

## Identification and Screening of Homozygous and Heterozygous Almond Progenies from Self-Pollinated Touno Cultivar Using PCR

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### Abstract

Self-incompatibility is one of the most important difficulties in almond production which reduce fruit set dramatically and makes orchard management difficult. Therefore, breeding almond to produce self-compatible genotypes is very important. In this research identification and screening of 86 almond progenies obtained from selfing Touno after the self-pollination by PCR reaction with specific primers of CEBAS<sub>f</sub> and AS1. PCR results confirmed the situation of self-compatible hybrids. In addition, it indicated that, frequencies of S<sub>f</sub> and S<sub>1</sub> was 100% and 50% in progenies respectively. Self-compatible hybrids had been identified that can be used in almond breeding programs particularly to development the monoculture of almond orchards. So to identify and screening homozygous self-compatibility almonds be capable of be another step towards creating monoculture of almond and use in breeding programs further.

**Keywords:** Almond, Hybrid, Self-compatible, Self-pollination

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### Introduction

Almond is a temperate zone fruit tree that is cultivated in many countries because of its nutritional value. Although the almond is one of the oldest crops used by humans, its specific environmental requirements have restricted its commercial production to specific areas of the world (Kester and Gradziel, 1996). Self-incompatibility is a wide-spread and heritable reproductive phenomenon in flowering plants, in which self-fertilization is prevented by rejection of pollen from the same plant. This is an evolutionary advantage because of its effectiveness in avoiding inbreeding and the encouragement of out-crossing (Socias i Company, 1990; Alonso and Socias i Company, 2005a). *Prunus* species, such as almond, are characterized by a

gametophytic type of self-incompatibility, which means that there is no pollen germination on the stigma (Yamashita *et al.*, 1987), or that tube growth stops, most often in the upper third of the style (Socias i Company *et al.*, 1976; Mousavi *et al.*, 2014). Self-incompatibility in almond is controlled by a single *S*-locus with multiple codominant alleles (Socias I Company *et al.*, 1976). This trait is expressed in the style by special glycoproteins (*S*-RNases) that arrest the growth of pollen tubes in self-incompatible cultivars (Socias I Company *et al.*, 1976; Boskovic *et al.*, 1998; Boskovic *et al.*, 2003).

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Nowadays, the use of molecular methods is very important for identification of self-compatible and self-incompatible cultivars. In addition, these methods are very reliable for determination of *S*-genotypes in almond (Alonso and Socias I Company, 2006; Mousavi *et al.*, 2011). So far, it has been shown that there are about 35 self-incompatible alleles ( $S_1, S_2, \dots, S_{35}$ ) and one self-compatible allele ( $S_f$ ) (Lopez *et al.*, 2006; Boskovic *et al.*, 2007; Mousavi *et al.*, 2011). Only a limited number of the numerous almond cultivars grown worldwide are self-compatible, the majority of which come from the Italian region of Apulia (Kester and Gradiziel, 1996). Prominent among these are Tuono and Genco, Filippo Ceo (Kester and Gradiziel, 1996), Mazzetto synonymous for Tuono (Lopez *et al.*, 2006), Falsa Barese, Ferrante and Palatina (Godini, 2002). As these cultivars were shown to be capable of transmitting their self-compatibility to their offspring (Socias I Company and Felipe 1988) using them has proved to be the most effective method for obtaining new self-compatible cultivars. Knowledge of the inheritance of self-compatibility is an essential step in the attainment of such an objective (Socias I Company *et al.* 1976). Duval *et al.* (2001) analyzed a progeny of 'Ferralise x Tuono'. 'Ferralise' shares the  $S_1$  allele with 'Tuono' ( $S_1S_f$ ) and has the same  $S_1S_3$  genotype 'Ferragnès'. All tested progeny seedlings, except one, were genotyped  $S_1S_f$  or  $S_3S_f$ , and were considered self-compatible. The transmission of self-compatibility from 'Tuono', is similar in the two progenies 'Ferralise x Tuono' and 'Ferragnès x Tuono'.

However, in some of the crosses with the female parent related to Cristomorto, the proportion of self-compatible individuals was higher than expected (Lopez *et al.*, 2006). Ortega and Dicenta (2008) studied the inheritance of self-compatibility in almond. They observed that frequencies of self-compatible descendants were in accordance with the accepted theory concerning the gametophytic system of *Prunus*. Their results confirmed the presence of a common allele

in the cultivars Genco, Tuono and Ferragnes (Ortega and Dicenta, 2008). So far, 35 *S* incompatibility alleles, in addition to the  $S_f$  self-compatibility allele, have been identified in almond using different molecular analyses, specifically, ribonuclease, *S* allele PCR and sequencing analysis (Lopez *et al.*, 2006). More than 154 almond cultivars have been genotyped (Lopez *et al.*, 2006) and 19 cross-incompatible groups have been established. The techniques used to identify genotypes as self-compatible can be more pointed; use of paper bags and flower covers in branches that contain flower and finally examine the fruit set (Socias i Company, 1990), evaluation of pollen tube growth in pistils using fluorescence microscopy (Mousavi *et al.*, 2014), and use of molecular methods including PCR (Lopez *et al.*, 2004). The PCR method to detect genotypes has been used by researchers such as Socias i Company (1990), Channuntapipat *et al.* (2001), Martin-Gomez (2003), Channuntapipat *et al.* (2003) and Alonso and Socias i Company (2006). Based on these results, main objective of this study was identification and screening of homozygous ( $S_fS_f$ ) from heterozygous ( $S_1S_f$ ) almond progeny hybrids obtained from self-pollination of Tuono using PCR technique.

## Materials and Methods

### Plant materials

In this experiment, 86 progenies from self-pollinated Tuono ( $S_1S_f$ ) almond were prepared in Seed and Plant Improvement Institute (SPII), Karaj, Iran.

### Genomic DNA extraction

Total DNA was extracted from young leaves collected in early spring by the method described by Murray and Thompson (1980), but modified and adapted to almond with the difference that 2  $\mu$ L of  $\beta$ -mercaptoethanol was added immediately after application of extraction buffer (100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA, 2% CTAB, 1% PVP, 0.2% mercaptoethanol, 0.1% NaHSO<sub>3</sub>). The purified total

DNA was quantified by gel electrophoresis, and its quality verified by use of a NanoDrop 1000 Spectrophotometer V3.7. DNA samples were stored at -20° C. Three independent extractions were performed for each sample.

#### PCR reaction

DNA samples with a concentration of 10 ng per  $\mu$ L were prepared for the PCR reaction (Lopez *et al.*, 2006). Amplification reactions were carried out in 10  $\mu$ L volumes containing: 0.9 mM PCR buffer, 0.6 mM  $MgCl_2$ , 0.9 mM dNTPs, 0.1 mM of each primer (forward and reverse; Table 1), 0.2 unit of SmarTaq

DNA polymerase (Cinnagen) and 1 ng of genomic DNA.

#### PCR amplification

The thermo cycles of the PCR program consisted of three minutes at 95 °C for primary denaturation, 35 cycles of 94 °C for 1 minute, 53 °C for 1 minute and then 72 °C for two minutes, followed by 10 min at 72 °C (Channuntapipat *et al.*, 2003). After PCR, the products were stored at 4 °C (refrigerator) until electrophoresis. Profiles of primers used in this study are shown in Table 1.

Table 1. Profile of primers used in this study

Marker (locus)	Sequence (5' → 3')	Primer	Band size (bp)	Visible allele	T annealing (°C)	Reference
SFF	GTGCCCTATCTAATTGTTGAC	CEBASf/AmyC5R	449	Sf	53	Channuntapipat <i>et al.</i> (2003)
AS1II	TATTTTCAATTTGTGCAACAATGG	AS1II/AmyC5R	1100	S1	60	

#### Electrophoresis of PCR products

Amplified PCR products were separated using 2% agarose gel electrophoresis (Biowittaker Maine, USA) using 0.5 X Tris–Boric acid–EDTA buffer.

The molecular sizes of the amplification products were estimated using a 100-bp DNA ladder (Fermentas). After agarose gel electrophoresis, the gel was stained with ethidium bromide (1  $\mu$ g/ml) and visualized under UV light using the method of Tamura *et al.* (2000).

#### Results

For identification of self-compatible homozygous from heterozygous progenies, 86 seedlings resulting from controlled hybridizations of Tuono were studied. The results showed that hybrids obtained from crosses made between Tuono had S genotype Sf/S1 as parents formed bands and these bands considering the especial primer indicated that hybrids were 100% self-compatible (Table 2) (Figs. 1, 2 and 3).

Table 2. Genotyping of S<sub>i</sub>, and S<sub>f</sub> alleles in progenies

Hybrid	CEBASf/AmyC5R	Second intron	Genotype
TT1	–	1100/450	S <sub>i</sub> /S <sub>f</sub>
TT2	–	1100/450	S <sub>i</sub> /S <sub>f</sub>
TT3	450	450/450	S <sub>f</sub> /S <sub>f</sub>
TT4	450	450/450	S <sub>f</sub> /S <sub>f</sub>
TT5	–	1100/450	S <sub>i</sub> /S <sub>f</sub>
TT6	–	1100/450	S <sub>i</sub> /S <sub>f</sub>
TT7	–	1100/450	S <sub>i</sub> /S <sub>f</sub>
TT8	–	1100/450	S <sub>i</sub> /S <sub>f</sub>
TT9	450	450/450	S <sub>f</sub> /S <sub>f</sub>
TT10	450	450/450	S <sub>f</sub> /S <sub>f</sub>
TT11	–	1100/450	S <sub>i</sub> /S <sub>f</sub>

Table 2. Continued

TT12	–	1100/450	$S_I/S_f$
TT13	450	450/450	$S_f/S_f$
TT14	–	1100/450	$S_I/S_f$
TT15	–	1100/450	$S_I/S_f$
TT16	–	1100/450	$S_I/S_f$
TT17	450	450/450	$S_f/S_f$
TT18	–	1100/450	$S_I/S_f$
TT19	–	1100/450	$S_I/S_f$
TT20	–	1100/450	$S_I/S_f$
TT21	–	1100/450	$S_I/S_f$
TT22	–	1100/450	$S_I/S_f$
TT23	–	1100/450	$S_I/S_f$
TT24	450	450/450	$S_f/S_f$
TT25	450	450/450	$S_I/S_f$
TT26	–	1100/450	$S_I/S_f$
TT27	450	450/450	$S_f/S_f$
TT28	450	450/450	$S_f/S_f$
TT29	–	1100/450	$S_I/S_f$
TT30	450	450/450	$S_f/S_f$
TT31	–	1100/450	$S_I/S_f$
TT32	–	1100/450	$S_I/S_f$
TT33	–	1100/450	$S_I/S_f$
TT34	450	450/450	$S_f/S_f$
TT35	–	1100/450	$S_I/S_f$
TT36	–	1100/450	$S_I/S_f$
TT37	–	1100/450	$S_I/S_f$
TT38	–	1100/450	$S_I/S_f$
TT39	–	1100/450	$S_I/S_f$
TT40	450	450/450	$S_f/S_f$
TT41	450	450/450	$S_f/S_f$
TT42	–	1100/450	$S_I/S_f$
TT43	–	1100/450	$S_I/S_f$
TT44	–	1100/450	$S_I/S_f$
TT45	450	450/450	$S_f/S_f$
TT46	–	1100/450	$S_I/S_f$
TT47	–	1100/450	$S_I/S_f$
TT48	–	1100/450	$S_I/S_f$
TT49	–	1100/450	$S_I/S_f$
TT50	–	1100/450	$S_I/S_f$
TT51	450	450/450	$S_f/S_f$
TT52	–	1100/450	$S_I/S_f$
TT53	–	1100/450	$S_I/S_f$
TT54	–	1100/450	$S_I/S_f$
TT55	–	1100/450	$S_I/S_f$

Table 2. Continued

TT56	—	1100/450	$S_I/S_f$
TT57	—	1100/450	$S_I/S_f$
TT58	—	1100/450	$S_I/S_f$
TT59	—	1100/450	$S_I/S_f$
TT60	—	1100/450	$S_I/S_f$
TT61	—	1100/450	$S_I/S_f$
TT62	450	450/450	$S_f/S_f$
TT63	450	450/450	$S_f/S_f$
TT64	—	1100/450	$S_I/S_f$
TT65	—	1100/450	$S_I/S_f$
TT66	—	1100/450	$S_I/S_f$
TT67	—	1100/450	$S_I/S_f$
TT68	—	1100/450	$S_I/S_f$
TT69	—	1100/450	$S_I/S_f$
TT70	—	1100/450	$S_I/S_f$
TT71	450	450/450	$S_f/S_f$
TT72	—	1100/450	$S_I/S_f$
TT73	—	1100/450	$S_f/S_I$
TT74	—	1100/450	$S_I/S_f$
TT75	—	1100/450	$S_I/S_f$
TT76	—	1100/450	$S_f/S_f$
TT77	450	450/450	$S_f/S_f$
TT78	—	1100/450	$S_I/S_f$
TT79	—	1100/450	$S_I/S_f$
TT80	—	1100/450	$S_I/S_f$
TT81	450	450/450	$S_f/S_f$
TT82	—	1100/450	$S_I/S_f$
TT83	450	450/450	$S_f/S_f$
TT84	—	1100/450	$S_f/S_I$
TT85	—	1100/450	$S_I/S_f$
TT86	—	1100/450	$S_f/S_I$

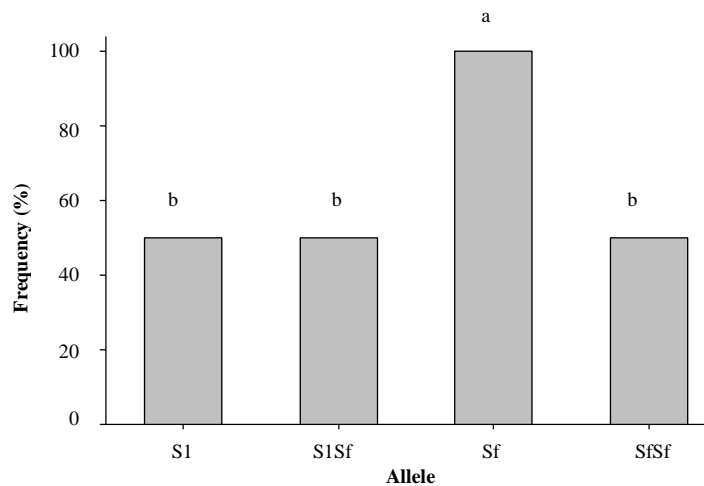


Fig. 1. Frequency of S and F alleles amplified in almond hybrids

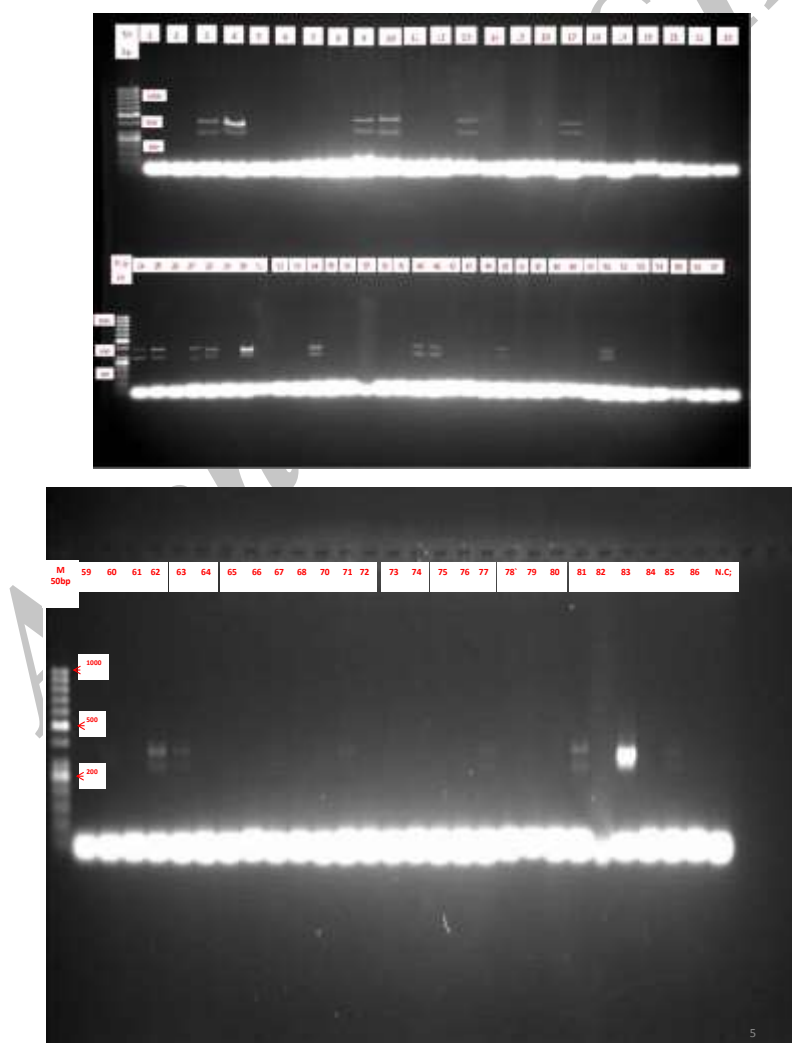


Fig.2. Bands of S and F alleles almond progenies using CEBAS<sub>f</sub> and AmyC5R

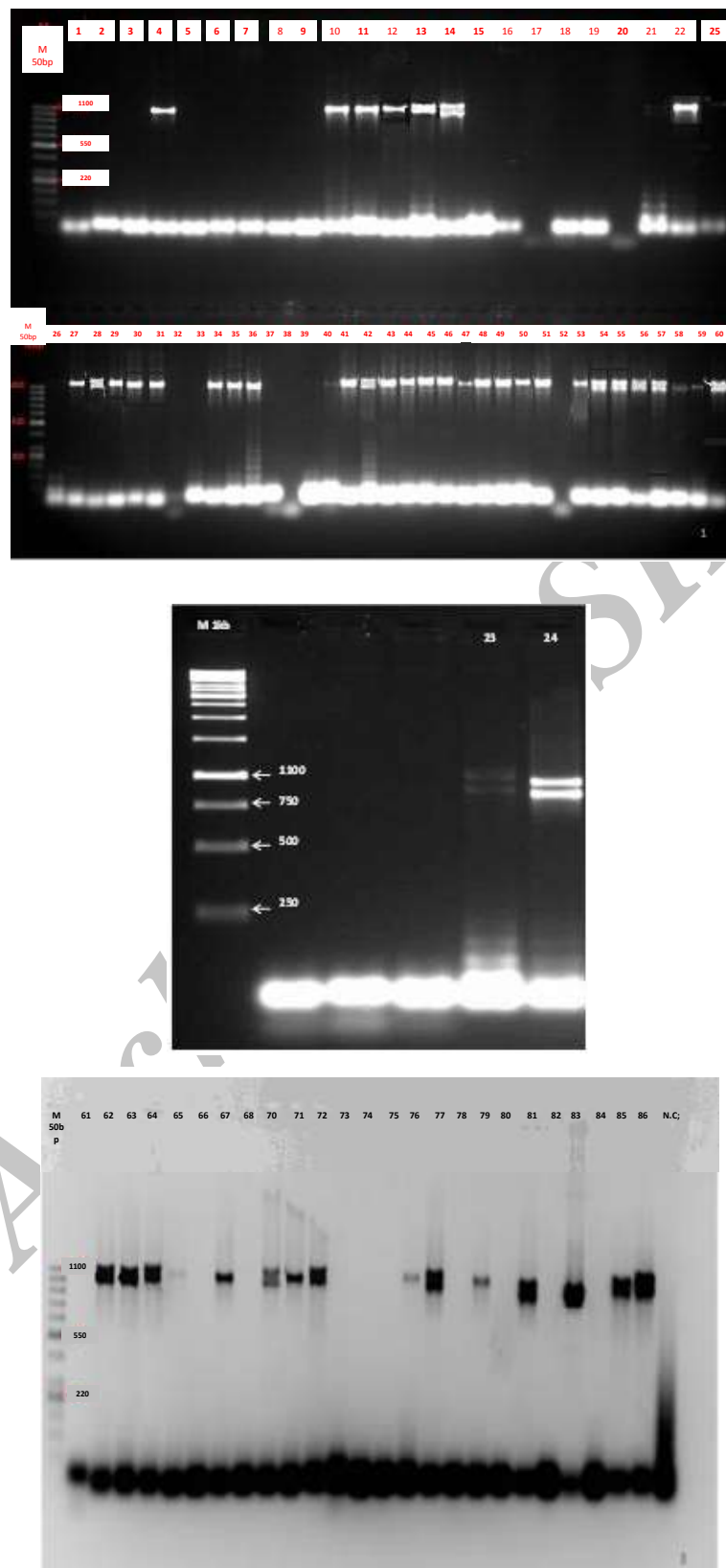


Fig.3. Bands of  $S_1$  allele of almond hybrids using  $AS_1$  and  $AmyC5R$

## Discussion

As indicated in Figs. 2 and 3 and Table 2, homozygous and heterozygous self-compatible progenies are completely segregated as also reported by Alonso and Socias i Company (2005b). In addition, the frequency of  $S_f$  and  $S_1$  alleles were 100 and 50%, respectively. Similar results were reported by Momenpour *et al.* (2011) who identified self-compatible  $S_f$  alleles in 48 almond hybrids using the PCR technique. Also, it is interesting to note that the heritability of self-compatible alleles observed here is consistent with Mendel's First Law, since the proportion of the self-compatible hybrids to total hybrids is 1: 2 (Fig. 1 and Table 2). If, in the female parent, there is an  $S_1$  allele, all offspring will be the self-compatible, because pollen of the  $S_1$  allele of the male parent is unable to growth in to the style tissue and the zygote will not formed; thus only pollen with  $S_f$  alleles is able to penetrate into the style and fertilize the ovule, so all offspring will have ( $S_fS_x$ ,  $S_1S_f$ )  $S$  genotype ( $S_x$ = uncertain). These results are consistent with Martin-Gomez *et al.* (2003), Chanuntapipat *et al.* (2003), Alonso and Socias i Company (2005a) and Kamali *et al.* (2009). In last part, it can be said PCR method was used in this study for identifying and screening homozygous and heterozygous almond hybrids from self-pollinated Touno cultivar, was a rapid and accurate method. Also similar results previously reported for identifying and determining the self-compatibility and incompatibility in the DNA genome of almond (Kodad *et al.*, 2008). The present research showed that of 86 almond hybrids analyzed by the polymerase chain reaction (PCR) using specific primers; all 86 hybrids formed self-compatibility band. The same results have been reported by Alonso and Socias i Company (2005b), Kodad *et al.* (2008) and Momenpour *et al.* (2011). In our study, the genotyping of homozygous and heterozygous self-compatibility of the 86 hybrids have been grouped (Table 2). Identified homozygous self-compatible

progenies in this study can be used in almond breeding programs in future. Thus the identification and screening for homozygous self-compatibility in almond represents another step towards creating an almond monoculture for use in future research programs.

## Conclusions

The screening of 86 almond offspring of self-pollinated Touno by PCR (specific CEBAS<sub>f</sub> and AS1 primers) indicated that the frequencies of  $S_f$  and  $S_1$  were 100% and 50%, respectively, in progeny. In future studies selected hybrids can be used in almond breeding programs particularly those aimed at development monoculture almond orchards.

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## شناسایی و غربالگری نتاج بادام هموزیگوت و هتروزیگوت از خود گرده افشانی رقم Touno رقم با استفاده از PCR

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### چکیده:

یکی از مشکلات تولید بادام خود ناسازگاری است که موجب کاهش شدید تشکیل میوه و نهایتاً ایجاد مشکل در مدیریت باغ می‌گردد. بنابراین اصلاح بادام به منظور ایجاد ارقام خود سازگار اهمیت بالایی دارد. در این آزمایش شناسایی و غربالگری دورگ های خودگشن هموزایگوس ( $S_f S_f$ ) از خودگشن هتروزیگوس ( $S_1 S_f$ ) در ۸۶ دورگ بادام حاصل از خودگشنی بادام تونو با استفاده از آغازگر اختصاصی (CEBASf/AmyC5R) و از روش PCR انجام شد. نتایج PCR وضعیت خودگشنی دورگ‌ها را تایید نمود. علاوه بر آن مشخص شد که فراوانی آلل‌های  $S_f$  و  $S_1$  به ترتیب ۵۰ و ۱۰۰ درصد در جمعیت هیبریدها بود. بنابراین در تحقیق حاضر هیبریدهای خودسازگار شناسایی شده‌اند که می‌تواند در آینده در برنامه‌های اصلاحی به ویژه در کشت‌های تک‌رقمی مورد استفاده قرار گیرند تا گامی دیگر در جهت شناسایی و معرفی ارقام خود سازگار هموزایگوس برای توسعه باغات تک رقمی و استفاده در برنامه‌های اصلاحی بعدی باشد.

**واژه های کلیدی:** بادام، هیبرید، خودسازگار، خودگشن.