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DNA-based *S*-genotyping of Japanese Plum and Pluot Cultivars to Clarify Incompatibility Relationships

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Abstract. **Diploid japanese plum (*Prunus salicina* Lindl.) cultivars are commonly self-incompatible. To date, 14 incompatibility alleles (*S*-alleles) have been identified and labeled with alphabetical (S_a-S_n) and 5 with numeric codes (S_1, S_3-S_6). We applied polymerase chain reaction amplification of the *S*-RNase alleles with degenerate and allele-specific primers in 10 japanese plum cultivars and two pluots of unknown incompatibility alleles. Besides DNA sequencing, an additional method for the exact length determination of the first intron region was used for the first time for *S*-genotype japanese plums. The S_3-allele was shown to correspond to S_k in the alphabetic nomenclature, S_4 to S_c, S_5 to S_e, and S_6 to S_f. The S_5-allele-specific primer can be used as a reliable marker for self-compatibility in japanese plum. 'Black Amber', 'October Sun', 'TC Sun', and 'Super Giant' share the S_bS_c genotype, which was confirmed by test crosses. These cultivars belong to the widest incompatibility group currently known in japanese plum. An additional incompatibility group (S_cS_h) was established, including 'Green Sun' and 'Queen Rosa', a cultivar formerly known as a universal donor. By incorporating all previous and recent results, a table was assembled including 49 cultivars assigned to I–VII incompatibility groups, to the self-compatible group and to the group O of unique genotypes. These data may considerably contribute to further growing and breeding activities.**

Self-incompatibility in the Rosaceae L. family is of the gametophytic type based on pistil *S*-ribonucleases (*S*-RNases) controlled by the highly polymorphic *S*-locus (de Nettancourt, 2001). If two different cultivars share identical *S*-genotypes, it presents an incompatible combination in each direction, i.e., pollen tubes are arrested in the middle part of the stylar tissue.

Because *Prunus* L. species are unable to bear fruit parthenocarpically and adequate fertilization is crucial to fruit set, the self-incompatibility genotypes of self-incompatible (SI) tree crops have been studied intensively. Several *S*-alleles and incompatibility groups were described on the basis of molecular studies in sweet cherry (*Prunus avium* L.) (Boškovic and Tobutt, 2001; Sonneveld et al., 2003; Tao et al., 1999), almond [*P. dulcis* (Mill.) D.A. Webb.] (Boškovic et al., 2003; Sánchez-Pérez et al., 2004; Tamura et al., 2000), apricots (*P. armeniaca* L. and *P. mume* Sieb. et Zucc.) (Halász et al., 2005; Yaegaki et al., 2001), and plums (Sutherland et al., 2004a).

Most commercial cultivars of japanese plum (*Prunus salicina*) are self-incompatible (Nyéki and Szabó, 1995; Ontivero et al., 2006; Sansavini et al., 1981). The first *S*-genotype (S_aS_b) of a japanese plum cultivar ('Sordum') was described by Yamane et al. (1999). Later, Beppu et al. (2002, 2003) demonstrated the diversity of *S*-haplotypes in japanese plum by molecular cloning of genomic DNAs and cDNAs with primers designed from the conserved sequences of rosaceous *S*-RNases (Tao et al., 1999; Yamane et al., 2001). They identified 14 different *S*-alleles (S_a-S_n) and found that *S*-RNase genes of japanese plum also contained two introns at the same sites as those of other *Prunus* species (Igic and Kohn, 2001). Both introns varied in size in an *S*-haplotype-specific manner.

Sapir et al. (2004) cloned five additional *S*-alleles from three commercially important japanese plum cultivars and labeled them with numeric codes (S_1, S_3–S_6). Four of the five clones were described as new alleles (S_3–S_6) and S_1 from 'Red Beaut' was shown to correspond to the S_a-allele identified by Yamane et al. (1999). Allele-specific primers were designed and used to analyze compatibility relationships among five cultivars.

The aim of this study was to identify self-incompatibility alleles in commercially significant plum and pluot cultivars with unknown incompatibility genotypes using polymerase chain reaction (PCR), DNA sequencing, or precise length determination of the PCR amplification products as well as to establish new and to clarify previously described incompatibility groups in this species.

Materials and Methods

Plant materials. Ten cultivars of japanese plum were analyzed ('Black Amber', 'Friar', 'Green Sun', 'October Sun, 'Santa Rosa', 'Shiro', 'Angeleno', 'Sweet Autumn', 'TC Sun' and 'Super Giant') from the orchard at Derecske, Hungary and two pluots—interspecific hybrids between *Prunus salicina* Lindl. and *P. armeniaca* L.—('Flavor Grenade' and 'Flavor King') from an orchard at Siófok, Hungary.

DNA extraction. Genomic DNA was extracted from fully expanded young leaves using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). DNA concentrations were measured using a spectrophotometer GeneQuant II RNA/DNA Calculator (Pharmacia Biotech., Budapest, Hungary).

S-Polymerase chain reaction analyses. PCR was conducted using the degenerate primers EM-PC2consFD and EM-PC3consRD for the amplification of the second intron region according to Sutherland et al. (2004b). To amplify the first intron, the fluorescently labeled forward primer PaConsI-F (Ortega et al., 2005; Sonneveld et al., 2003) was used in combination with the reverse primer EM-PC1consRD (Ortega et al., 2005). Allele-specific PCR primers were used to selectively detect the S_1-(IB2 and A6), S_3-(IW5 and A5), S_4-(IZ1 and IZ4), S_5-(IZ2 and IZ5), and S_6-alleles (PruC2

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and IW2) (Sapir et al., 2004; Tao et al., 2000; Yamane et al., 1999). PCR was carried out in a PTC 200 thermocycler (MJ Research, Budapest, Hungary) using the programs described for the consensus (Ortega et al., 2005; Sutherland et al., 2004b) and allele-specific primers (Sapir et al., 2004). Approximately 20 to 80 ng of genomic DNA was used for PCR amplification in a 25 µL reaction volume containing 1 × PCR buffer (Sigma, Budapest, Hungary) with the final concentrations of 10 mm Tris-HCl (pH 8.3), 50 mm KCl, 1.5 mm $MgCl_2$, 0.2 mm of dNTPs, 0.4 µm of the adequate primers, and 0.625 U of *Taq* DNA polymerase (Sigma). PCR products were separated by electrophoresis in 2% TAE agarose gels for 2 h at 100 V and DNA bands were visualized by ethidium bromide staining. Fragment lengths were estimated by comparison with the 1-kb DNA ladder (Promega, Madison, Wis.). To determine the exact size of the first intron region products under 500 bp, fluorescently labeled amplicons were run in an automated sequencer ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Budapest, Hungary).

Cloning, sequencing, and analyses of DNA sequences. Some PCR products were cloned into a pGEM-T Easy vector (Promega) and sequenced with the previously described sequencer. For each allele, the nucleotide sequences of three clones were determined in both directions. DNA sequences were submitted to the GenBank/EMBL/DDBJ database under accession numbers DQ790372 (second intron region of S_k-RNase from 'Friar'), DQ790373 (second intron region of S_h-RNase from 'Green Sun'), DQ790374 (second intron region of S_h-RNase from 'Friar'), DQ790375 (first intron region of S_b-RNase from 'Black Amber'), and DQ790376 (first intron region of S_c-RNase from 'Black Amber'). Homology searches were performed using the BLASTN program at NCBI (Altschul et al., 1990), and partial S_h- and S_c-RNase sequences were aligned manually.

Crosspollination test. Crosses between cultivars with the putatively same *S*-genotype ('Black Amber' × 'TC Sun', 'Black Amber' × 'Super Giant', 'Super Giant' × 'TC Sun', 'October Sun' × 'Super Giant') were carried out in an orchard at Derecske, Hungary, in 2006. Flowers were emasculated just before anthesis and then the previously collected and dried pollens were transferred to the stigmas. Open flowers and late buds were removed to prevent self- and crosspollination. After 6 weeks, percentage of fruit set was determined.

Results

All analyzed cultivars showed two different fragments on agarose gels except 'Shiro' after the amplification with consensus primers for the second intron (Fig. 1). Six fragments of different lengths were obtained from 12 cultivars; fragment lengths could be determined by comparing them with the 1-kb DNA ladder (Table 1). Two different fragment lengths exactly sized with an automated sequencer could also be obtained in each tested plum accession. Seven different fragment sizes were described (Table 1).

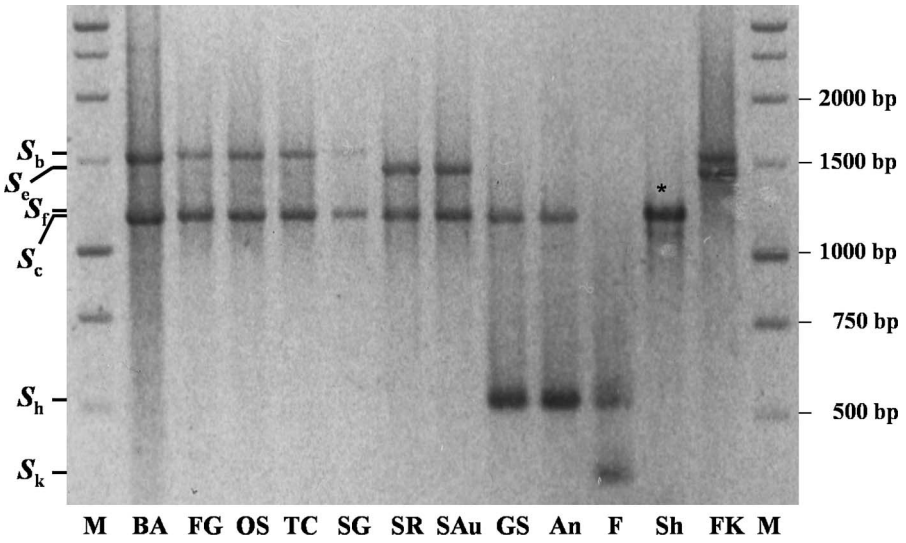

Fig. 1. *S*-RNase gene-specific polymerase chain reaction for 12 cultivars using the EM-PC2consFD and EM-PC3consRD consensus primers to amplify the second intron region of the gene. M: 1-kb ladder, BA: 'Black Amber' (proposed genotype: S_bS_c), FG: 'Flavor Grenade' (S_bS_e), OS: 'October Sun' (S_bS_c), TC: 'TC Sun' (S_bS_c), SG: 'Super Giant' (S_bS_c), SR: 'Santa Rosa' (S_cS_e), SAu: 'Sweet Autumn' (S_cS_e), GS: 'Green Sun' (S_cS_h), An: 'Angeleno' (S_cS_h), F: 'Friar' (S_hS_k), Sh: 'Shiro' (S_f–), FK: 'Flavor King' (S_bS_e). *Combined bands.

Five SI cultivars ('Black Amber', 'October Sun', 'TC Sun', 'Super Giant', and 'Flavor Grenade') had identical fragment sizes for the second (≈1550 and ≈1200 bp) and the first (343 and 367 bp) intron regions. Therefore, fruit set evaluation was carried out after controlled pollinations between some of those cultivars. Crosspollination among the tested cultivars has not resulted in fruit set or the percentage of the fruit set was negligible (Table 2). Two other SI cultivars, 'Green Sun' and 'Angeleno', possessed equally sized fragments for the first (343 and 388 bp) and second (≈1200 and ≈550 bp) intron regions. Two self-compatible (SC) cultivars, 'Santa Rosa' and 'Sweet Autumn', also shared an identical pattern with 343- and 372-bp-long fragments for the first intron region and ≈1200- and ≈1450-bp-long fragments for the second intron.

Allele-specific primers designed by Sapir et al. (2004) for the S_4-allele amplified a fragment of the relevant size in 7 SI and 2 SC cultivars (Fig. 2A). Primers specific for the S_5-allele resulted in successful amplification only in three SC cultivars ('Santa Rosa', 'Sweet Autumn', and 'Flavor King') (Fig. 2B). We could not detect any PCR product with primers for the S_1-allele; however, primers for S_3 worked in the cultivar Friar and primers for the S_6 in 'Shiro' (data not shown). This cultivar showed only one thick band in the agarose gel when the fragments PCR amplified with consensus primers were detected (Fig. 1), although first intron amplification resulted in two differently sized fragments (295 and 327 bp).

Genomic DNA fragments amplified by the EM-PC2consFD and EM-PC3consRD primers (Sutherland et al., 2004b) from 'Green Sun' and 'Friar' as well as fragments amplified by the PaConsI-F (Sonneveld et al., 2003) and EM-PC1consRD (Ortega et al., 2005) primer set from 'Black Amber' were cloned and sequenced. BLAST homology searches revealed that 'Green Sun' and 'Friar' carried the S_h-allele (Fig. 3A). The other allele cloned from the cultivar 'Friar' was clarified to be identical with the S_k-allele (data not shown). Sequence analysis of 'Black Amber' confirmed that this cultivar carried the S_b- (data not shown) and S_c-alleles (Fig. 3B) following the nomenclature established by Beppu et al. (2002, 2003). Alignments revealed that the identity was 100% between the partial sequence of the S_k-allele cloned from 'Friar' and that retrieved from the GenBank database (sequenced from 'Stark Gold') or between the S_b cloned from 'Black Amber' and that found in the GenBank database ('Sordum') (data not shown). In 'Friar' S_h- and 'Black Amber' S_c-alleles, single nucleotide polymorphisms (SNP) were

Table 1. Sizes of the polymerase chain reaction amplification products for the first and second intron regions (bp) and correspondences between the two previously established allele nomenclatures.

Allele[z]	Allele[y]	First intron	Second intron
S_b	–	367	1550
S_c	S_4	343	1200
S_e	S_5	372	1450
S_f	S_6	295	1250
S_h	–	388	550
S_k	S_3	382	350

[z]According to the allele nomenclature described by Beppu et al. (2002).
[y]According to the allele nomenclature described by Sapir et al. (2004). S_1 was previously shown to correspond to S_a and S_2 was omitted from the original allele series established by Sapir et al. (2004).

Table 2. Test crosses made at Derecske, Hungary, in 2006 to confirm cross-incompatible genotypes.

Parents			No. of pollinated flowers	Fruit set (%)
Male	×	Female		
Black Amber	×	TC Sun	83	0
Black Amber	×	Super Giant	86	0
October Sun	×	Super Giant	110	0.9
Super Giant	×	TC Sun	171	0

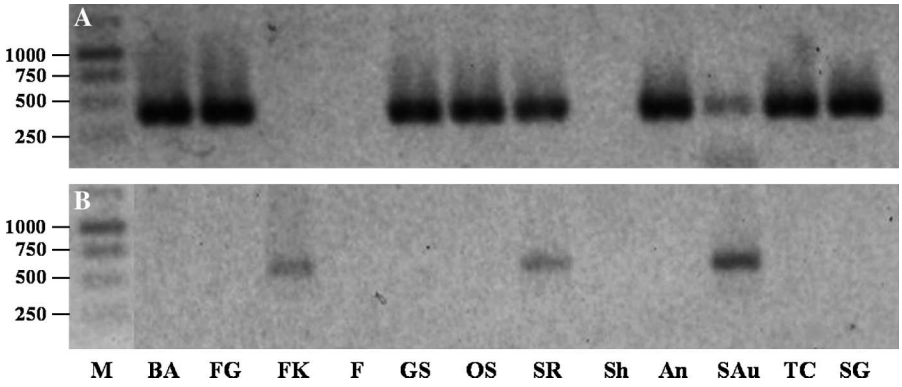

Fig. 2. *S*-polymerase chain reaction analysis using allele-specific primers. (**A**) IZ1 + IZ4 for amplifying S_4- and (**B**) IZ2 + IZ5 for detecting S_5-allele. Codes for samples are the same as in Figure 1.

detected; however, all SNPs occurred within the intron regions (Fig. 3A, B).

The genotypes proposed for all 12 cultivars analyzed in this study and the 37 analyzed previously are arranged in Table 3 following the practice in sweet cherry. Cultivars could be assigned to seven incompatibility groups, each of them containing two to eight cultivars. Nine self-compatible cultivars and 12 cultivars in the group O of unique genotypes can be widely used as universal pollen donors.

Discussion

Consensus and allele-specific PCR amplification of the *S*-RNase gene fragments has already proved to be an effective and rapid method for identifying *S*-genotypes in cherry (Sonneveld et al., 2003), almond (Ortega et al., 2005; Tamura et al., 2000), japanese apricot (Tao et al., 2002; Yaegaki et al., 2001), apricot (Halász et al., 2005), peach [*P. persica* (L.) Batsch] (Hegedűs et al., in press), and japanese plum (Beppu et al., 2002, 2003; Sapir et al., 2004). In this study, we could identify five different *S*-genotypes in japanese plum and pluot cultivars not studied earlier and possessing high economic value both in the U.S. and in the Mediterranean region (Bassi and Pirazzoli, 1998; Faust and Surányi, 1999). This is the first study in which detecting *S*-alleles with a degenerate primer set amplifying the second intron region of the *Prunus S*-RNase gene (Sutherland et al., 2004b) was successful in *S*-genotyping japanese plum cultivars, and also the precise fragment length determination method elaborated by Ortega et al. (2005) was adopted to

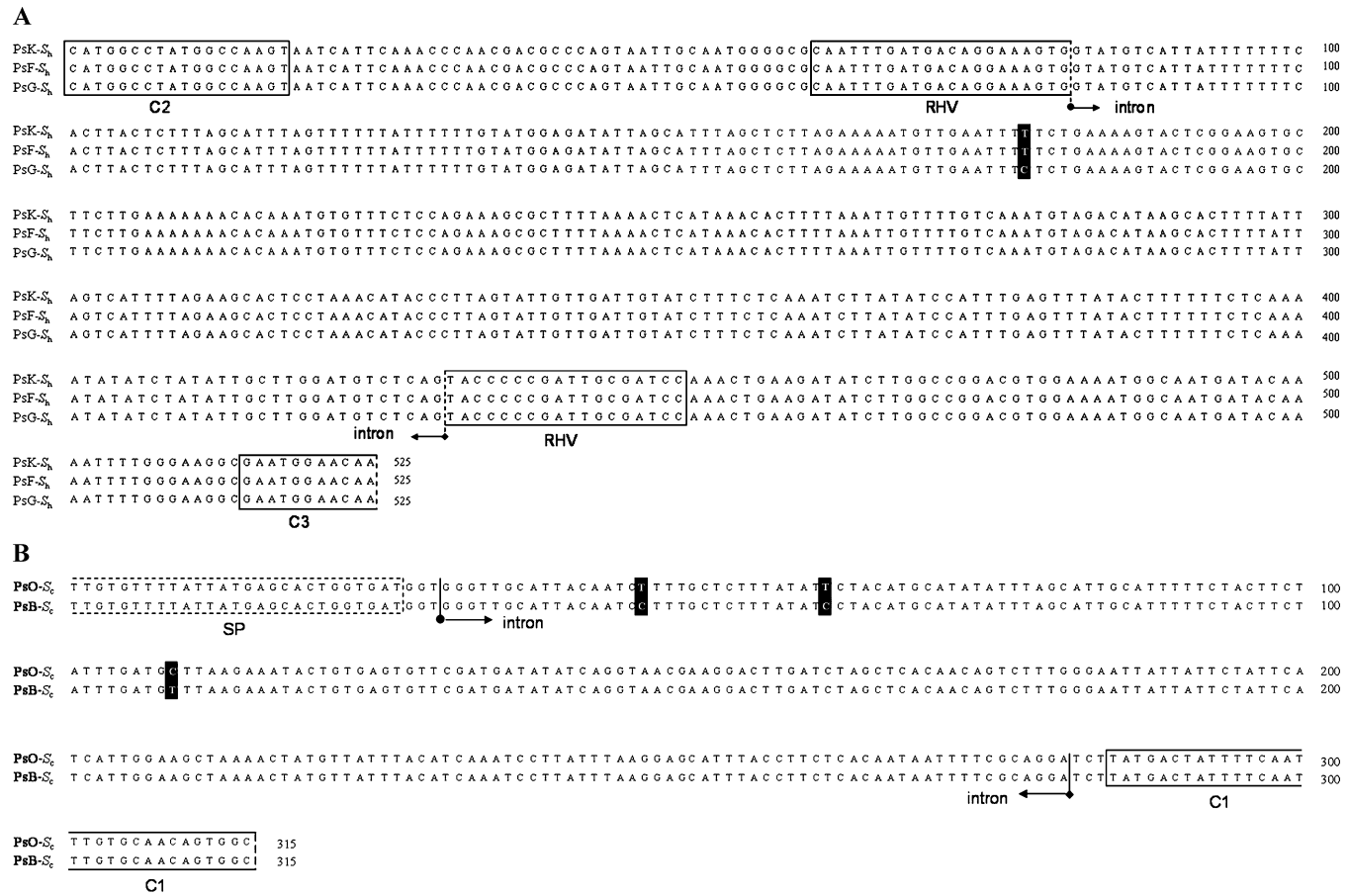

Fig. 3. Alignment of nucleotide sequences of the second intron region from *P. salicina* 'Kelsey' (PsK-S_h, accession no. AB084148), 'Friar' (PsF-S_h, DQ790374), and 'Green Sun' (PsG-S_h, DQ790373) S_h-alleles (**A**) as well as the first intron region from *P. salicina* 'Oishiwasesumomo' (PsO-S_c, AB084144) and 'Black Amber' (PsB-S_c, DQ790376) S_c-alleles (**B**). Black boxes indicate different residues between the aligned sequences. The conserved regions (C1, C2, and C3) and the hypervariable region (RHV) are boxed as indicated by Ushijima et al. (1998) and dashed box shows the predicted signal peptide sequence (SP) as determined by Tao et al. (1999). Speckle and diamond denote the starting and ending borders of the deduced intron sequences, respectively.

Table 3. *S*-genotypes and predicted incompatibility groups of Japanese plum cultivars assembled by the incorporation of former and recent results.

Group	Cultivar	*S*-genotype	Reference
Self-compatible	Beauty	S_cS_e	Beppu et al. (2002)
	Black Diamond	S_e—[z]	Sapir et al. (2004)
	Flavor King	S_bS_e	Studied in this work
	Late Santa Rosa	S_cS_e	Beppu et al. (2002)
	Royal-Zee	S_cS_e[z]	Sapir et al. (2004)
	Santa Rosa	S_cS_e	Beppu et al. (2002) and this work
	Rio	S_aS_e	Beppu et al. (2002)
	Simka	S_eS_k	Beppu et al. (2003)
	Sweet Autumn	S_cS_e	Studied in this work
Group I	Burmosa	S_aS_b	Beppu et al. (2003)
	Red Beaut	S_aS_b	Beppu et al. (2002)
	Sordum	S_aS_b	Yamane et al. (1999)
Group II	Laroda	S_bS_c	Beppu et al. (2002)
	Oishinakata	S_bS_c	Beppu et al. (2002)
	Taiyo	S_bS_c	Beppu et al. (2002)
	Black Amber	S_bS_c	Studied in this work
	Flavor Grenade[y]	S_bS_c	Studied in this work
	October Sun	S_bS_c	Studied in this work
	TC Sun	S_bS_c	Studied in this work
	Super Giant	S_bS_c	Studied in this work
Group III	Frontier	S_bS_f	Beppu et al. (2003)
	Gran Colle	S_bS_f	Beppu et al. (2003)
	Verna Delicious	S_bS_f	Beppu et al. (2003)
Group IV	Queen Anne	S_bS_h	Beppu et al. (2002)
	Yonemomo	S_bS_h	Beppu et al. (2002)
Group V	Bakemonosumomo	S_bS_i	Beppu et al. (2002)
	Kasahara Hatankyou	S_bS_i	Beppu et al. (2003)
Group VI	Abandancia	S_fS_h	Beppu et al. (2003)
	Kelsey	S_fS_h	Beppu et al. (2002)
	Kelsey Paulista	S_fS_h	Beppu et al. (2003)
Group VII	Angeleno[y]	S_cS_h	Studied in this work
	Green Sun	S_cS_h	Studied in this work
	Queen Rosa	S_cS_h	Beppu et al. (2003)
Group O—unique genotypes	Bonnie	S_gS_h	Beppu et al. (2003)
	Botan	S_aS_m	Beppu et al. (2003)
	Combination	S_gS_l	Beppu et al. (2003)
	Formosa	S_bS_d	Beppu et al. (2002)
	Friar	S_hS_k	Studied in this work
	Harypickstone	S_bS_k	Beppu et al. (2003)
	Honey Rosa	S_bS_g	Beppu et al. (2003)
	Lantz	S_bS_l	Beppu et al. (2003)
	Oishiwasesumomo	S_cS_d	Beppu et al. (2002)
	Shiro	S_f—[z]	Studied in this work
	Starkgold	S_gS_k	Beppu et al. (2003)
	Summer Queen	S_cS_f	Beppu et al. (2003)
	Tecumseh	S_fS_j	Beppu et al. (2003)
	Terada	S_aS_f	Beppu et al. (2002)
	White plum	S_fS_g	Beppu et al. (2002)
	Wickson	S_kS_f[z]	Sapir et al. (2004)

[z]Translated from numeric into alphabetical allele nomenclature.
[y]Requires further confirmation.

improve discrimination between the first intron amplification products, which are characterized by restricted size variability.

Because the degenerate consensus primer sets amplified both *S*-alleles carried by the tested cultivars, they can be proposed for further *S*-genotyping studies not only in cherry, almond, and apricot, but also in japanese plum. The success of these robust primers may partly be because two *P. salicina* alleles were also used for primer designing (Sutherland et al., 2004b).

The S_e-haplotype was shown to be responsible for the self-compatibility trait (Beppu et al., 2002, 2005). We can hypothesize that the fragments of 372 (first intron) and ≈1450 bp (second intron) lengths correspond to the S_e-allele because they are present in all SC cultivars, whereas the allele indicated by the 343-bp- and ≈1200-bp-long fragments of 'Santa Rosa' also appears in seven SI cultivars. Because *S*-genotype of 'Santa Rosa' was determined as S_cS_e by Beppu et al. (2002), this fragment putatively corresponds to the S_c-allele.

Besides the 14 alphabetically labeled *S*-alleles (Beppu et al., 2002, 2003; Yamane et al., 1999), five additional alleles were isolated and labeled with numeric codes and four of them were considered to be new alleles (Sapir et al., 2004). Allele-specific primers for all five alleles were designed (Sapir et al., 2004), which were also applied in this study to reveal correspondences between the two nomenclatures. Allele-specific primers confirmed that the S_4-allele corresponded to S_c and S_5 to S_e. Because both primers worked in 'Santa Rosa', its previously identified S_cS_e genotype should be translated into S_4S_5 in the numeric label system. Furthermore, the S_5-allele-specific primer can be used as a reliable marker for determining self-compatibility in japanese plum accessions.

The only band detected after the second intron amplification of 'Shiro' may be the result of alleles with very similar intron sizes. The presence of two different alleles as expected under gametophytic self-incompatibility was clarified by the first intron amplification results. One of these alleles is the S_6, as confirmed by the relevant allele-specific primer. BLAST searches revealed that partial sequence of the S_6-allele was identical to S_f. By DNA sequencing and BLAST searches, the *S*-genotype for 'Friar' was determined to be S_hS_k, a haplotype combination, which has not been detected in any other cultivar, thereby 'Friar' may be considered as a universal pollen donor because their pollen should be fully or semicompatible on all cultivars of currently known genotypes. Allele S_k was clarified to be identical with S_3 (Table 1) using allele-specific primers (Sapir et al., 2004) and DNA sequence alignment. Alignments of the partial sequences of S_h- and S_c-RNase alleles have revealed one and three SNPs, respectively, in the intron regions, which may be the result of the more rapid mutation rate of introns (Sutherland et al., 2004a). However, these changes will not influence the function of the coding regions, because it was evident from the failure in fruit set after crosspollination (Table 2) between some of the group II cultivars carrying the S_c-allele with three SNPs in their first intron (Fig. 3B).

We have found two incompatible groups among the analyzed cultivars; one of them consists of 'Black Amber', 'October Sun', 'TC Sun', and 'Super Giant'. Their mutual incompatibility was confirmed by test crosses. DNA sequencing of their first intron region and BLAST searches allowed us to determine their genotype as S_bS_c similarly to 'Laroda', 'Oishinakata', and 'Taiyo' genotyped by Beppu et al. (2003). These seven cultivars form the widest incompatibility group presently known in japanese plum. Furthermore, PCR results (Fig. 1; Table 1) suggest that 'Flavor Grenade', a pluot cultivar also belongs to this group, but it requires confirmation. This would be interesting as pluots with interspecific origin have more complex genetic background, although with predominantly plum parentage (Ahmad et al., 2004).

The second incompatibility group identified in the present study contains 'Green Sun' and 'Queen Rosa' (S_cS_h), which was previously considered as a unique genotype (Beppu et al., 2003). The second intron region of the S_h-RNase allele containing the hypervariable region was sequenced from the cultivar 'Green Sun,' which together with the use of allele-specific primers clarified that this cultivar also had the S_cS_h genotype. The inclusion of 'Angeleno' to this incompatibility

group seems possible on the basis of its PCR analysis with consensus primers (Fig. 1; Table 1); however, for there to be no doubts, clarification from test crosses or sequence analyses are required. Because the S_c-allele is shared between the two identified incompatibility groups, cultivars belonging to these groups are semicompatible with each other. Semicompatibility is also of major horticultural significance, because cultivars may be low-potency pollinizers for each other as was described in the case of apple (Goldway et al., 1999).

All five numerically labeled alleles were shown to be identical with some of the alleles initially described and labeled with alphabetic codes; hence, until now, only 14 alleles could have been identified in japanese plum cultivars indicating a quite restricted allele pool as compared with apple, almond, sweet cherry, or even apricot (Hegedűs et al., in press). It suggests that further alleles are expected to be identified as putatively as the one in 'Shiro' other than the S_f.

Table 3 shows the genotypes not only of cultivars analyzed in this article, but also of those genotyped by Beppu et al. (2002, 2003), Sapir et al. (2004), and Yamane et al. (1999). This is the first study to assign these cultivars to incompatibility groups presenting a table similar to those available in an updated form for cherry (Boškovic and Tobutt, 2001) and almond cultivars (Boškovic et al., 2003). By incorporating all known data, Table 3 consists of 49 cultivars arranged to I–VII incompatibility groups to the group O encompassing cultivars of unique genotypes, which can be considered as ''universal pollen donors,'' and an additional group accumulating all SC cultivars. This information may provide valuable guidelines for horticulturists to design successful combinations for breeding programs and to aid cultivar association in plum orchards all over the world.

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