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Cross-incompatibility in the cultivated almond (*Prunus dulcis*): updating, revision and correction

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ABSTRACT

Most almond cultivars are self-incompatible, and so to obtain a yield they need to be pollinated with cross-compatible cultivars. For an efficient use of these cultivars in growing and breeding, over the years many have been *S*-genotyped. This information has been included in consecutive tables of cross-incompatibility groups, which are essentially an update of the previously proposed. However, the information of these tables has not always been reconciled and their inconsistencies have not been corrected. In this work the *S*-genotypes of 15 Spanish almond local cultivars, included in a research program for the preservation of almond biodiversity, were determined for the first time. For this, PCR with consensus primers for the *Prunus S-RNases,* PCR using *S-RNase* allele specific primers designed herein, and also cloning and sequencing were performed. As a result, a new *S-RNase* allele numbered as S_{53} could be identified, and a compilation table of almond cross-incompatibility groups is provided including the information from this and previous studies. In this novel Table 12 new cross-incompatibility groups have been established, two former groups were omitted, and those cultivars with inconsistent genotypes have been removed. The information of this table will facilitate the use of a very high number of almond cultivars in research and breeding. Determination of the *S*-genotype might be considered as an important addition to a set of identity markers of almond genetic resources, what is of particular interest in the characterization of the agricultural biodiversity.

Keywords: *Prunus dulcis*, Self-incompatibility, Cross-incompatibility, S-genotyping, S-RNases, agricultural biodiversity

30 **1. Introduction**

31 Most almond cultivars [*Prunus dulcis* (Mill.) D.A. Webb] are self-incompatible, what means that
32 to produce a yield they need to be cross-pollinated with cultivars having a different incompatibility
33 genotype (*S*-genotype). Thus, determination of the *S*-genotype is mandatory to choose cross-compatible
34 cultivars in almond growing and breeding. Likewise, *S*-genotyping facilitates the use of almond genetic
35 resources for the recovering and preservation of biodiversity.

36 Self-incompatibility in this species is of the gametophytic type and it is controlled by the *S* locus,
37 which has at least two independent genes expressed in pistil or pollen (Tao and Iezzoni, 2010). The pistil
38 *S* gene is expressed as glycoproteins with ribonuclease activity known as S-RNases, which arrest the
39 growth of incompatible pollen in the style (McClure et al., 1989; Tao et al., 1997). The S-RNases from
40 several *Prunus* fruit tree species, including almond, have been molecularly characterized (Ushijima et
41 al., 1998; Sonneveld et al., 2003; Vilanova et al., 2005; Ortega et al., 2006; Sutherland et al., 2009). This
42 characterization revealed that the S-RNases have five conserved regions (C1-C5), a hypervariable region
43 (RHV) and two polymorphic introns. One of the introns is located between the signal peptide and C1,
44 and the other one resides within the RHV region between C2 and C3.

45 The *S*-genotype of several almond cultivars has been determined using different approaches. At
46 first, controlled crosses among a few cultivars allowed deducing their *S*-genotype (Crossa-Raynaud and
47 Grasselly, 1985; Kester et al., 1994). Later, these and other almond cultivars were *S*-genotyped by
48 isoelectric focusing of stylar proteins followed by staining for ribonuclease activity (Bošković et al.,
49 1997; Certal et al., 2002; Bošković et al., 2003). More recently, consensus primers amplifying the *S-*
50 *RNases* of other *Prunus* (Sonneveld et al., 2003; Sutherland et al., 2004) proved to be very efficient in
51 almond (Ortega et al. 2005; Halász et al. 2010). Other primers have also been used to assess the *S*-
52 genotype in almond (Channuntapipat et al., 2003; López et al., 2006; Barckley et al., 2006; Halász et
53 al., 2008; Kodad et al., 2008; Kodad et al., 2010; Curró et al., 2015). The information obtained with the
54 different methods and primer sets indicated has been included in different versions of a table of almond
55 cross-incompatibility groups (Kester et al., 1994; Bošković et al., 2003; Barckley et al., 2006; Ortega et
56 al., 2006; Kodadand Socias i Company, 2009; Hafizi et al., 2013). However, to date no attempt has been
57 made to reconcile the information of all these tables neither to correct possible inconsistencies.

58 The aims of this study were to determine for the first time the *S*-genotype of 15 almond cultivars
59 included in a research program for the preservation of almond biodiversity, and to construct a
60 compilation table of almond cross-incompatibility, by updating and correcting previous information.
61

62 **2. Materials and methods**

63 *2.1. Plant material*

64 Samples of young leaves were collected from trees of 15 Spanish almond cultivars previously un-
65 genotyped for self-incompatibility, which were part of a genetic resources open field genebank at
66 "Institut de Recerca i Tecnologia Agroalimentàries" (IRTA) in Constantí (Tarragona, Spain).
67 Additionally, leaf samples from other 15 almond cultivars and from one selection grown at the
68 experimental field of CEBAS-CSIC in Santomera (Murcia, Spain) were used as reference for the almond
69 *S*-RNase alleles S_1-S_{29} and S_f described in Ortega et al. (2005) (Table 1).
70

71 **Table 1**
72 Almond cultivars used as reference for the *S*-RNase alleles S_1-S_{29} and S_f.

Cultivar	*S* genotype	Reference[1]	Source[2]
A2-198	S_fS_f	D	CB
Avellanera Gruesa	$S_{22}S_{26}$	O	CB
CEBAS-1	S_4S_{13}	B3	CB
Cristomorto	S_1S_2	CG, B1	CB
Ferragnès	S_1S_3	CG, B1	CB
Fina del Alto	$S_{28}S_{29}$	O	CB
Fournat de Brezenaud	$S_{24}S_{27}$	O	CB
Gabaix	$S_{10}S_{24}$	O	MB
IXL	S_7S_8	B1	CB
La Mona	$S_{23}S_{25}$	O	CB
Marcona	$S_{11}S_{12}$	B2	CB
Padre	S_1S_{18}	B3	UC
Primorskyi	S_5S_9	B3	CB
Ramillete	S_6S_{23}	B3	CB
Rumbeta	$S_{11}S_{21}$	B3	CB
Titan	S_8S_{14}	O	CB

73 [1]B1, Bošković et al. (1997); B2, Bošković et al. (1998); B3, Bošković et al. (2003); CG, Crossa-Raynaud and
74 Grasselly (1985); D, Dicenta et al. (2002); O, Ortega et al. (2005)
75 [2]CB, CEBAS-CSIC, Murcia, Spain; UC, University of California, Davis, USA; MB, IRTA-Mas Bové, Spain
76

77 *2.2. DNA extraction and determination of S-genotypes by PCR*

78 Genomic DNA was extracted from fresh leaf samples using the CTAB protocol described in
79 Sonneveld et al. (2001). *S*-RNases were identified by PCR using the consensus primers EM-PC2consFD
80 + EM-PC3consRD (Sutherland et al., 2004), and PaConsI-F + EM-PC1consRD (Sonneveld et al., 2003;
81 Ortega et al., 2005), which amplify the second and first intron of *Prunus S-RNases*, respectively.
82 Moreover, in some particular cases, PCR analysis with primers designed in the present study to
83 specifically amplify almond S_{10}-RNase was performed. The specific primers S10-C2-F and S10-C3-R
84 (Table2) were designed from the almond S_{10}-*RNase* DNA sequence published in EBI database under the
85 accession number AM231659, and were used in combination with the primers IC-F and IC-R
86 (Sonneveld et al., 2003), which served as an internal control to identify false negatives in the PCR.

87 PCR reaction and cycling parameters for the second intron primers were as indicated in Ortega et
88 al. (2005). However, for the first intron the PCR reaction described in Ortega et al. (2005) was cycled
89 here as in Sonneveld et al. (2003). PCR with the allele-specific primers S10-C2-F and S10-C3-R was
90 set up as described in Sonneveld et al. (2003), while cycling conditions were modified and consisted of
91 2 min at 94ºC, 35 cycles of 10 s at 94ºC, 2 min at 60ºC and 3 min at 68ºC, and a final extension of 10
92 min at 68ºC.

93 The PCR products obtained were run on agarose for 1.5 h at 100 V alongside 1 Kb Plus DNA
94 ladder (Invitrogen, Carlsbad, CA, USA). TAE agarose gels were made at 1.5% for second intron and
95 specific primers, and at 2% agarose for the first intron primers. In all cases, staining was performed by
96 adding to each of the PCR products and to the DNA ladder 5x Orange loading buffer (580 mM sucrose,
97 1 mg/ml Orange G) containing 250x GelRed (Biotium, Hayward, CA) to a final concentration of 1x.
98 The gels were photographed using the automated image acquisition software GeneSnap (Syngene,
99 Cambridge, UK).

100

101 *2.3. Cloning and sequencing of S-RNase alleles*

102 PCR products with different sizes to those of the reference alleles S_1-S_{29} and S_f were ascribed to
103 potentially new *S*-RNase alleles. In most cases, they were cloned and sequenced after amplification from
104 the signal peptide (SP) to the conserved region C5, as indicated in Ortega et al. (2006). However, when

amplification from SP to C5 was not possible, the S-RNases were amplified from C1 to C5 regions as indicated in Ortega et al. (2006), but with an annealing temperature of 58ºC.

The PCR products obtained were purified using the QIA Quick PCR purification kit (Qiagen, Hilden, Germany) and later quantified as indicated in Ortega et al. (2006). Then, the products were cloned into a pCR-Blunt-II-TOPO vector (Invitrogen) and transformed into One Shot Chemically Competent *Escherichia coli* cells (Invitrogen) following the recommendations of the manufacturer. Positive transformants were identified by PCR with the same primer sets used to obtain the fragments cloned, and isolation of the plasmids was carried out with the QIAprep Spin Miniprep Kit (Qiagen). For each *S*-RNase allele, three positive plasmids were sent for sequencing using the M13 primers to STAB VIDA (Caparica, Portugal).

2.4. Analysis of DNA sequences

For each of the *S*-RNase alleles cloned a consensus nucleotide sequence, including the intron(s), was obtained after assembling the sequences of the three plasmids with SeqManII software (DNASTAR, Madison, USA). The identity of these sequences was ascertained by comparison of the deduced amino acid sequences with those available in public databases after blastp search (protein-protein BLAST) in the NCBI web site (https://www.ncbi.nlm.nih.gov). Sequences with percentages of identity higher than 90% were aligned using MegAlign software (DNASTAR).

2.5. Revision and updating of almond cross-incompatibility groups

A table of almond cross-incompatibility was constructed using the results of the present work and the plentiful data reported by other authors (Ortega et al., 2006; López et al., 2006; Barckley et al., 2006; Kodad et al., 2008; Valizadeh et al., 2009; Kodad and Socias i Company, 2009; Kodad et al., 2009; Halász et al., 2010; Kodad et al., 2010; Fernández i Martí et al., 2010; Martínez-García et al., 2011; Mousavi et al., 2011; Hafizi et al., 2013; Curró et al., 2015). This new table was not intended to be merely an update of the earlier ones, but a compilation or review table in which all the self-incompatible almond cultivars *S*-genotyped to date were to be included, and thus it was made after an accurate correction of the inconsistencies detected in previous works.

133

134 **3. Results**

135 *3.1. Identification of S-genotypes*

136 In most cases the cultivars analyzed herein were *S*-genotyped only by using the sets of primers
137 above indicated. However, in two particular cases additional cloning and sequencing of *S-RNases* were
138 necessary to determine the *S*-genotype.

139 PCR analysis for the second intron followed by horizontal electrophoresis on agarose gels yielded
140 two bands of different sizes in all cases; whilst with the primers for the first intron no amplification or a
141 triplet band was observed for some cultivars (Table 2). In most cases the sizes of the bands obtained
142 with first and second intron consensus primers were identical to those in the reference cultivars, and
143 thus the *S*-genotypes could be straightaway assessed. Moreover, in the case of the alleles S_5 and S_{10}
144 specific primers for the S_{10} allele designed in the present work were necessary to obtain conclusive
145 results since, as reported by Ortega et al. (2005), the bands of the alleles S_5 and S_{10} were difficult to
146 distinguish after second intron PCR, and they were not detectable on agarose with first intron primers.
147 Thus, the specific primers for S_{10}-*RNase* successfully enabled to detect the S_{10} haplotype when a band
148 of 200 bp appeared in agarose gels. In contrast, the S_5 haplotype was assessed when a band of 330 bp
149 was obtained with second intron primers and only a band slightly above 1000 bp (corresponding to
150 amplification with IC primers) was observed after S_{10} specific PCR (Table 2, Fig. 1).

151 Bands of different sizes to those of the reference cultivars were only observed in the cultivars
152 'Mollar de la Princesa' and 'Parque Samá', what indicated they may correspond to new *S*-RNases, and
153 thus they were cloned and sequenced.

154

155 **Table 2**

156 PCR product sizes obtained with second and first intron consensus primers, S_{10}-RNase specific PCR

157 scores, and *S*-genotypes assessed to 15 Spanish almond cultivars.

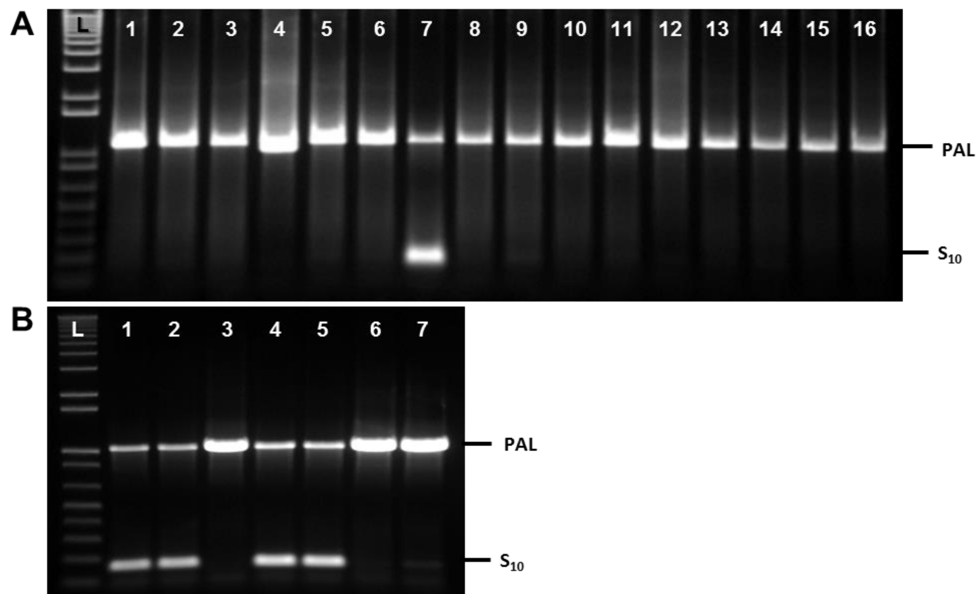
Cultivar	C2-C3 (bp)	SP-C1 (bp)[a]	S_{10}-*RNase*[b]	*S*-genotype assessed
Angones	300, 1130	n.a., 400	+	$S_{10}S_{22}$
Asperilla	300, 1360	n.a., 380	+	$S_{10}S_{27}$
Belardino	450, 400	270, 400		S_2S_{11}
Caima	350, 1300	n.a., 200	–	S_5S_{12}
Carreró	1300, 1360	200, 380		$S_{12}S_{27}$
Esperanza Forta	1300, 1130	200, 400		$S_{12}S_{22}$
Mollar de la Princesa	875, 570	380, 360		$S_{24}S_{53}$
Mollar de Tarragona	750, 1300	590/700/870, 200		S_1S_{12}
Nano	1300, 340	200, 300		$S_{12}S_{28}$
Parque Samá	750, 1280	590/700/870, 380		S_1S_{35}
Pauet	570, 300	350, n.a.	+	S_6S_{10}
Pep de Juneda	750, 300	590/700/870, n.a.	+	S_1S_{10}
Rof	330, 690	n.a., 375	–	S_5S_{23}
Tardaneta	330, 1300	n.a., 200	–	S_5S_{12}
Verd	1300, 1360	200, 380		$S_{12}S_{27}$

158 [a]n.a.: no amplification

159 [b]The sequences of the primers used are: S10-C2-F: 5'-GTACTCTTGGGTGGTTAATTGCC-3'; S10-C3-R: 5'-G

160 TTTGGTTTCAATTCTAGGAGACA GA-3'

161



162

163

164 **Fig. 1.** Allele-specific PCR for S_{10}-RNase. Lanes marked with L correspond to 1Kb Plus DNA ladder. (A) 1:
165 Cristomorto (S_1S_2), 2: Ferragnès (S_1S_3), 3: CEBAS-1 (S_4S_{13}), 4: Primorskyi (S_5S_9), 5: Ramillete (S_6S_{23}), 6: IXL
166 (S_7S_8), 7: Gabaix ($S_{10}S_{24}$), 8: Marcona ($S_{11}S_{12}$), 9: Titan (S_8S_{14}), 10: Padre (S_1S_{18}), 11: Rumbeta ($S_{11}S_{21}$), 12:
167 Avellanera Gruesa ($S_{22}S_{26}$), 13: La Mona ($S_{23}S_{25}$), 14: Fournat de Brezenaud ($S_{24}S_{27}$), 15: Fina del Alto ($S_{28}S_{29}$),
168 and 16: A2198 (S_fS_f). (B) 1: Angones ($S_{10}S_{22}$), 2: Asperilla ($S_{10}S_{27}$), 3: Caima (S_5S_{12}), 4: Pauet (S_6S_{10}), 5: Pep de
169 Juneda (S_1S_{10}), 6: Rof (S_5S_{23}), and 7: Tardaneta (S_5S_{12}).

170 *3.2. Analysis of the S-RNase sequences obtained*

171 Two *S*-RNase alleles were successfully cloned and sequenced from 'Mollar de la Princesa' and 172 'Parque Samá', obtaining DNA sequences of 1198 bp (SP-C5) and 1647 bp (C1-C5), respectively.

173 After BLAST searches in NCBI database, the deduced amino acid sequence of the S-RNase from 174 'Parque Samá' was identical to almond S_{35}-RNase under the accession number ABW04811, and showed 175 99% of identity with almond S_{34}-RNase (ABW04810). Unlike this, the amino acid sequence obtained 176 from 'Mollar de la Princesa' showed only 93% of identity with almond S_6-RNase (AEI69728 and 177 CAJ77725), 92% identity with MSRN-2 RNase from *Prunus mume* (BAB55854), 92% identity with S_6 178 from almond (AAM22178), and 92% identity with S_e from almond (AAZ67031). In fact, it should be 179 highlighted here that according to Bošković et al. (2003) and Barckley et al. (2006), S_e-RNase 180 corresponds to S_6-RNase. Thus, BLAST searches confirmed the *S-RNase* cloned and sequenced from 181 'Mollar de la Princesa' was new, and then it was accordingly labelled as S_{53}*-RNase*. DNA sequences of 182 the *S-RNases* here obtained were accepted in the EMBL-EBI nucleotide sequence database under the 183 accession numbers LN624640 (S_{53}) and LN624641 (S_{35}).

184 In detail, the alignment of the deduced amino sequence of S_{53}*-RNase* from 'Mollar de la Princesa' 185 with those sequences in database showing a percentage of identity above 90% revealed that it was 186 different from all the other sequences in RC4 and mainly in the region between RC4 and C5 (Fig. 2). 187 This alignment also showed that the sequences for almond S_6-RNase included in databases and 188 published were not identical among them. Thus, S_6-RNase under the accession number AEI69728 189 (Hafizi et al., 2013) differed from all the other S_6-RNase sequences aligned in two glycine residues, one 190 located in SP region and the other between RHV and C3 (Fig. 2). Likewise, S_e (=S_6) with accession 191 number AAZ67031 differed from the other S_6 sequences in a leucine located in SP region (Fig. 2).

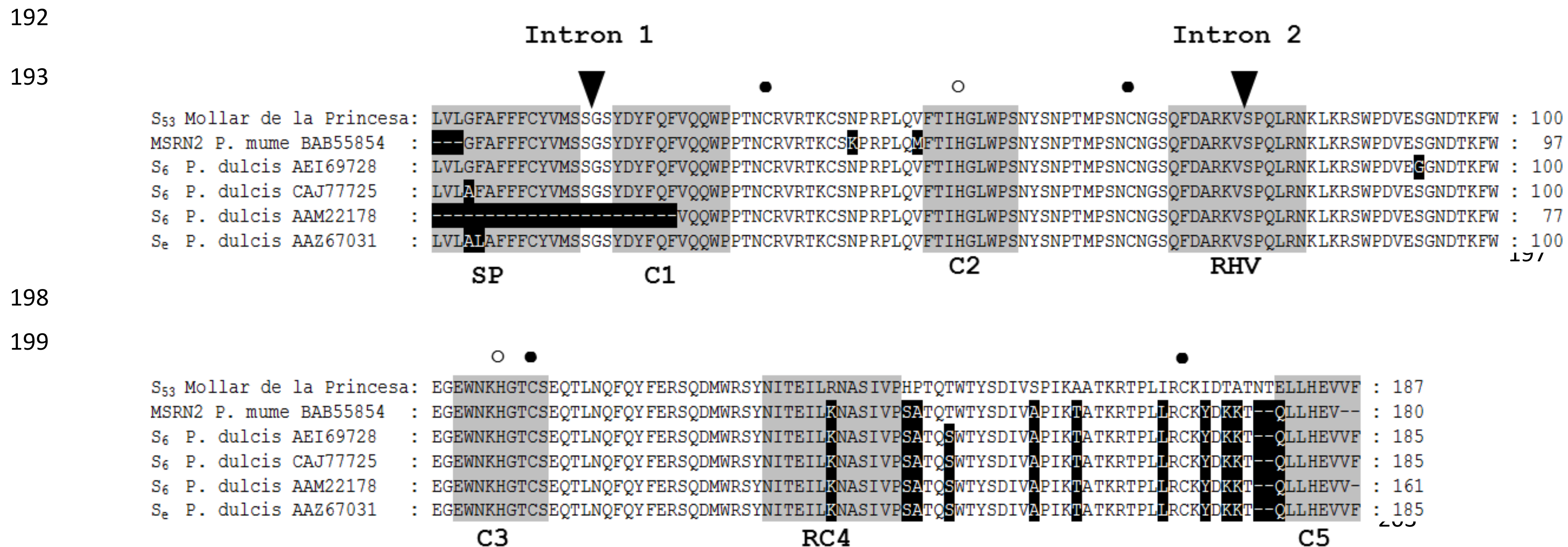


Fig. 2. Alignment with ClustalW method of the deduced amino acid sequence of S_{53}-*RNase* from Mollar de la Princesa with sequences in NCBI database with a percentage of identity higher than 90% after Blast search. Amino acids differing from S_{53} are shaded in black. Signal peptide (SP), C1-C5 conserved regions and the hypervariable region (RHV) described in the Rosaceae by Ushijima et al. (1998) are shaded in grey. Location of the introns is indicated by arrowheads. Fully conserved cysteine and histidine residues in *S*-RNases are indicated with filled and open circles, respectively.

213 *3.3. Updating and reconciliation of almond cross-incompatibility groups*

214 Using data from the present and previous studies, a new table of almond cross-incompatibility 215 including all self-incompatible almond cultivars genotyped to date was created (Table 3). In this table 7 216 new groups (XLIII, XLV, XLVI, XLVII, XLVIII, XLIX, and L) were established with the results of *S*-217 genotyping and cloning of this study. Likewise, after compilation of the results of previous works, other 218 5 new groups (XXXIII, XL, XLI, XLII and XLIV) were created. In detail, group XLIV was established 219 after verifying that amino acid sequence of S_g-RNase KM225270 (Curró et al., 2015) is identical to S_{18}-220 RNAse sequence AM231667 (Ortega et al., 2006). In addition, groups IX ($S_{7A}S_8$) and XXXI (S_7S_{48}), 221 respectively established in Bošković et al. (2003) and in Hafizi et al. (2013), were omitted. In addition, 222 and following previous nomenclature, group O contains those cultivars with a unique *S*-genotype, which 223 are consequently considered as "universal" pollinators.

224

225 **4. Discussion**

226 *4.1. Identification of S-genotypes*

227 Despite it was indicated that the 15 local Spanish almond cultivars here studied had not been 228 genotyped for the *S*-locus before, it should be explained that the cultivar 'Verd' had previously being 229 included in a review article of almond *S*-genotypes as $S_{11}S_{12}$ (López et al., 2006). This genotype had 230 been assigned after isoelectric focusing of stylar ribonucleases (López, 2004). However, the same 231 authors considered the *S*-genotype assigned was doubtful and encouraged to verify it by PCR (López et 232 al., 2006). In fact, the *S*-genotype herein deduced for 'Verd' was $S_{12}S_{27}$ (Table 3), different for one of 233 the *S-RNase* alleles to that previously proposed. It is important to remark that the $S_{12}S_{27}$ genotype and a 234 kernel shape similar to that of 'Marcona' almond, make 'Verd' a good pollinator of 'Marcona' ($S_{11}S_{12}$) 235 in its original growing region (Northern Castellón province, East of Spain), what had been denied before.

236 Even though consensus primers amplifying almond *S-RNases* have proven to be useful to 237 accurately identify and distinguish a wide range of alleles (Ortega et al., 2006; Halasz et al., 2010; this 238 work), allele specific PCR was found essential to ascertain the presence of several almond *S-RNases* 239 (Halasz et al., 2010; Curró et al. 2015; present study: Table 2, Fig. 1). This is usually due to a very 240 similar size of the PCR products amplified. However, sometimes reference cultivars were not included

241 and the consensus primers used had some limitations, as it was the case in Channuntapipat et al. (2003)
242 and Curró et al. (2015).
243
244 *4.2. Table of cross-incompatibility groups*
245 This new table (Table 3) not only contains genuine information from self-incompatible cultivars
246 previously un-genotyped, but also includes several corrections on formerly established groups, and thus
247 constitutes a comprehensive revision in this field that provides an updated and compiled dataset useful
248 for almond breeding and growing.
249 In some cases, omission and correction of previous information was necessary in order to include
250 consistent data in Table 3. Thus, group IX ($S_{7A}S_8$) established in Bošković et al. (2003), including only
251 'Jeffries' cultivar, was omitted because this cultivar has been reported to express unilateral
252 incompatibility (Kester et al. 1994; Connell, 2000). According to these reports, 'Jeffries' is cross-
253 incompatible with cultivars from other groups, what contravenes the principle of a table of cross-
254 incompatibility groups, in which each cultivar within a group should be cross-compatible with any other
255 cultivar from a different group.
256 On the other hand, former group XXXI (S_7S_{48}) from Hafizi et al. (2013) was eliminated, since it
257 was established on the basis of an erroneous identity of the DNA sequences obtained from 'Sefied' and
258 'Monagha' cultivars. This was found after the alignment of S_{48}-RNase sequences from these cultivars
259 reported by the Hafizi et al. (2013) under the accession numbers JX067632 and JX067633. These
260 sequences showed many differences, indicating these cultivars have a different genotype and therefore
261 they should belong to different groups. Interestingly, 'Sefied' and 'Monagha' have also being *S*-
262 genotyped by other authors as (S_7S_{56}) after cloning and sequencing of their *S-RNases* (E. Ortega,
263 personal communication) (Table 3).
264 Regarding group O, the particular case of the cultivar 'Rof' needs to be remarked since, due to its
265 male sterility (Vargas and Romero, 1978), it should not be considered as a universal pollinator despite
266 of being in this group. However, it may still be interesting to know its *S*-genotype for different reasons:
267 to complete its agronomical characterization, to choose suitable pollinator cultivars, and also for
268 phylogenetic studies.

269

270 **5. Conclusions**

271 In this work the *S*-genotypes of 15 Spanish almond cultivars were determined for the first time
272 mainly using consensus and allele specific primers, and also by cloning and sequencing. New primers
273 herein designed and specifically amplifying almond S_{10}-*RNase* proved to be very useful to complete *S*-
274 genotyping, and a new *S-RNase* allele numbered S_{53} could be identified after sequencing. With the
275 information here obtained and that from previous studies, a compilation table of almond cross-
276 incompatibility groups including 296 cultivars has been created. This new table will facilitate the use of
277 a high number of almond cultivars in research and breeding. The *S*-genotype might be considered as an
278 important addition to a set of identity markers of almond genetic resources, what is of particular interest
279 in the characterization of the agricultural biodiversity.

280

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288

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388 **Table 3**

389 Cross-incompatibility groups (CIG) in the cultivated almond.

CIG	Cultivars[a]	*S*-genotype
I	Galaxy, Golden State, Grace, IXL, Long IXL, Mckinlay's Magnificent, Nonpareil, Riedenhoure, Shahrodi-16, Shahrodi-18, Tardy Nonpareil, West Steyn	S_7S_8
II	Ballico, Bulbuente, Garbi, Glorieta, Languedoc, Shahrekord-E1[b], Texas (=Mission), Wawona	S_1S_5
III	Baxendale, Durango, Granada, Harvey, Le Grand, Mono, Robson, Sauret 2, Thompson, Wassum, Woods Colony	S_5S_7
IV	Aldrich, Jenette, Merced, Ne Plus Ultra, Norman, Pearl, Price, Ripon, Rosetta, Sano, Yalda-1[b], Yalda-2[b]	S_1S_7
V	Carmel, Jubilee, Livingston, Monarch, Reams, Sauret 1, Tioga	S_5S_8
VI	Avalon, Bigelow, Blue Gum, Butte, Dottie Won, Duro Amarelo, Folsom, Grace, G-99 [b], Kutsch, Monterey, Northland, Plateau, Rivers Nonpareil, Sultana	S_1S_8
VII	A-2[b], Eureka, Kapareil, Shahrodi-15, Solano, Sonora, Vesta	S_8S_{13}
VIII	Azar, Ferragnès, Ferralise, Mourisca, Shahrodi-12	S_1S_3
XIX	Harpareil, Jordanolo	S_7S_{14}
X	Drake, Kochi, Shahrodi-17, Smith XL, Tétényi Kedvenc	S_6S_8
XI	Abizanda, Fritz, Peerless, Ruby, Rumbeta-2, Selvatica Favata	S_1S_6
XII	Anxaneta, Tarragones	S_2S_9
XIII	Ardéchoise, Coop, Desmayo Largueta, Pep de Juneda, Zahaf	S_1S_{10}
XIV	Achaak, Alnem88, Ferrastar, Kerman-20[b], Poost Nazok Naeen	S_2S_{10}
XV	Pajarera-2, Pestañeta (=Pestanhieta)	$S_{12}S_{23}$
XVI	Malagueña, Muel, Pau, Planeta Fina, Planeta Roja, Verdeta	$S_{22}S_{23}$
XVII	Garrigues, Pajarera-1	$S_{13}S_{27}$
XVIII	AS1, Marcona Flota	S_8S_{12}
XIX	Belle d'Aurons, Peraleja	S_3S_{23}
XX	Bartre, Castañera	S_3S_5
XXI	Masbovera, Moncayo, Tarraco	S_1S_9
XXII	Casanova, Coelhinha, Ferraduel, K-11-40[b], Shahrodi-8, Zarghan-7[b]	S_1S_4
XXIII	Chellastone, Milow, Monaghay-e-Najafabad	S_7S_{13}
XXIV	Parada, Pestañeta Menuda	$S_{11}S_{22}$
XXV	A-92, Khorshidi, Pierce	S_8S_{23}
XXVI	Alzina, Garondes	$S_{fa}S_{36}$
XXVII	Eriane, Szigetcsépi 55 (3/9)	$S_{11}S_{31H}$
XXVIII	Nikitskyi, Óriás Kagyló (4/4)	$S_{36}S_{37}$
XXIX	Cristomorto, Cuore (S_1S_2), Sahand, Zarghan-8[b]	S_1S_2
XXX	Aï, Shahrodi-6	S_3S_4
XXXI	Tokyo, Yazd-11[b], Yazd-13[b]	S_6S_7
XXXII	Carretas Bajas, Yazd-2[b], Yazd-103[b]	S_4S_{12}
XXXIII*	K-16-8[b], Shokoufeh	S_3S_8
XXXIV	G-1[b], Mashhad-30[b]	S_4S_7
XXXV	K-10-15 [b], Zanjan-2[b]	S_8S_9
XXXVI	Harir[b], Shekoofe [b]	S_4S_{24}
XXXVII	K-1-16[b], Shamshiri[b]	S_7S_{24}
XXXVIII	Mashhad-17[b], Safari[b]	$S_{62}S_{63}$

Table 3 *(Continued)*

CIG	Cultivars[a]	*S*-genotype
XXXIX	Monagha[b], Sefied[b]	S_7S_{56}
XL*	CEBAS I, Verdeal	S_4S_{13}
XLI*	Shahrodi-4, Tardive de la Verdiere	S_3S_9
XLII*	Bonita, Don Peppino	S_1S_{21}
XLIII*	Angones, Bronte 1	$S_{10}S_{22}$
XLIV*	Lisciannarisa[c], Nivera Selvaggia[c]	$S_{18}S_{21}$
XLV*	Del Cid, Esperanza Forta	$S_{12}S_{22}$
XLVI*	Colorada, Nano	$S_{12}S_{28}$
XLVII*	Nambaredda, Pauet, Szigetcsépi 58	S_6S_{10}
XLVIII*	K-12-4[b], Mollar de Tarragona	S_1S_{12}
XLIX*	Carreró, G-25, Verd	$S_{12}S_{27}$
L*	Caima, Tardaneta	S_5S_{12}
O	A5 Shahrekord (S_2S_{12}), Abdolla ($S_{54}S_{57}$)[b], Angelica (S_5S_{33}), Asperilla ($S_{10}S_{27}$), Atascada (S_5S_{22}), Atocha ($S_{13}S_{22}$), Avellanera Gruesa ($S_{22}S_{26}$), Bagher (S_8S_{11})[b], Barunissa ($S_{35}S_{51}$), Belardino (S_2S_{11}), Bertina (S_6S_{11}), Bianculidda di Pezzino ($S_{18}S_{51}$)[c], Biota (S_5S_{13}), Boa Costa (S_8S_{21}), Bonita de São Brás (S_8S_{22}), Carrion (S_5S_{14}), Castilla (S_6S_{22}), Cavalera ($S_{10}S_{31}$), Chiatta ($S_{13}S_{38}$), Colossal (S_7S_{11}), Cupani Piccola (S_7S_{51}), Don Filippo ($S_{21}S_{30}$), Fascionello ($S_{18}S_{52}$)[c], Fina del Alto ($S_{28}S_{29}$), Fournat de Brezenaud ($S_{24}S_{27}$), Fura Saco (S_4S_{23}), G-11 ($S_{14}S_{22}$), G-2 (S_5S_{56})[b], G-11 ($S_{14}S_{22}$)[b], Gabaix ($S_{10}S_{24}$), Gr-16 (S_2S_{60})[b], Haj Mirzaei (S_4S_9)[b], Haji Badam (S_9S_{46}), Hajmirzaei (S_9S_{23}), Harriot (S_6S_{14}), Holouei ($S_{13}S_{55}$)[b], ITAP-1 ($S_{fa}S_{11}$), Jiménez Salazar ($S_{21}S_{26}$), Jordi (S_5S_6), K-10-11 (S_9S_{24})[b], Kerman-1 ($S_{22}S_{24}$)[b], Kerman-19 ($S_{11}S_{23}$)[b], Kerman-5 (S_4S_{21})[b], Khorshidi (S_4S_8)[b], La Mona ($S_{23}S_{25}$), Liso ($S_{10}S_{23}$), Mamaei ($S_{61}S_{63}$)[b], Marcona ($S_{11}S_{12}$), Marcona de San Joy ($S_{22}S_{27}$), Marie Dupuy (S_6S_{39}), Mashhad-13 ($S_{12}S_{24}$)[b], Mashhad-40 ($S_{57}S_{59}$)[b], Mashhad-8 (S_4S_{27})[b], Mashhad-91 (S_2S_{24})[b], Menut ($S_{10}S_{13}$), Moldavskyi 810 (S_6S_{28}), Moldavskyi 812 ($S_{27}S_{31H}$), Mollar (S_8S_{24}), Mollar de la Princesa ($S_{24}S_{53}$), Monagha-dirgol ($S_{54}S_{60}$)[b], Mullisa Piccola ($S_{10}S_{11}$), Nerone (S_6S_{18})[c], Nivera Manza (S_2S_5), Óriás Kagyló (4/3) (S_6S_{25}), Padre (S_1S_{18}), Padre Santo (S_3S_{10}), Pané-Barquets (S_1S_{34}), Parque Samá (S_1S_{35}), Piatta Mollisa (S_6S_{27}), Pizzuta d'Avola ($S_{18}S_{23}$)[c], Planeta de las Garrigues ($S_{22}S_{35}$), Ponç ($S_{fa}S_{27}$), Poostnazok ($S_{55}S_{59}$)[b], Pou d'Establiments ($S_{12}S_{33}$), Pozdnyi ($S_{24}S_{39}$), Primorskyi (S_5S_9), Rabie (S_7S_{27})[b], Ramillete (S_6S_{23}), Redonda de Palma (S_3S_{25}), Retsou (S_2S_3), Rizsangi (S_2S_{13}), Rof**(S_5S_{23}), Rumbeta ($S_{11}S_{21}$), Scumissa (S_1S_{25}), Shahrekord-Ar ($S_{56}S_{63}$)[b], Shahrekord-G2 ($S_{10}S_{56}$)[b], Shahrodi-21 ($S_{13}S_{24}$), Sher Badam ($S_{13}S_{45}$), Shiraz-46 ($S_{11}S_{24}$)[b], Shirbadam (S_2S_4)[b], Somerton (S_1S_{23}), Szigetcsépi 55 (52/18) ($S_{28}S_{38}$), Szigetcsépi 92 ($S_{22}S_{36}$), Tejada-2 (S_1S_{22}), Tejari ($S_{24}S_{54}$)[b], Tendra Amarga ($S_{21}S_{23}$), Tétényi Bőtermő (S_8S_{31H}), Tétényi Keményhéjú (S_6S_{13}), Tío Martín ($S_{23}S_{27}$), Titan (S_8S_{14}), Totsol (S_8S_{31}), Verruga (S_5S_{10}), Vinoslivyi ($S_{11}S_{36}$), Vivot ($S_{fa}S_{23}$), Winters (S_1S_{14}), Yazd-15 ($S_{13}S_{63}$)[b], Yazd-17 ($S_{57}S_{58}$)[b], Yazd-21 (S_6S_{57})[b], Yosemite (S_8S_{10}), Zanjan-1 ($S_{24}S_{63}$)[b]	

[a]Underlined cultivars are those genotyped in this work.
[b]Cultivars genotyped by Mousavi et al. (E. Ortega, personal communication).
[c]Cultivars genotyped by Curró et al. (2015) in which the S_g allele has been replaced by its synonymous S_{18}.
*CIGs marked with an asterisk are those established in the present work.
**Male sterile cultivar. Thus, unlike the other cultivars included in group O, it should not be considered as a universal pollinator.