malayeru.ac.ir

In addition, almond is a predominantly self-incompatible species, although some of the self-compatible cultivars have been described (Halasz *et al.,* 2006). Self-incompatibility is a gametophytic type and controlled by a single *S* locus with multiple codominant alleles and expressed within the styles of flowers as *S*-RNAs glycoproteins (Halasz *et al.,* 2006; Wirthensohn *et al.,* 2011). These glycoproteins are responsible for the inactivation of self-pollen tube growth in most species of genus *Prunus,* including almond (Socias I Company and Alonso, 2004; Alonso and Socias I Company, 2006), apricot (Hajilou *et al.,* 2006), sweet cherry (Wunsch *et al.,* 2004) and plum (Yamane *et al.,* 1999). Ortega *et al.,* (2005) characterized the alleles S_1–S_{23}, and also S_f, with respect to the length polymorphisms of their first and second intron products by the use of two novel consensus primer pairs (EM-PC2consFD/ EM-PC3consRD and PaConsI-F/EM-PC1consRD).

Twenty six *S*- alleles of incompatibility (from S_1 to S_{25} and S_{7A}), a part from the self-compatible dominant allele S_f, have been identified in the cultivated almond species. Two methods have mainly been used to determine the S genotype: Controlled pollination (in the field or in the laboratory) and *S-RNase* analysis (in the laboratory) (*Boskovic et al.* 2003*)*, although other new technologies based on the DNA analysis have recently been developed (Lopez *et al*. 2006; Mousavi *et al.,* 2010; Mousavi *et al.,* 2014). Since information about *S*-haplotypes is very important to ensure fruit set, *S-RNase* gene specific primer PCR analysis has been developed to determine the S-haplotypes of various *Rosacea*e fruit tree species, such as almond (Ortega et al. 2005; Mousavi *et al.,* 2010; Mousavi *et al.,* 2014)**.**

Rahemi *et al.,* (2010) studied the *S*-alleles in 96 wild almonds and related *Prunus* species from 10 taxonomic groups. They used six sets of primers including three degenerate primer pairs (PaConsI-F(FAM)/EMPC1consRD, PaConsI-F(FAM)/EM-PC3consRD, EM-PC2consFD/EM-PC3consRD), one general primer pair AS1II/AmyC5R, one allele specific primer pair (CEBASf/AmyC5R), and one set of multiplex primers (AS1II/CEBASf/AmyC5R). Results showed that the number of amplified bands (155) and their size ranges were higher than in previous reports. The primers, including the allele specific (CEBASf/AmyC5R), did not amplify any self-compatibility allele (*Sf*) among the evaluated samples . Sizes of amplified alleles were compared with previous reports in almond and labeled accordingly. Alleles S_9, S_2, S_{13}, and S_{25} had the highest frequencies (12.26, 8.39, 7.74, and 7.74 percent, respectively) (Rahemi *et al.,* 2010). Alleles S_{16}, S_{17}, S_{18}, S_{19}, S_{22}, and S_{28} were not observed in the examined samples and alleles *S*15 and *S*26 had a low frequency (0.65). The dendrogram revealed that *S*-alleles were more similar within a taxonomic group than other groups (Rahemi *et al.,* 2010).

Wirthensohn *et al.,* (2011) determined the self-incompatibility (SI) genotypes of 25 Australian almond cultivars by PCR analysis of genomic DNA using a combination of specific primers based on the intron regions and primers based on the conserved regions of Rosaceous S-RNase genes. DNA fingerprinting of the cultivars was achieved through microsatellite fragment analysis and comparison with European and American cultivars to determine the genetic diversity within Australian almond accessions. The results showed a diverse range of incompatibility groups within Australian cultivars and fingerprinting which reflected their ancestry, a combination of American and European backgrounds (Wirthensohn *et al.,* 2011).

Mousavi *et al*., (2014) studied the S-RNase alleles of 70 almond accessions that were identified by PCR using combinations of the consensus primers PaConsI-F, EM-PC1consR, EM-PC2consF, EM-PC3consR and EM-PC5consRD. Sixteen cultivars of different origins were also included in the analysis as a reference

for 30 S-RNase alleles already characterized in this species. In most cases, the results showed two bands matching the size of the already known S-RNase alleles. However, in 13 accessions, some bands differed in size and were considered to correspond to new S-RNases. Nine new S-RNase alleles were cloned and sequenced from seven almond cultivars. The results from Mousavi *et al.*, (2014) showed that Iranian almond cultivars had variation in S-alleles. Therefore, most cultivars had an S-genotype different from those of the established cross-incompatibility groups in almond. They were included in the universal group O. The alleles S_1, S_2, S_4, S_7, S_{12} and S_{24} had the highest frequencies, whereas S_{25}, S_{38}, S_{41} and S_{42} had the lowest frequencies in Iranian almonds. According to these results, S-alleles are more diverse in almonds originated in different geographical regions, and thus, could be considered as genetic markers in studies of genetic diversity. Identification of S-alleles in almond cultivars is important for orchard design and for designing crosses and choosing parents in breeding programs. Pollen-pistil compatibility relationships among some almond cultivars and genotypes have been poorly characterized.

The aim of this study was to evaluate the agronomic traits and identify self-incompatibility alleles in 71 almond cultivars and genotypes.

Materials and Methods

Plant material

This experiment was performed at, Agriculture Research Center of Malayer and Horticulture and Landscape Department of Malayer University. The plant materials used were sampled from three trees in each sample of different geographical scales. They derived mainly from the almond collections of Kamal-Abad (Karaj, Iran). This area is located in Alborz province, at 36°08′27″ N latitude, 50°03′26″ E longitude and 1,270 m above the sea level, with an annual average temperature of 13.8°C and an annual average precipitation of 260 mm. In this study, 61 Iranian and 10 foreign almond cultivars and genotypes were investigated. The trees were nine years old, healthy, and had a full crop.

Morphological traits

Some morphological important traits of the assayed cultivars and genotypes, which were used in this study, were measured according to the Gulcan (1985) descriptor. To measure vegetative and fruit characteristics, at least 50 mature fruits from each tree were hand-harvested, the hulls removed and nuts dried for four weeks at room temperature. For each sample, some important fruit traits were analyzed.

Ten leaves were collected from the mid-shoot portion and used for measurements of leaf area (mm^2) using the leaf area meter devise (Leaf Area Meter-England, WinDIAS3 software).

Identification of S-alleles

DNA extraction

Total DNA was extracted using CTAB extraction method that based on the procedure described by Doyle and Doyle (1987).

PCR amplification

S-RNase alleles in Iranian of almond cultivars were identified by PCR, using the primer set studied for single-PCR was AS1II (forward) and AmyC5R (reverse), which represent common sequences in the almond self-incompatibility *S-RNases* and AmyC5R (reverse) and CEBASf (forward) primer, which is specifically designed for the self-compatibility allele (S_f) (Sánchez-Pérez *et al.*, 2004). All primers had the annealing temperature of 53 ◦C. Primers were synthesized by Invitrogen™ Life Technologies. Their sequences (5′ to 3′) are as follows:

AS1II:TATTTTCAATTTGTGCAACAATGG;

AMYC5R:CAAAATACCACTTCATGTAACAAC;:

CEBASf: AGATCTATCTATATCTTAAGTCTG.

In addition, two primers set were used for the multiplex-PCR:

a-AS1II (forward), CEBASf (forward), and AmyC5R (reverse)

b -A1Sc1 (forward), A1Sd2 (forward) and AmyC5R (reverse)

PCR reaction

PCR reactions were performed in a 25 µl volume with the reaction mixtures containing 16 mM (NH4)2SO4, 67 mM Tris-HCl pH 8.8, 0.01% Tween-20, 2 mM MgCl2, 0.1 mM of each dNTP, 0.2 µM of each primer, one unit of Taq DNA Polymerase (Ecogen S.R.L., Barcelona, Spain), and 90 ng of genomic DNA. The cycling parameters were: one cycle of 95°C for three minutes; 35 cycles of 94°C for one minute, 53ºC for one minute, and 72°C for two minutes, followed by a ten minute final extension (Fallah *et al.*, 2014)

Electrophoresis of PCR products

Amplified PCR products were separated by electrophoresis on 1.5% agarose gels (1 × TAE buffer), stained with ethidium bromide (0.5 µg/mL), and visualized under the UV light using a 10Kb Plus DNA Ladder (Invitrogen^{TM} Life Technologies, Barcelona, Spain) as molecular size standard (Fallah *et al.*, 2014)

Data analysis

Values of traits including minimum, maximum and mean were measured. The frequency histograms for each trait were represented by using the mean values of the years of the study. Morphological data were statistically analyzed in the frame of completely randomized design (CRD) with SAS software version 9.1. The mean values were compared by Duncan's Multiple Range Tests.

Molecular weight of each of the potential specific bands was calculated using a 10Kb Plus DNA Ladder (Invitrogen^{TM} Life Technologies) as molecular size standard and the software program Gene Tools (SynGene. GeneTools Analysis Software -Version 3.02.00 - Serial No. 7458*5213).

Results

Morphological traits

The analysis of variance showed significant differences at 1% level among the studied cultivars and genotypes for all traits (Table 1), and the mean values for each characteristic were different (Table 1 and 2). Fig. 1 showed the distribution of the 71 almond cultivars for the some vegetative, phonological and fruit traits such as flowering time (Fig. 1a), growth vigor (Fig. 1b), foliage density (Fig. 1c), kernel color (Fig. 1d), shell hardness (Fig. 1e) and ripening time (Fig. 1f).

The flowering time of the almond cultivars and genotypes spread between the earliest ('Gazvin1') and the latest ('Shahrood 12'), (Table 2, Fig. 1a), considering the mean values of each cultivars and genotypes. The almond cultivars and genotypes followed a bimodal distribution (Fig. 1a).

For growth vigor trait, the majority of studied cultivars belonged to the intermediate group (Table 2, Fig. 1b), although some of the cultivars had weak and strong growth habit (Table 2, Fig. 1b). Foliage density trait in 71 studied cultivars and genotype showed that most of the cultivars had a dense growth habit (Table 2, Fig. 1c). The remaining cultivars were medium and low foliage density, respectively (Table 2, Fig. 1c). Results showed measurements of 1163.52 mm^2 (average), 3086.06 mm^2 (maximum in Mirpanjeh Tehran cultivars) and 547.24 mm^2 (minimum in Nonpareil cultivars), respectively (Table 2). The variability of nut weight in the all assayed cultivars was very

high, as observed in fruits from 1.09 (Shekofeh) to 7.10 g (Shale Gazvin) (Table 2). In all cultivars and genotypes, an average of nut weight was 3.59 g (Table 2). In this study, all fruit resulted form open pollination.

Results showed that 'Dir Ras-e-Savojbolagh' had a maximum nut width of 38.00 mm and a mínimum value of 14.00mm in cultivars (Table 2). An average nut width of 21.99 mm was calculated in all assayed almond cultivars (Table 2). The mean value of kernel weight of all the cultivars and genotypes was 1.19 grams (Table 2). Range of kernel weights was between 0.43g ('Tuono1') and 2.09g ('Number 3-12'). The rest of cultivars were between these ranges (Table 2). The average of kernel percent in examined almonds was 43.23 %. High and low amount of this trait were observed in 'Price' (76.60%) and 'Hybrid holo badam' (20.00%) cultivars, respectively (Table 2). The results revealed that the mean value of double kernels percent in assayed almonds ranged from 0.00% to 55.00%, although the average of this trait was 16.80% (Table 2). The influence of the environment on the production of double kernels is well known (Spiegel-Roy and Kochba, 1974; Kester and Gradiziel, 1996).

As can be seen in Fig. 1e, the majority of studied cultivars were hard shell and the rest genotypes were intermediate, soft, paper and extra hard shell, respectively (Fig.1e). Shell hardness in 'Hybrid holo badam' was extra hard (cracking by hammer), while 'Price', 'Z-10'and 'Number 16-8'cultivars, were extra soft (paper) (Table 2). The ripening date in all assayed cultivars and genotypes showed a normal distribution between the earliest ('Number 9-7') and the latest cultiavars ('Dir Rase Savojbolagh') (Fig.1f). The majority of assayed cultivars showed the medium ripening date. Our results agreed with previous studies by Grasselly and Crossa-Raynaud (1980) and Sanchez –Perez *et al.*, (2007).

Table 1. Analysis of variance of some agronomic traits studied for 71 almond cultivars and genotypes .

SOV	DF	Mean square
Blooming time	70	16.10**[1]
Tree vigour	70	4.77**
Foliage density	70	9.23**
Leaf area	70	547485.84**
Length/width leaf	70	0.68**
Nut weight	70	6.25**
Nut length	70	0.88**
Nut width	70	0.57**
Kernel weight	70	0.48**
Kernel color	70	0.14**
Double Kernel	70	2156.86**
Kernel percent	70	600.44**
Softness of shell	70	10.53**
Ripening time	70	7.50**
Error	142	-
Total Error	212	-
%Coefficient of Variation 0.00 to 8.049%		

[1] n.s., * and ** non-significant and significant respectively at the 5% and 1% level by Duncan's test.

Table 2. Some important traits on superior almond cultivars and genotypes in this study.

Genotypes	Blooming time	Tree vigor	Foliage density	Leaf area	Length/width leaf	Nut weight	Nut length	Nut width	Kernel weight	Kernel color	Double Kernel	Kernel percent	Softness of shell	Ripening time
	Code	Code	Code	mm^2	-	gr	mm	mm	gr	Code	%	%	Code	Code
Genco	ML[1]	W[9]	I	1029.25	32.00	2.14	20.70	20.10	1.03	Li[14]	35.00	48.13	I	M
D-124	ML	I[10]	Lo[12]	1063.23	34.20	4.33	49.00	27.00	1.40	Li	0.00	25.00	I	E
Ruby	L	W	Lo	1319.07	32.20	2.00	30.00	20.00	1.07	I	8.00	63.00	I	M
Mission	VL[3]	S[11]	S	940.62	33.90	3.30	25.00	19.00	1.62	I	0.00	38.27	I	M
Nonpareil	M[4]	W	Lo	547.24	40.50	1.22	29.40	18.50	0.81	Li	01.00	66.14	So[15]	E
Price	M	I	I	746.41	33.10	1.77	33.90	17.00	1.20	I	20.00	76.60	P[16]	M
Ne Plus ultra	L	I	Lo	1132.89	34.00	2.04	35.60	19.40	1.04	Li	5.00	51.86	So	E
D-99	ML	I	Lo	1129.26	31.00	2.13	32.20	22.00	1.36	Li	10.00	63.00	H[17]	E
Peerless	EM[5]	W	D[13]	857.60	30.10	3.20	33.50	23.00	1.13	I	31.40	35.40	H	M
Mirpanjeh Tehran	M	I	D	3086.06	23.00	3.65	42.00	19.00	1.30	Li	50.00	55.00	So	L
Pakotahe Talaghan-1	ML	W	D	1610.34	36.20	4.23	36.00	24.00	1.40	Li	40.00	40.00	I	M
V- 13-34	L	W	D	1116.99	27.40	5.11	42.60	32.50	1.89	I	33.05	36.00	H	L
Orumieyh-68	L	W	D	1509.51	27.20	5.76	39.00	30.00	1.40	I	20.31	24.31	I	L
Yazd-60	E[6]	I	Lo	1279.04	35.80	3.70	31.00	20.00	1.40	I	5.50	65.00	H	E
Pakotahe Talaghan-2	M	W	D	1410.29	32.30	5.11	42.60	32.50	1.88	Li	23.00	48.94	I	L
Dir RaseSavojbolagh	L	W	Lo	809.88	29.30	3.16	50.00	38.00	0.96	Li	21.00	46.74	H	VL
V- 16-8	L	W	Lo	1109.05	31.50	3.45	33.30	17.40	1.76	Li	0.00	60.00	P	E
Barg Doroshte Hamedan	M	I	I	2076.93	28.70	4.70	46.00	25.00	1.80	Li	5.00	50.00	H	L
Shale Gazvin	E	I	D	1176.92	29.30	7.10	35.00	23.00	1.80	Li	20.00	50.00	H	M
Barg Siyahe Gazvin	ML	I	D	1379.02	31.60	2.42	30.00	14.00	1.50	Li	22.00	60.00	I	M
Hybrid holo badam	M	I	Lo	1654.18	37.30	3.40	32.00	19.00	0.70	I	2.50	20.00	EH[18]	L
Gazvin 1	EE[7]	W	Lo	1393.85	34.80	4.80	35.00	25.00	1.69	Li	22.00	58.00	H	E
Shekofeh	L	W	D	1105.13	31.10	1.09	22.50	16.80	0.62	I	19.90	60.00	So	M
Tuono1	M	S	D	886.55	10.23	2.27	25.50	15.40	0.43	Li	18.00	27.00	H	E
Shahroodi 121	EL[8]	I	D	1512.32	38.70	6.21	39.70	27.40	1.96	I	0.00	32.00	H	M
Tuono2	M	S	D	1424.21	14.60	3.96	24.08	22.70	0.77	Li	22.00	30.00	H	E
Shahroo12	EL	I	D	1077.70	12.73	6.21	32.70	25.40	0.81	I	23.00	38.00	H	M
Tuono3	M	I	Lo	1013.11	17.60	3.15	26.08	22.00	1.01	Li	20.00	30.00	H	E

1. Medium Late; 2. Late; 3. Very Late; 4. Medium; 5. Early Medium; 6. Early; 7. Extra Early; 8. Extra Late; 9. Weak; 10. Intermediate; 11. Strong; 12. Low; 13. Dense; 14. Light; 15.Soft; 16. Paper; 17. Hard; 18. Extra Hard

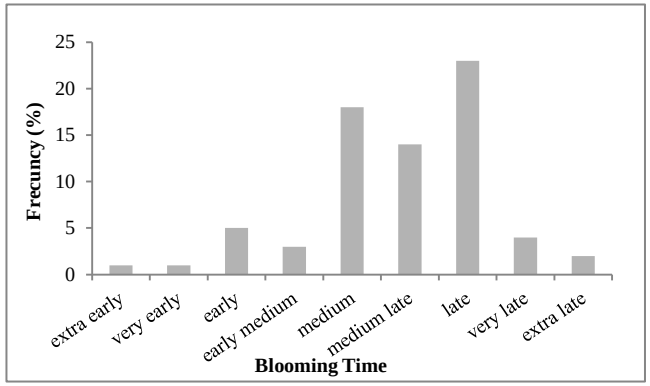

Fig. 1a. Frequency of flowering time in 71 evaluated almond cultivars and genotypes.

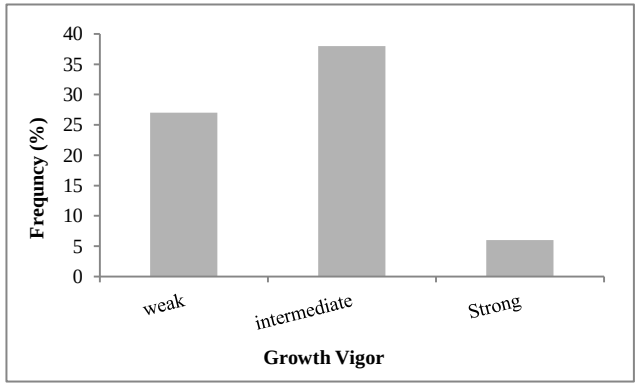

Fig. 1b. Frequency of Tree vigour in 71 evaluated almond cultivars and genotypes.

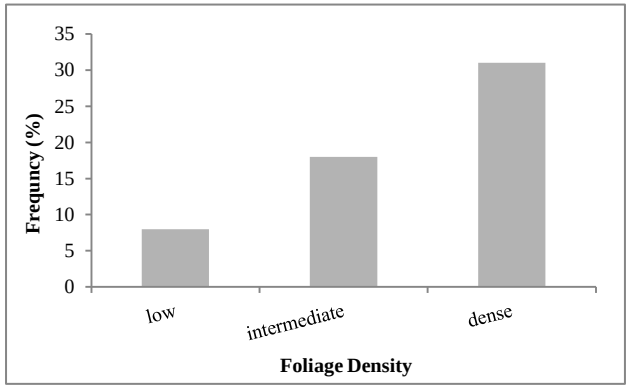

Fig. 1c. Frequency of foliage density in 71 evaluated almond cultivars and genotypes.

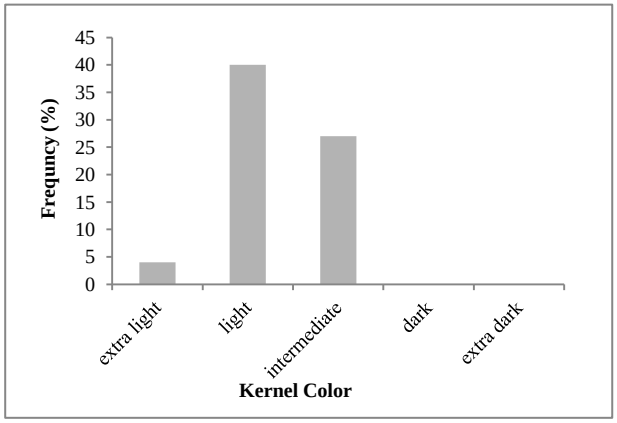

Fig. 1d. Frequency of kernel color in 71 evaluated almond cultivars and genotypes.

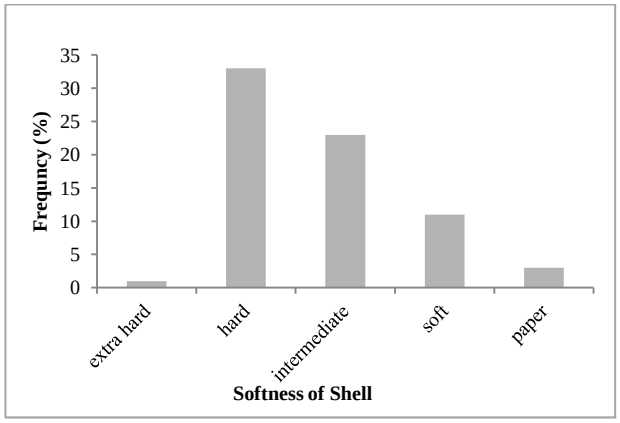

Fig. 1e. Frequency of shell hardness in 71 evaluated almond cultivars and genotypes.

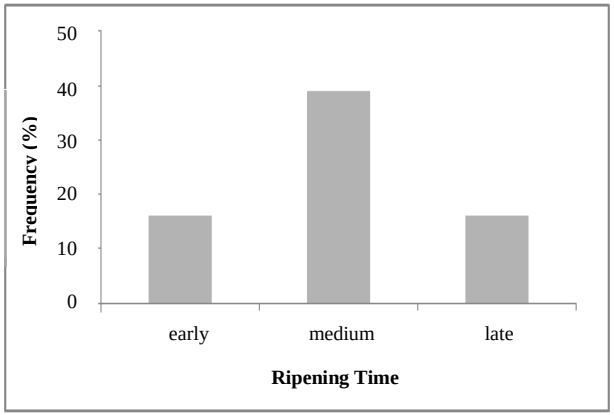

Fig. 1f. Frequency of ripening time in 71 evaluated almond cultivars and genotypes.

Fig .1 Distribution of fercquency for the some vegetative, phonological and fruit traits in 71 almond cultivars: flowering time (Fig. 1a), growth vigor (Fig. 1b) , foliage density (Fig. 1c), kernel color (Fig. 1d), shell hardness (Fig. 1e) and ripening time (Fig. 1f).

Identification of self-incompatibility alleles

PCR using combined primers AS1II and AmyC5R in the assayed almond cultivars and genotypes showed amplification of ten self-incompatibility alleles (S_1, S_2, S_3, S_5, S_6, S_7, S_8, S_{10}, S_{12}, and *S* unknown) and three S_f allels (Fig. 2, Tables 3 and 4). In this case, S1 and S unknown had more frequencies in comparison with other S-alleles. Differences in the sizes of the PCR amplified fragments (from 382-2412 bp) permitted identification of these self-incompatibility alleles. Additionally, S-genotypes of the new assayed almond selections were determined (Tables 3 and 4).

S_9, S_{13}, S_{27}, S_{19}, S_{22}, S_{23}, S_{25}, and S_{29}, alleles were not detected (Table 4). DNA fragment sizes from the amplified bands corresponding to each S-allele agreed in most cases with the available data (Table 4). In addition, new bands were found with different sizes in some cultivars and genotypes. Future study should be done using controlled pollination and sequencer. The source of the self-fertile allele, S_f, is thought to be P. webbii, which grows wild in the regions of southern Italy and from which 'Genco' and some other cultivars originate (Marchese *et al*., 2008). PCR using combined primers AmyC5R (reverse) and CEBASf (forward) in all the accessions showed amplification of self-compatibility allele (S_f) (Table 3and 4). However, the PCR-amplified fragment of the self-compatibility allele S f was of a similar size (1.2 bp) to the fragment amplified from the S_3 allele. As expected, the amplified new product (0.4bp) was present in self-compatible cultivars 'Lauranne', 'Antoneta', 'Marta', 'Tuono', 'Genco', 'R1000' and 'Guara' as well as in the 'Spanish-230'cultivar.

Discussion

The leaf characters of evaluated cultivars were similar to studies by Sabeti (1994) and Baninasab and Rahemi (2007). Leaf length to leaf with ratio is an important trait in almond cultivars, and it is related to the photosynthesis rate for increasing of carbohydrate synthesis (Sanchez –Perez *et al*., 2007) In this research, the results indicated that in all studied cultivars, the average, maximum and minimum of this trait was 31.25, 45.50 (Z-3 cultivars) and 10.23 (Tuono1), respectively (Table 2).

Results showed significant differences in the weight in-shell between both pollination types in almond cultivars. The differences between these mean values (2.7g for self-pollination, and 2.8 g for open pollination) were not important from a commercial point of view, since this weight difference was mainly due to the shell. Also, our results agreed with those of Socias I Company *et al.,* (2004), who observed a significantly higher average nut weight (i.e. weight in-shell) in fruits from open pollination.

Results of our research were in concurrence with previous studies (Kester *et al.,* 1977; Spiegel-Roy and Kochba, 1974; Dicenta *et al.,* 1993). The majority of studied cultivars and genotypes showed a light kernel color (Fig. 1d). Dark and extra dark kernel colors were not observed in any assayed cultivars, although some cultivars showed an intermediate color and had extra light color in this trait (Fig. 1d). Kernel percent is very important in almond breeding programs and breeders have to consider the parents with high kernel percentage in their crosses (Kester *et al.,* 1977; Dicenta *et al.,* 1993). The shell hardness was controlled by major genes, hard shell being dominant, although when cultivars with intermediate shell hardness were crossed, the inheritance was more complex. It was proposed that shell hardness and in-shell/kernel ratio were quantitative traits with an intermediate heritability. Arteaga and Socias i Company (2001) obtained the lowest heritability (around 0.3) for shell hardness.

The results of multiplex-PCR using three

primers, AS1II / CEBASf / AmyC5R, differentiated in a single reaction, the ten self-incompatibility alleles and the S_f allele. Except for the S_f allele, the lengths of the amplified fragments generated in the multiplex-PCR were identical to those observed by single-PCR using the two primers (AS1II / AmyC5R). Thus, multiplex PCR is a very powerful, easy and inexpensive in early selection and primary study of self-incompatibility and self-compatibility alleles in almond cultivars (Sanchez –Perez *et al*., 2004; Mousavi *et al*., 2014). Pairs of primers developed by Tamura *et al*., (2000) (AS1II and AmyC5R) for the conserved regions of the S-alleles were quite efficient in the identification of a number of these S-alleles. Martınez-Gomez *et al*. (2003) demonstrated the specificity of these PCR primers for the identification of eight S alleles in almond cultivars assayed in single PCR reactions. The self-compatibility allele present in 'Tuono', 'Lauranne' is S_f, found in cultivars from Puglia, Italy ('Tuono' and 'Genco') and introduced into new self-compatible cultivars through breeding (Dicenta and Garcıa, 1993). The identification of the S-genotypes in almond cultivars is essential in breeding programs to maximize the efficiency of crosses (Mousavi *et al*., 2010; Mousavi *et al*., 2011a; Mousavi *et al*., 2011b; Mousavi *et al*., 2014). Self-compatibility permits high yields even following poor cross-pollination conditions (Mousavi *et al*., 2014). A PCR-based early identification of self-compatible progeny seedlings those with the S_f allele permits drastic reduction of the number of plants/trees that need to be grown to first flowering, where their compatibility could be assessed by traditional methods (Zeinolabedini *et al*., 2012). In addition, the multiplex PCR is an easy low-cost tool that permits identification of other new S-alleles using multiple primers sets (Sanchez –Perez *et al*., 2004).

Table 3. Identification of the S-alleles in the some almond cultivars assayed and the reference cultivars by specific primers based on the first intron conserved region sequences of the S-alleles.

Almond cultivar/ genotype *	Origin	Size (bp)	Incompatibility Genotype / S-alelle	Almond cultivar/ genotype *	Origin	Size (bp)	Incompatibility Genotype / S-alelle
Azar	Iran	800/1700	S_2/S_9	Tuono	Italy	1100/1200	S_1/S_f
Bahar-e- Hamedan	Iran	1650/2000	$S_?/S_8$	V-1-21	Iran	694/2000	$S_?/S_8$
Barg Doroshte Hamedan	Iran	572/772	$S_?/S_?$	V-2-27	Iran	740/934	$S_?/S_?$
Barg Siyahe Gazvin	Iran	1900/2000	$S_?/S_8$	V-2-7	Iran	750/1100	$S_?/S_1$
D-124	Iran	604/1200	$S_?/S_3$	V-2-29	Iran	800/1100	S_2/S_1
D-99	Iran	382/1100	$S_?/S_1$	V-3-12	Iran	800/980	$S_2/S_?$
Dir RaseSavojbolagh	Iran	640/900	$S_?/S_?$	V-3-17	Iran	388/1200	$S_?/S_3$
Genco	Iran	1100/1200	S_1/S_f	V-4-6	Iran	394/1200	$S_?/S_3$
Hybrid Holo Badam	Iran	880/1540	$S_?/S_?$	V-5-6	Iran	1361/1900	$S_?/S_?$
Kerman-5	Iran	800/880	$S_2/S_?$	V-5-17	Iran	600/2000	S_5/S_8
Kerman-20	Iran	760/800	S_{10}/S_2	V-8-4	Iran	600/600	$S_?/S_5$
Mashhad-10	Iran	800/1600	$S_2/S_?$	V-9-2	Iran	518/1600	$S_?/S_?$
Mashhad-4	Iran	1100/2100	S_1/S_4	V-9-7	Iran	740/1900	$S_?/S_?$
Mashhad-6	Iran	550/1000	$S_?/S_{12}$	V-9-32	Iran	822/800	$S_?/S_7$
Mashhad-7	Iran	1100/1200	S_1/S_3	V-11- 10	Iran	1030/2000	$S_?/S_8$
Mashhad-9	Iran	610/1250	$S_?/S_?$	V-12-26	Iran	783/1100	$S_?/S_1$
Mirpanjeh Tehran	Iran	600/1600	$S_5/S_?$	V-13-34	Iran	1600/1650	$S_?/S_?$
Mission	USA	600/1100	S_5/S_1	V-14-24	Iran	700/1100	S_7/S_1
Ne Plus ultra	USA	700/1100	S_7/S_1	V-16-3	Iran	614/600	$S_?/S_5$
Nonpareil	USA	700/2000	S_7/S_8	V-16-8	Iran	1250/1270	$S_?/S_?$
Orumieyh-54	Iran	760/800	$S_?/S_2$	V-16-25	Iran	800/1100	S_7/S_1
Orumieyh-68	Iran	--/640	$S_?/S_?$	Yazd-13	Iran	860/2000	S_6/S_7
Orumieyh-98	Iran	--/1260	$S_?/S_?$	Yazd-60	Iran	760/800	$S_?/S_2$
Pakotahe Razan	Iran	689/1250	$S_?/S_?$	Yazd-103	Iran	560/1000	S_4/S_{12}
Pakotahe Talaghan-1	Iran	700/1100	S_7/S_1	Yazd-318	Iran	550/1000	$S_?/S_{12}$
Peerless	Iran	860/1100	S_6/S_1	Yazd-444	Iran	760/800	$S_?/S_2$
Gazvin1	Iran	760/800	$S_?/S_2$	Zarghan -3	Iran	658/640	$S_?/S_?$
Price	USA	700/1100	S_7/S_1	Zarghan -7	Iran	1100/2100	S_1/S_4
Ruby	USA	860/1100	S_6/S_1	Zarghan -8	Iran	800/1100	S_2/S_1
Sahand	Iran	800/1100	S_2/S_1	Zarghan -10	Iran	600/1100	S_5/S_1
Shahroodi 12	Iran	1100/1200	S_1/S_3	Zarghan -26	Iran	1284/1260	$S_?/S_?$
Shale Gazvin	Iran	1000/1700	$S_?/S_?$	Zarghan -36	Iran	800/1200	S_2/S_3
Shekofeh	Iran	1300/1650	$S_?/S_?$				

Table 4. Incompatibility groups of the some almond cultivars assayed and the reference cultivars by specific primers based on the first intron conserved region sequences of the alleles.

Incompatibility group	Almond cultivars*	S-genotypes
I	Nonpareil, IXL, Long IXL, Riedenhoure, Tardy Nonpareil	S_7S_8
II	Mission, Zarghan -10, Granada*, Harvey, Mono, Shahrekord-E1	S_1S_5
III	Granada, Harvey, Mono, Robson, Sauret 2, Thompson, Woods Colony	S_5S_7
IV	Ne Plus Ultra, Price, Pakotahe Talaghan-1, V-14-24, V-16-25, Aldrich, Merced, Ne Plus Ultra, Norman, Pearl, RiponYalda-1, Yalda-2	S_1S_7
V	V-5-17, Carmel, Carrion, Jubilee, Livingston, Monarch, Reams, Sauret 1, Tioga	S_5S_8
VI	Bigelow, Butte, Dottie Won, Grace, Kutsch, Monterey, Northland, Rivers, Nonpareil, Sultana	S_1S_8
VII	Eureka, Kapareil, Solano, Sonora, Vesta, A-2	S_8S_{13}
VIII	Mashhad-7, Shahrood 12_1, Shahrood 12_2, Jeffries, Ferragnès, Ferralise	S_1S_3
IX	Jeffries, Nonpareil, IXL	$S_{7A}S_8$
X	Harpareil, Jordanolo, Monagha	S_7S_{14}
XI	Jubilee,Reams	S_8S_{15}
XII	Revers Nonpareil, Bigelow,	S_8S_{16}
XIII	Drake, Smith XL	S_6S_8
XIV	Peerless, Ruby, Fritz, Peerless, Rumbrta-2	S_1S_6
XV	Anxaneta, Tarragones, A230	S_2S_9
XVI	Azar, Anxaneta, Tarragones, Ardéchoise, Desmayo Largueta	S_1S_{10}
XVII	Achaak, Ferrastar, Kerman-20, G-5	S_2S_{10}
XVIII	Pajarera-2, Pestanhieta	$S_{12}S_{23}$
XIX	Malagueña, Planeta Fina, Planeta Roja	$S_{22}S_{23}$
XX	Garrigues, Pajarera-1	$S_{13}S_{27}$
XXI	Sefid, Monaghay-e-Najafabad	S_7S_{13}
XXII	Khorshidi, Pierce	S_8S_{23}
XXIII	Tajeri, Holoei	$S_{10}S_{29}$
XXIV	Shekoofe, Azar-1, Fournat de Breze-naud	$S_{24}S_{27}$
XXV	Sahand, V-2-29, Zarghan -8, Cristomorto	S_1S_2
XXVI	K-1-16, Shamshiri	S_7S_{24}
XXVII	Mashhad-4, Zarghan -7, K-11-40, Zarghan-7, Ferraduel	S_1S_4
XXVIII	Neyriz-1, Mashhad-13, Zanjan-1	$S_{12}S_{24}$
Table 4. Continued	Mashhad-30, G-1	S_4S_7
XXX	Yazd-103, Carretas Bajas	S_4S_{12}
XXXI	Yazd-13, Tokyo	S_6S_7
XXXII	Ai, Azar-2	S_3S_4
XXXIII	Masbovera, A200	S_1S_9
O	Barg Siyahe Gazvin ($S_?S_8$), Barg Doroshte Hamedan($S_?S_?$), Bahar-e- Hamedan ($S_?S_8$), D-124($S_?S_8$), D-99($S_?S_1$), Dir aseSavojbolagh($S_?S_3$), Hybrid Holo Badam ($S_?S_?$), Kerman-5 ($S_2S_?$), Mashhad-10($S_2S_?$), Mashhad-6($S_2S_?$), Mashhad-9($S_?S_?$), Mirpanjeh Tehran($S_5S_?$), Orumieyh-54($S_?S_2$), Orumieyh-68($S_?S_?$), Orumieyh-98($S_?S_?$), Pakotahe Razan($S_?S_?$), Pakotahe Talaghan-2($S_?S_?$), Gazvin 1($S_?S_2$), Shale Gazvin($S_?S_?$), Shekofeh($S_?S_?$), V-1-21($S_?S_8$), V-2-27($S_?S_?$), V-2-7($S_?S_1$), V-3-12($S_2S_?$), V-3-17($S_?S_3$), V-4-6($S_?S_3$), V-5-6($S_?S_?$), V-8-4($S_?S_5$), V-9-2($S_?S_?$), V-9-7($S_?S_?$), V-9-32($S_?S_7$), V-11- 10($S_?S_8$), V-12-26($S_?S_1$), V-13-34($S_?S_?$), V-16-3(S_7S_5), V-16-8($S_?S_?$), Yazd-60($S_?S_2$), Yazd-318($S_?S_{12}$), Yazd-444($S_?S_2$), Zarghan -3($S_?S_?$), Zarghan -26($S_?S_?$), Zarghan -36(S_2S_3)	
self-compatible cultivars	Genco (S_1S_f), $Tuono_1$, $Tuono_2$, $Tuono_3$ (S_1S_f),	

*Reference cultivars are written underlined (Adapted from Boskovic *et al.,* (2003); Ortega *et al.,* (2006); Valizadeh and Ershadi , (2009); Mousavi *et al.,* (2011 and 2014)).

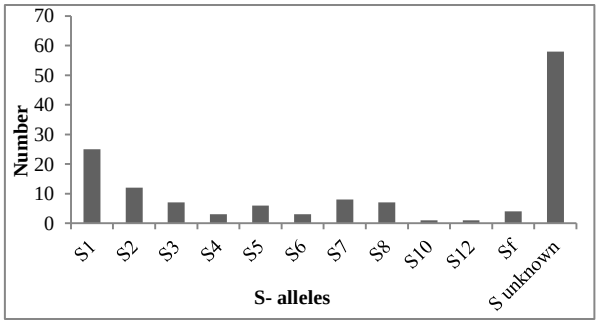

Fig. 2. Frequency of self-incompatibility and S_f alleles in 71 almonds cultivars studied in this reserch.

Conclusions

The results of this study showed that cultivars and genotypes including 'Dir Ras–e-Savojbolagh', 'D-124', 'D-99', 'Shahrood 12', 'Tuono', 'Nonpareil', 'Price', 'Mirpanj-e-Tehran', 'Pakotahe-e- Taleghan' , 'V-13-34', 'V-16-8, 'V-11-10', 'Zarghan 10', 'Uromiyeh 68', 'Barg dorosht-e-Hamedan' and 'Yazd 60' were better than the other cultivars and genotypes in terms of important traits such as late flowering. Ultimately, 58 new bands were found with different sizes in cultivars, which have to be studied using controlled pollination and sequencer. Idenitification of the new S-RNases should be verified by cloning and sequencing before this information is used for orchard design and for parental choice in breeding programmers.

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