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EVALUATION OF SELF-INCOMPATIBILITY (SI ALLELES) AMONG SOME GENOTYPES IN ALMOND (*Prunus dulcis*)

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ABSTRACT
Almond (*Prunus dulcis*.) is one of the most important nut fruits worldwide and its seed has been recognized as a health nutrient supply for human consumption. In this study, this study aimed to study different aspects of self-incompatibility (SI) in almond (*Prunus dulcis*) using sequence quality and levels of species discrimination (A multi-approach was used to study different aspects of self-incompatibility (SI) in almond (*Prunus dulcis*) The results are discussed in view of the evolution of the gametophytic SI system and the models proposed for its mechanism. Eight SI alleles were tested, in which 4 showed amplifications as homogenous or heterogeneous alleles in one or more almond trees. Comparing all alleles, some almond trees show self-incompatibility. Prunus Saint Catherine sampled from Saint Catherine showed the highest number of SI alleles (3) followed by *P. Non-pareil, P. ferdal and P. texas* (2), *P. Saint Catherine wadi shrezz, P. Wadi40, P.Zuweid2, P. perles (mal), P. IXL and P.* Zuweid3 showed a unique SI allele, while *P.* Saint Catherine and *P.* Zuweid1 were completely clear of any SI alleles (Table 21). Even the alleles of each SI type shows different molecular size and appear in different individuals, but they all affecting the selfing percentage and cause infertility and the urgent need to cross pollinate the tree to expect fruits. P. Saint Catherine needs more extensive study to reveal the complete SI alleles that can be found.

KEY WORDS: *Prunus dulcis*, Almond, Self-incompatibility.

INTRODUCTION

Most of the fruit tree species in *Prunus*, including almond, sweet cherry (*P. avium*), Japanese apricot (*P. mume*), and Japanese plum (*P. salicina*), show gametophytic self-incompatibility (GSI). In GSI, the compatibility of a cross is determined by the haploid genome of the pollen and the diploid genome of the pistil (de Nettancourt, 2001). There are different SI systems, the most widespread of which is gametophytic SI (GSI). GSI is present in several families including Rosaceae, which represents most of the fruit trees. Almond is an economically important member of the Rosaceae, most cultivars being self-incompatible. The successful breeding of self-fertile almond cultivars has long been an important breeding goal. The first almond S-proteins associated with GSI have only recently been identified as ribonucleases (Boˇskovic´ *et al*., 1997b; Self-incompatibility (SI) is a genetic mechanism that prevents inbreeding and promotes outcrossing. This mechanism comprises recognition of self- or self-related pollen, by cells of the pistil, followed by rejection of the incompatible pollen, through aborted development, either immediately after pollination on the stigma surface or at a subsequent stage during pollen tube growth into the stigma or style (de Nettancourt, 2001).

Almond cultivars traditionally cultivated are self-incompatible. However, due to the several advantages of growing self-compatible almonds (Dicenta *et al*., 2002a). In almond, self-incompatibility is under the genetic control of a gene on chromosome 6 with at least 40 alleles (S1 to S39, and Sf) which encode glycoproteins known as S-RNases. When expressed in the style of an ower, S-RNases recognise and degrade RNA from pollen tubes that have grown from pollen grain with matching S alleles Ballester *et al.*, 1997.

MATERIALS & METHODS

The current study was conducted in the Plant Biotechnology laboratory located in the Egyptian Deserts Gene Bank, North Sinai Research Station, Desert Research Centre, and the Molecular Genetics laboratory located in the Faculty of Agriculture, Ain Shams University during the period from 2010-2016.

Sampling and Collection

Samples Classified in this study into three groups: Group 1 includes wild almonds; Group 2 includes cultivated almonds collected samples from North Sinai Governorate, this area has excellent plant productivity, height nut quality and shrubs have a good mechanism for drought tolerance; and characterized by high tolerance for extreme environmental conditions, which have been collected from natural habitats and, Group 3 includes cultivated almonds collected from the Northern Coast of Egypt at this point, the choice of almond plant genetic resources for further genetic study. His name was in the samples with collection sites and/or local names.

A total of 12 varieties in this study (Table 1) the samples were obtained from young leaves and branches of the three Almond genetic resources, gene Bank Sheikh Zuweid research station belonging to the Desert Research Centre and the directorate for Agriculture and South Sinai.

Samples leaves were washed three times in sterile distilled water, and placed in ice and kept at 60 to 80C until used

TABLE 1: *Prunus dulcis* collected cultivars and wild species data including the origin, parentage and main agronomic characteristics.

No.	Varieties	Collection sites	Shell	Flowering	GPS	
					Latitude	Longitude
S1	*Prunus Saint Catherine Monastery1*	Saint Catherine Monastery South Sinai	Farkk	Late	28.557149	33.974250
S2	*Prunus Saint Catherine Monastery2*	Saint Catherine Monastery - South Sinai	Sami hard	Middle	28.556816	33.974785
S3	*Prunus Saint Catherine wadi shrezz*	Saint Catherine - *wadi shrezz* -South Sinai	Farkk	Middle	28.560364	33.957002
S4	*Prunus Wadi40*	Wadi El-Arabeen Saint Catherine South Sinai	Hard	Late	28.551731	33.949981
S5	*Prunus Zuweid1*	El-Sheikh Zuweid North Sinai El-Gora	Hard	Middle	30.952595	34.307769
S6	*Prunus Zuweid2*	El-Sheikh Zuweid North Sinai	Soft	Middle	31.222419	34.117095
S7	*Prunus Non-pareil*	Marsa Matrouh	Paper	Middle	31.349200	27.150591
S8	*Prunus ferdal*			Late	31.350141	27.138680
S9	*Prunus texas*	Groppi farm – General Authority for afforestation	Semi-hard	Late	Groppi farm – General Authority for afforestation	
S10	*Prunus perles (mal)*		Semi-hard			
S11	*Prunus IXL*		Soft	Middle		
S12	*Prunus Zuweid3*	El-Sheikh Zuweid Research Station, North Sinai	Soft	late	31.236689	34.117520

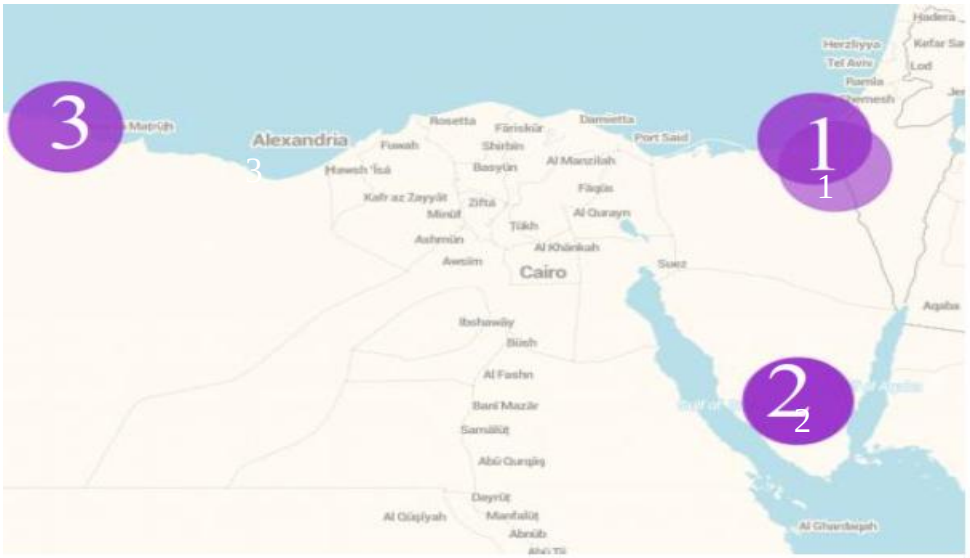

FIGURE 1: General map of Egypt, three remotely distant locations were defined: (1) North Sinai, (2) South Sinai and (3) Northern Cost.

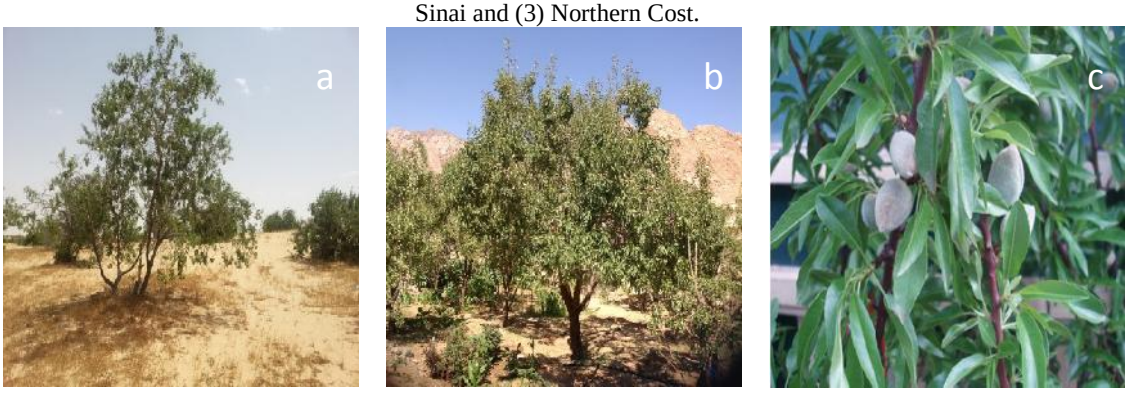

FIGURE 2: Almond trees in S. Catherine are shown (a: adult trees, b: sweet Almond, c: Almond leaves and immature fruits)

Plant Description

A small, deciduous tree, 3–8 m high with unarmed spreading horizontal branches and grayish brown bark and an open spreading crown. Leaves are alternate, medium green, lanceolate to elliptic lanceolate 3–9 cm by 1.2.5cm, sparsely pilose when young becoming glabrescent with broadly cuneate to rounded base, shallowly densely serrate, margin and acute to shortly acuminate apex. Flowers are usually borne laterally on spurs or short lateral branchlets or laterally on long shoots. Flowers solitary, white to pink, actinomorphic, pentamerous, 2–5 cm across, on 3–4 mm pedicels, appearing before the foliage. Hypanthium cylindrical and glabrous plate outside. Sepals are 5 broadly oblong to broadly lanceolate, margin pubescent, apex obtuse. Petals are 5 white or pinkish, oblong to obovate-oblong, base tapering to a narrow claw, apex obtuse to emarginate. Stamens elongated unequal in length. Ovary perigynous and densely tomentose. Style longer than stamens. Fruit an obliquely oblong to oblong-ovoid drupe 3–6 cm by 2–4 cm, pubescent, the tough mesocarp splitting at maturity to expose the endocarp

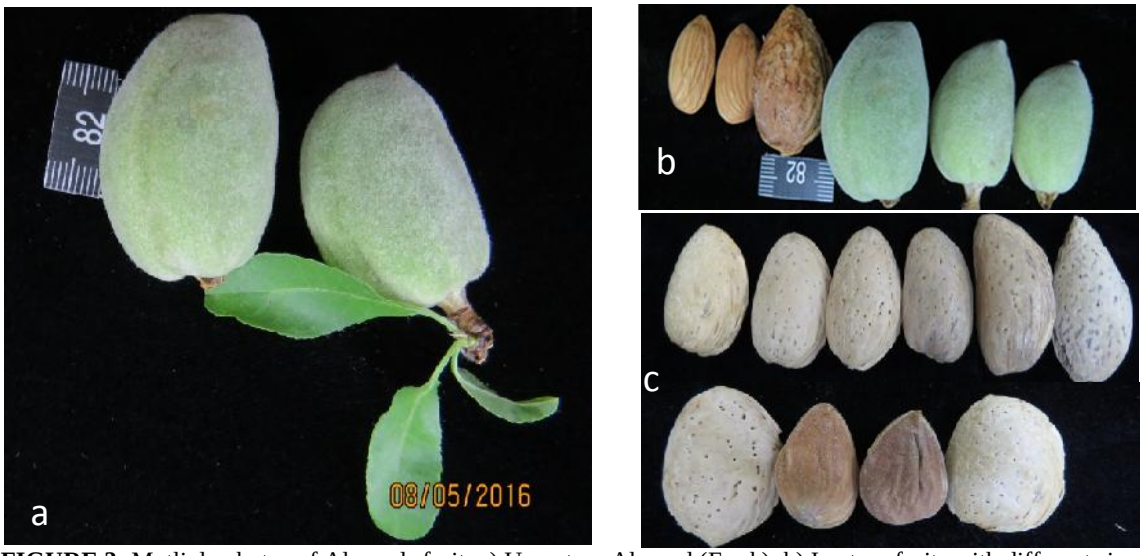

FIGURE 3: Mutliple photos of Almonds fruit: a) Unmature Almond (Farrk); b) Imature fruits with different sizes; c and d) Hard Almonds.

DNA extraction

Genomic DNA extraction:

Three grams of young leaves were ground to powder in liquid nitrogen using a mortar and pestle- were used for DNA extractions. The DNA extractions were carried out using SIGMA®-ALDRICH GenElute^{TM} Plant Genomic DNA Miniprep Kit (Cat# G2N350) which described as follows:

1. The plant tissue was ground into powder under liquid nitrogen.
2. Up to 100 mg tissue were transferred to a 1.5 or 2.0 ml centrifuge tube.
3. LP (lysis) buffer of 450µl were added to 4µl of 100mg/ml RNase A were added to each sample.
4. Samples were incubated at 65 ^{o}C for 15 min (vortex the tube 2-3 times during the incubation). The incubation time was extended when the samples were difficult to be lyses.
5. DA buffer (150µl) were added and mixed thoroughly, and then sample was incubated on ice for 5 minutes.
6. The lysate of all samples were applied to the Shredder Spin-column and centrifuged at 0.154g for 3 min, the supernatant was transferred to the Shredder Spin Column, then centrifuged at 0.154 g for 1 min.
7. The flow-through fraction was transferred to a new 1.5ml eppendorf tube.
8. Seven hundred and fifty µl (or 1.5 volume) of the P Binding buffer were added, and then was mixed thoroughly.
9. The mixture was transferred to the spin column, and then centrifuged at 2800 g for 1 min. The flow-through was discarded. Sample volume (more than 750 µl) was simply loaded and spin again.
10. Five hundred µl of the G Binding buffer were added into the spin column, and then centrifuged at 7800 g for 30 sec. The flow-through was discarded.
11. Six hundred µl washing buffer were added to the spin-column, and then centrifuged at 7800g for 30 sec., the flow-through was discarded, twice.
12. The samples were centrifuged for an additional 1 minute at 7800g and transferred the spin column to a sterile 1.5 micro-centrifuge tube.
13. One hundred µl to 200 µl elution buffer were added and then the samples were incubated at room temperature for 1 minute.
14. The samples were centrifuged at 11250 g for 1 min. The buffer in the micro centrifuge tube contained the DNA sample.
15. DNA was quantified using 1:10 diluted DNA elution buffer using Eppendorf® spectrophotometer x 100 device.
16. Agarose gel electrophoresis was performed to test the extracted genomic DNA with 1% agarose (Bioline)

eluted in 1X TBE buffer (stock solution 1) and stained by ethidium bromide staining (Sigma). Five µl of each sample was loaded while electrophoresis conditions were set to 100 V for 30 min. The technique will be described in details later on.

Stock solution 1: Tris-Boric-EDTA 5X stock buffer in 1 L.

Reagent	Quantity (1 L)	Final Concentration
Tris base (Invitrogen 15504020)	121.1 g	1 M
Boric Acid (MERCK)	61.8 g	1 M
EDTA (disodium salt: Sigma E5134)	73.4 g	0.02 M

- pH was adjusted to 8.3 using 1 M HCl.
- Prepare with 1 L RNase-free dd.H_2O.
- Dilute 100 mL to 1 L to make gel running buffer (1X).
- Store for up to 6 months at room temperature.

Agarose Gel Electrophoresis:

Gel preparation (1%):

1. One gram of agrose powder (Bioline) was added to 100 ml 1X TBE buffer solution.
2. Solution was heated in microwave oven for 2 min, while gently swirled and improve solubility.
3. One X Ethidium Bromide was added to the cooled solution (~ 60 ^oC) and mixed gently.
4. Solution was casted immediately into the gel tray while the comb was already inserted until completely solidified (~ 15 min).

Sample Preparation:

1. DNA samples were prepared in micro-centrifuge tubes (0.2 ml) as 1 µl 10X glycerol dye (stock solution 2) added to 10 µl extracted DNA for each sample and pipetted gently within the tube.
2. Under the buffer, 5 µl of prepared sample were loaded to a well along with 2.5 µl DNA ladder (GeneRuler ™ 100 bp, Fermentas, SM1143).

Stock solution 2: Glycerol dye mix 10X stock buffer in 10 ml.

Reagent	Quantity (1 L)	Final Concentration
Glycerol (MERCK)	5 ml	50 %
Bromo Phenol Blue (Sigma B8026)	5 mg	0.05 %
Xylene Cyanol (Sigma X4126)	5 mg	0.05 %
10 X TBE (Stock solution 1)	1 ml	1 X

- Prepare up to 10 ml using RNase-free dd.H_2O.
- Dilute 10 mL to 1 ml to make loading dye buffer (1 X).
- Store for up to 2 years at 4 ^oC temperature.

Electrophoresis:

1. The device tank HU10 (Consort NV, UK) was filled with 1 x TBE buffer.
2. The combs were removed by wiggling back and forth and then lifting up.
3. Based on 4 V / cm between cathode and anode electrodes, the connected power supply was adjusted to 100 V. Ampere reading was used to determine the buffer stability and freshness.
4. The electrophoresis ran for approximately 30 min, and stopped when the loading dye reached a pre-established position (2/3 of the gel length).

Gel Visualization and Documentation:

1. Gel was located to a trans-illuminator with dark room device to provide short wave length UV light (254 nm) generated by mineral-light model R-52.
2. Supplied camera (1.3 Mega Pixel) connected to the dark room was used to digitalize and capture the gel images.

SI Alleles Genotyping

1. A master mix was prepared and distributed depending on the PCR reactions number (sample/locus). Green GoTaq® Flexi DNA polymerase (Promega, M8295) was used in 25 µl reactions. In a sterile, nuclease-free micro-centrifuge tubes (0.2 ml), the following components were combined on ice:

Reagent	Stock concentration	Reaction concentration
GoTaq Flexi buffer	5x	1x
$MgCl_2$	25 mM	4 mM
dNTPs	10 mM each	0.20 mM each
Forward primer	10 µM	1 µM
Reverse primer	10 µM	1 µM
Go Taq	5 U/µl	1.25 U
DNA Stock	-	50 ng
dd.H_2O	-	up to 25 µl total volume

2. Several multiple alleles were detected using conventional PCR, however some were multiplexed (1 forward + 2 reverse primers) in order to amplify successfully. Primers used in the current study were the following:

Allele	Primer name	5'-Sequence-3'	*Tm*	Expected size
S1	S1F	CTCTTTAGCATTTTAGTTTTTAG	55	488
	S1R	CTGAGACATCCAAGCAATATAG		
S2	S2F	TGAGTCCAAACCGGTGC	65	
	S2R	GACTGCGTACGAATTCTC		
S3	S3F			
	S3R			
S8	S8F	CAAATGGTCCTTCAGGTTTTC	65	648
	S8R	GACTGCGTACGAATTCTG		
S23	S23F	ATTGTCATCTGAAGACCATATAC	60	437
	S23R	TGAGACATCCAAGCAATATATAC		

3. Micro-centrifuge tubes were placed in a Techne™ 96 thermocycler. The PCR program was designed considering the Tm of each primer pair, the general PCR program was:

Step	Temperature	Time	Cycles
Initial denaturation	94 °C	4 min	1
Denaturation	94 °C	1 min	35
Annealing	Tm °C	1 min	
Extension	72 °C	1 min	
Final extension	72 °C	7 min	1

4. PCR products were separated by 1.5 % (1.5 g /100 ml) agarose gel electrophoresis and visualized with 1 X Ethidum Bromide. Ten µl of PCR reactions contain the 1 X GreenGoTaq® Flexi buffer were loaded onto the gel directly after amplification, additionally one lane was loaded with 2.5 µl of GeneRuler™ 100 bp DNA Ladder Plus (Fermentas, SM1153). Gel preparation, electro phoretic conditions and gel documentation were the same as described before (point 3.2.2).

5. All gels were analyzed using Total Lab V120. The different fragment sizes were scored in an allelic data form 6. (Capital letter for each detected allele) and analyzed using Microstat to calculate dissimilarity matrix and phylogenetic trees.

RESULTS & DISSCUSIONS

Self-incompatibility alleles (SI alleles)

1-Primer AS1II/CEBASf/AmyC5R

Total of 2 SI alleles were detected, one of 933 bp and the other 645 bp. Each was unique and homozygous in almond tree number 2 and 4, respectively (Table 2 and Fig. 4).

TABLE 2. Allelic data scored for AS1II/CEBASf/AmyC5R SI primer.

		Genotypes (Primer AS1II/CEBASf/AmyC5R)											
Allele	**MW**	**OTU 1**	**OTU 2**	**OTU 3**	**OTU 4**	**OTU 5**	**OTU 6**	**OTU 7**	**OTU 8**	**OTU 9**	**OTU 10**	**OTU 11**	**OTU 12**
A1	**933**	0	1	0	0	0	0	0	0	0	0	0	0
B1	**645**	0	0	0	1	0	0	0	0	0	0	0	0
Genotype		-	A	-	B	-	-	-	-	-	-	-	-
		-	A	-	B	-	-	-	-	-	-	-	-

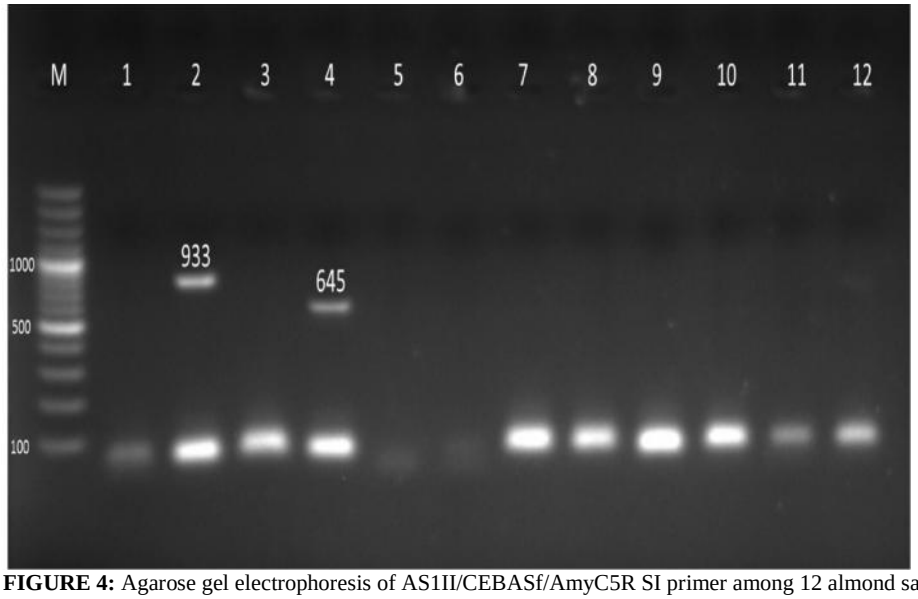

FIGURE 4: Agarose gel electrophoresis of AS1II/CEBASf/AmyC5R SI primer among 12 almond samples.

2-Primer S3

Total of 2 SI alleles were detected, one of 847 bp and the other 241 bp. Each was unique and homozygous in almond tree number 2 and 7, respectively (Table 3 and Fig. 5).

TABLE 3. Allelic data scored for S3 SI primer.

		Genotypes (Primer S3)											
Allele	**MW**	**OTU 1**	**OTU 2**	**OTU 3**	**OTU 4**	**OTU 5**	**OTU 6**	**OTU 7**	**OTU 8**	**OTU 9**	**OTU 10**	**OTU 11**	**OTU 12**
A3	**847**	0	1	0	0	0	0	0	0	0	0	0	0
B3	**241**	0	0	0	0	0	0	1	0	0	0	0	0
Genotype		-	A	-	-	-	-	B	-	-	-	-	-
		-	A	-	-	-	-	B	-	-	-	-	-

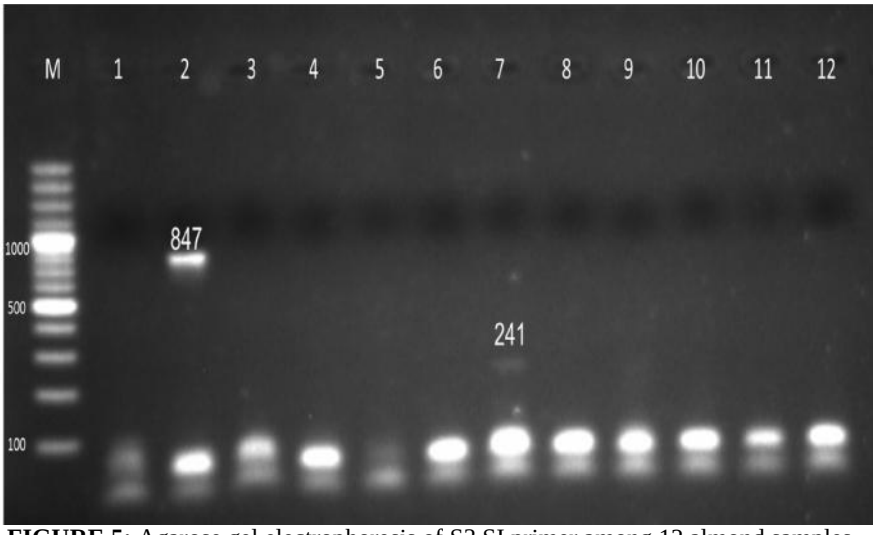

FIGURE 5: Agarose gel electrophoresis of S3 SI primer among 12 almond samples.

3- Primer S8

Total of 2 SI alleles were detected, one of 111 bp and the other 105 bp. Each present as homozygous in all almond trees except number 1 and 3, respectively (Table 4 and Fig. 6).

TABLE 4. Allelic data scored for S8 SI primer.

		Genotypes (Primer S8)											
Allele	**MW**	**OTU 1**	**OTU 2**	**OTU 3**	**OTU 4**	**OTU 5**	**OTU 6**	**OTU 7**	**OTU 8**	**OTU 9**	**OTU 10**	**OTU 11**	**OTU 12**
A8	**111**	0	0	1	1	0	1	1	1	0	1	0	0
B8	**105**	0	1	0	0	0	0	0	0	1	0	1	1
Genotype		-	B	A	A	-	A	A	A	B	A	B	B
		-	B	A	A	-	A	A	A	B	A	B	B

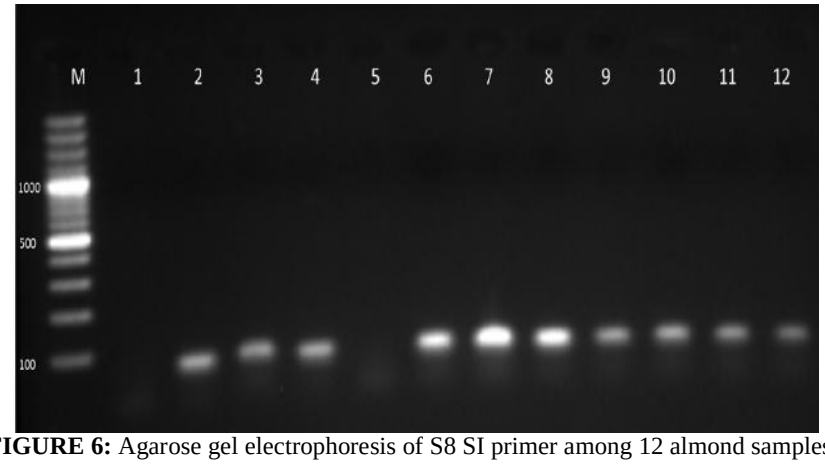

FIGURE 6: Agarose gel electrophoresis of S8 SI primer among 12 almond samples.

4-Primer S23

Single SI alleles were found of 386 bp. Only detected as homozygous in almond tree number 9 (Table 5 and Fig. 7).

TABLE 5. Allelic data scored for S23 SI primer.

Genotypes (Primer S23)													
Allele	**MW**	**OTU 1**	**OTU 2**	**OTU 3**	**OTU 4**	**OTU 5**	**OTU 6**	**OTU 7**	**OTU 8**	**OTU 9**	**OTU 10**	**OTU 11**	**OTU 12**
A23	**386**	0	0	0	0	0	0	0	0	1	0	0	0
Genotype		-	-	-	-	-	-	-	-	A	-	-	-
		-	-	-	-	-	-	-	-	A	-	-	-

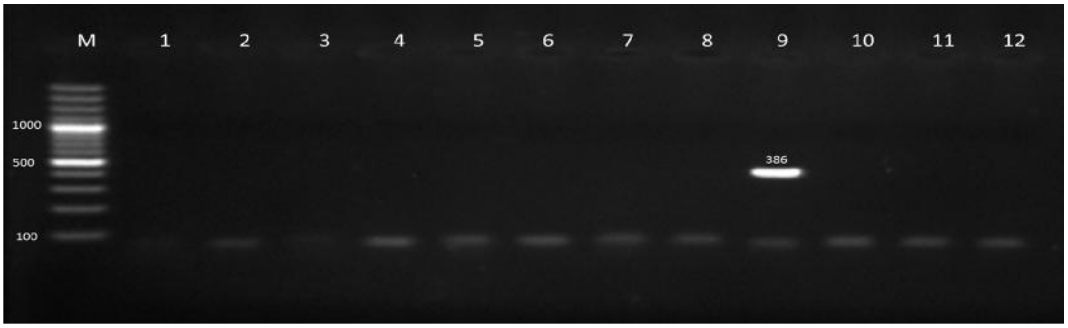

FIGURE 7: Agarose gel electrophoresis of S23 SI primer among 12 almond samples.

Eight SI alleles were tested, in which 4 showed amplifications as homogenous or heterogeneous alleles in one or more almond trees. Comparing all alleles, some almond trees show self-incompatibility. S2 sampled from Saint Catherine showed the highest number of SI alleles (3) followed by S7, S8 and S9 (2), S3, S4, S6, S10, S11 and S12 showed a unique SI allele, while S1 and S5 were completely clear of any SI alleles (Table 6). Even the alleles of each SI type shows different molecular size and appear in different individuals, but they all affecting the selfing percentage and cause infertility and the urgent need to cross pollinate the tree to expect fruits. S2 need more extensive study to reveal the complete SI alleles that can be found.

TABLE 6. Genotyping of 4 out of 8 detected SI alleles for 12 Almond trees.

		Genotypes											
SI allele	**No. allele**	**OTU 1**	**OTU 2**	**OTU 3**	**OTU 4**	**OTU 5**	**OTU 6**	**OTU 7**	**OTU 8**	**OTU 9**	**OTU 10**	**OTU 11**	**OTU 12**
S1	**2**	0	A_1A_1	0	0	0	0	0	B_1B_1	0	0	0	0
S3	**2**	0	A_3A_3	0	0	0	0	B_3B_3	0	0	0	0	0
S8	**2**	0	B_8B_8	A_8A_8	A_8A_8	0	A_8A_8	A_8A_8	A_8A_8	B_8B_8	A_8A_8	B_8B_8	B_8B_8
S23	**1**	0	0	0	0	0	0	0	0	$A_{23}A_{23}$	0	0	0

Allelism is one of the most striking characteristics of the S-locus. Rare S-alleles have reproductive advantage because pollen bearing such alleles is less likely to land on a stigma with the same allele and many S-alleles may thus be maintained in a finite population (Wright 1939). Since over dominant selection is thought to operate in S-RNases disfavoring SC, species with SI tend to contain high numbers of specificities (S-alleles), sometimes as many as a hundred (Bernatsky *et al*., 1988). In fact, classical studies on the numbers of S-alleles in populations have revealed huge allelic diversity at the S-locus in homomorphic SI species and have inspired diverse theoretical research (Wright 1965; Nagylaki 1975; Yokoyama and Hetherington 1982; Uyenoyama 1991). Although Rosaceae and Solanaceae express similar S-locus products in pistils, the Rosaceae S-RNases sequenced so far have only one hypervariable region (RHV) and one conserved region (RC4) not present in Solanaceae. According to Ushijima *et al*. (1998), rosaceaous species appear to have diverged more recently than Solanaceae, and after divergence of the sub-families. Accordingly, our results show a high diversity of S-phenotypes in a restricted population. The new S-allele, Sx, found only in cultivars from one collection (Algarve), supports the idea that division of a population into a number of partially isolated groups in which alternative descendent haplotypes may undergo substitution and subsequent evolution enhances the rate of S-allele diversification (Uyenoyama and Newbigin, 2000).

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staff of Egyptian Deserts Gene Bank for their cooperation and friendly atmosphere.

CONCLUSION

The results are discussed in view of the evolution of the gametophytic SI system and the models proposed for its mechanism.

This study can present new views for the genetic structure of Egyptian almond. In conclusion, evaluation and conservation of wild almond germplasm are important for the almond breeding programs. Large genetic variations could provide the opportunity to select and breed new varieties with adaptability to different environments.

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