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PCR-based method for identifying the *S*-genotypes of Japanese pear cultivars

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Abstract Japanese pear (*Pyrus pyrifolia* Nakai), a member of the Rosaceae, shows gametophytic self-incompatibility that is controlled by the *S*-locus. The *S*-genotype of Japanese pear cultivars is an important factor for crossing and breeding. We report a rapid reliable method to identify these *S*-genotypes. It consists of PCR amplification of the S-RNase gene from genomic DNA and subsequent digestion of the PCR fragments with *S*-allele-specific restriction endonucleases. Using this method, we determined the unknown *S*-genotypes of nine Japanese pear cultivars and selected self-compatible varieties from the offspring of the self-compatible cultivar, 'Osa-Nijisseiki'.

Key words Japanese pear · PCR · Rosaceae · *S*-alleles · Self-incompatibility

Introduction

Japanese pear (*Pyrus pyrifolia* Nakai; Rosaceae) is self-incompatible and requires artificial cross-pollination to set fruit. Classical crossing experiments have shown that Japanese pear has gametophytic self-incompatibility (GSI) controlled by a single multiallelic locus, the *S*-locus (Kikuchi 1929), which has seven *S*-alleles (S_1–S_7) (Terami et al. 1946). In GSI, fertilization is inhibited when the *S*-allele of the pollen matches one of the two *S*-alleles of the pistil.

Japanese pear is an important commercial fruit tree crop in Japan, and breeders have been challenged to develop a new disease-resistant cultivar that has high quality fruit (Kajiura and Sato 1990). They have also been trying to breed a self-compatible cultivar with excellent fruit in order to cut down on artificial pollination, which requires much labor and many workers. To carry out these experiments they have to know the *S*-genotypes of the male and female parents as well as those of their offspring. It takes several years to determine the *S*-genotypes using crossing experiments, and the results obtained are sometimes ambiguous because of the effects of many environmental and physiological factors. Another problem is that the self-incompatibility phenomenon is not clear in Japanese pear. In fact, following pollination, more than 70% of the fructification is defined as 'compatible' and less than 30% as 'incompatible' in crossing experiments.

To overcome these drawbacks of crossing, various molecular biological methods have been devised to identify the *S*-genotypes. We established a method for identifying the *S*-genotype of Japanese pear cultivars that uses two-dimensional polyacrylamide-gel electrophoresis (2D-PAGE) (Ishimizu et al. 1996) and identified the *S*-genotype of the cultivar 'Hosui' as S_3S_5. 'Hosui' is often used as a parent when breeding a new cultivar, but its *S*-genotype could not be determined by crossing experiments (Ishimizu et al. 1998 a). The 2D-PAGE method is rapid (3 days) and reliable, but has not become widespread among Japanese pear breeders because the manipulation required is very complicated and involves great skill.

Recently, the nucleotide sequences of cDNAs encoding seven S-RNases of Japanese pear and of the introns inserted in their coding regions were determined

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(Norioka et al. 1995; Ishimizu et al. 1998 b; Ishimizu et al., unpublished results). Seven cDNAs of apple (*Malus* × *domestica* Borkh.), a member of the Rosaceae, have also been cloned and sequenced (Broothaerts et al. 1995; Janssens et al. 1995; Sassa et al. 1996). Their nucleotide sequences were found to be closely related to those of the Japanese pear (Norioka et al. 1996; Sassa et al. 1996; Ishimizu et al. 1998 b). Alignment of the amino-acid sequences showed some conserved regions as well as a hypervariable (HV) region that has highly diversified amino-acid and nucleotide sequences (Ishimizu et al. 1998 b). The intron inserted within the HV region also shows considerable diversification (Ishimizu et al., unpublished results).

We have designed a new method for identifying the *S*-alleles of Japanese pear that is based on the variations in the HV region and its intron. It consists of a polymerase chain reaction (PCR) and *S*-allele-specific digestion of PCR-amplified fragments with restriction endonucleases. Using this method, we determined the *S*-genotypes of nine Japanese pear cultivars and selected self-compatible varieties from the offspring of the self-compatible cultivar 'Osa-Nijisseiki' ($S_2S_4^{sm}$; sm = stylar-part mutant in the S_4-locus).

Materials and methods

Plant materials

Young leaves of Japanese pear cultivars were collected in the spring of 1997 at the Tottori Horticultural Experiment Station and the Fruit Tree Research Station, Ministry of Agriculture, Forestry and Fisheries of Japan. The leaves were rapidly frozen in liquid nitrogen and stored at −170°C until used.

Isolation of genomic DNA

Young leaves (0.1 g) of each test cultivar were ground in liquid nitrogen using a mortar and a pestle. Genomic DNA was extracted and purified with an ISOPLANT DNA isolation kit (Nippon gene) according to the manufacturer's instructions with a slight modification. In this study, 2-mercaptoethanol and polyclar-AT (polyvinylpyrrolidone, GAF Chemicals Co.) were added to the extraction buffer of the kit at the respective final concentrations of 5% and 3%.

PCR amplification

Synthesis of the oligonucleotide primers for PCR amplification was based on the conserved nucleotide sequences, 'FTQQYQ' (TTTACGCAGCAATATCAG) and 'anti-IIWPNV' ($AC^A/_GTTCGGCCAAATAATT$), in the seven Japanese pear S-RNases (Norioka et al. 1995; Ishimizu et al. 1998 b) and seven apple S-RNases (Broothaerts et al. 1995; Janssens et al. 1995; Sassa et al. 1996). The PCR was conducted with an Expand high-fidelity PCR system (Boehringer Mannheim) according to the manufacturer's instructions using 50 ng of genomic DNA from the young leaves. The reaction was run in a GeneAmp PCR System (Perkin Elmer) programmed as follows: 1 cycle of 120 s at 94°C; 10 cycles of 15 s at 94°C, 30 s at 48°C, and 120 s at 70°C; 20 cycles of 15 s at 94°C, 30 s at 48°C, and 150 s at 70°C; 1 cycle of 420 s at 70°C. The PCR products were analyzed by PAGE (6% gel).

Digestion of PCR fragments with restriction endonucleases

The restriction endonucleases employed were purchased from Takara Shuzo (*Nde*I, *Hin*cII, and *Acc*II) and New England Biolabs (*Sfc*I, *Ppu*MI, and *Alw*NI). Each enzyme (1–5 U) and 10× the digestion buffer specific to each enzyme were added to the PCR reaction mixture, and the whole digested for 2 h at 37°C. The digests were analyzed by PAGE (7% gel).

Results

New method for identifying the *S*-genotype of Japanese pear cultivars

We designed a pair of primers based on the two consensus sequences, FTQQYQ and anti-IIWPNV. The expected sizes of the PCR fragments from each S-RNase gene are given in Table 1. When PCR amplification was conducted for the four cultivars ['Imamuraaki' (S_1S_6), 'Nijisseiki' (S_2S_4), 'Hosui' (S_3S_5), and Okusankichi' (S_5S_7)] whose *S*-alleles are known, DNA fragments of the expected sizes (Table 1) were produced, and no extra fragment was amplified (Fig. 1). The PCR fragments derived from the S_3- and S_5- alleles comigrated as a single band on this gel [see 'Hosui' (S_3S_5) in Fig. 1]. The amplified fragments were 350–370-bp long, an extremely long fragment of about 1400 bp being amplified in 'Nijisseiki' (Fig. 1). This unique fragment corresponds to the S_2-RNase gene which has

Table 1 Expected sizes of PCR fragments and their *S*-allele-specific restriction endonuclease digests (bp)

S-allele	PCR fragment	*Sfc*I (S_1 specific)	*Ppu*MI (S_3, S_5 specific)	*Nde*I (S_4 specific)	*Alw*NI (S_5 specific)	*Hin*cII (S_6 specific)	*Acc*II (S_6, S_7 specific)
S_1	367	135 and 232	367	367	367	367	367
S_2	1347	1347	1347	1347	1347	1347	1347
S_3	376	376	110 and 266	376	376	376	376
S_4	368	368	368	228 and 140	368	368	368
S_5	376	376	110 and 266	376	113 and 263	376	376
S_6	347	347	347	347	347	214 and 133	234 and 113
S_7	352	352	352	352	352	352	250 and 102

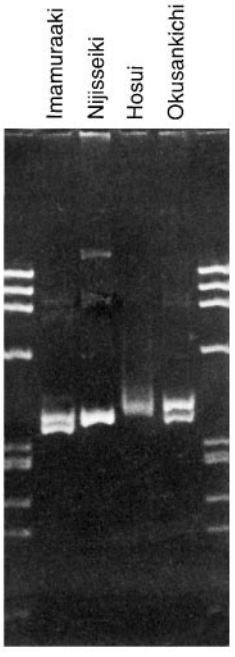

Fig. 1 Analysis of PCR fragments from four *S*-genotype-known Japanese pear cultivars: 'Imamuraaki' (S_1S_6), 'Nijisseiki' (S_2S_4), 'Hosui' (S_3S_5) and 'Okusankichi' (S_5S_7). The sizes of the molecular-marker bands on both sides are, from top to bottom 1353, 1078, 872, 603, 314, 281 and 234 bp. Fragments were electrophoresed in a 6% polyacrylamide gel

a longer intron (1153 bp) than the introns (about 170 bp) of the other six S-RNase genes (Ishimizu et al., unpublished results).

To assign the PCR fragments to their respective *S*-alleles, we searched for the *S*-allele-specific cleavage sites of the restriction endonucleases in their nucleotide sequences. The sites of *Sfc*I, *Nde*I, *Alw*NI and *Hin*cII were unique in the respective PCR fragments from the S_1-, S_4-, S_5- and S_6-alleles. The *Ppu*MI site was present only in the PCR products from the S_3- and S_5-alleles, and that of *Acc*II in the products from the S_6- and S_7-alleles. The expected fragment sizes after digestion with each restriction endonuclease are shown in Table 1. When the fragments amplified from the four known *S*-genotype cultivars were digested with each of the six *S*-allele-specific restriction endonucleases (Fig. 2), fragments whose sizes coincided with the expected ones (Table 1) were obtained. The procedures used to identify each *S*-allele (S_1–S_7) are described in detail below.

S_1-allele: a fragment of 367 bp is amplified by PCR then digested with *Sfc*I, producing 135- and 232-bp fragments [see 'Imamuraaki' (S_1S_6) in Figs. 1 and 2(a)].
S_2-allele: an extremely long fragment of 1347 bp is amplified by PCR but is not digested by any of the restriction enzymes employed [see 'Nijisseiki' (S_2S_4) in Figs. 1 and 2].
S_3-allele: a fragment of 376 bp is amplified by PCR then digested with *Ppu*MI, producing 110- and 266-bp fragments; it is not digested with *Alw*NI [see 'Hosui' (S_3S_5) in Figs. 1, 2(b), and 2(d)].
S_4-allele: a fragment of 368 bp is amplified by PCR then digested with *Nde*I, producing 140- and 228-bp fragments [see 'Nijisseiki' (S_2S_4) in Figs. 1 and 2 (c)].
S_5-allele: a fragment of 376 bp is amplified by PCR then digested with *Ppu*MI and *Alw*NI which respectively produce fragments of 110 and 266 bp, and of 113 and 263 bp [see 'Hosui' (S_3S_5) and 'Okusankichi' (S_5S_7) in Figs. 1, 2(b), and 2(d)].
S_6-allele: a fragment of 347 bp is amplified by PCR then digested with *Hin*cII and *Acc*II which respectively produce fragments of 133 and 214 bp, and of 113 and 234 bp [see 'Imamuraaki' (S_1S_6) in Figs. 1, 2(e), and 2(f)].
S_7-allele: a fragment of 352 bp is amplified by PCR then digested with *Acc*II, producing 102- and 250-bp fragments; it is not digested with *Hin*cII [see 'Okusankichi' (S_5S_7) in Figs. 1, 2(e), and 2 (f)].

If a cultivar has an *S*-allele other than the S_1--S_7-alleles, a PCR fragment whose electrophoretic behavior does not correspond to any of the seven *S*-alleles will be

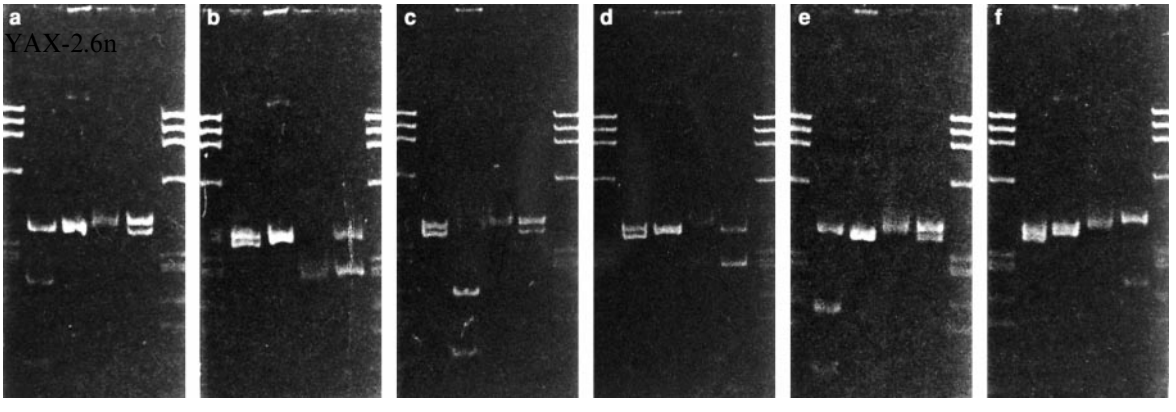

Fig. 2a–f Analyses of the PCR fragment digests with restriction endonucleases. The restriction endonucleases employed were (**a**) *Sfc*I, (**b**) *Ppu*MI, (**c**) *Nde*I, (**d**) *Alw*NI, (**e**) *Hin*cII, (**f**) *Acc*II. The lanes in each gel show, from left to right, the molecular marker, 'Imamuraaki', 'Nijisseiki', 'Hosui', 'Okusankichi', and the molecular marker. The band sizes of the molecular markers are, from top to bottom, 1353, 1078, 872, 603, 314, 281, 234, 194 and 118 bp. Fragments were electrophoresed in 7% polyacrylamide gels

detected. But we have to confirm the nucleotide sequence of the PCR fragment before we assign it to a new *S*-allele, because there is a possibility that a point mutation occurs only at the restriction-enzyme site and the other nucleotide sequence is unchanged.

Identification of the *S*-genotypes of Japanese pear cultivars by the PCR-based method

This method for identifying *S*-alleles was used on nine Japanese pear cultivars whose *S*-genotypes were unknown. Analysis findings for the PCR fragments from the nine cultivars are shown in Fig. 3. Fragments of about 360 bp are present in all of them. A fragment of about 1400 bp was produced only in the cultivar 'Yasato', indicative that it has the S_2-allele. Results for PCR fragments digested with restriction endonucleases are shown in Fig. 4. On the basis of the results shown in Figs. 3 and 4, we identified the *S*-genotypes of the nine cultivars (Table 2). Two *S*-alleles were identified for each cultivar, except 'Akibae' in which only the S_5-allele-specific fragment was detected. 'Akibae' is a self-compatible cultivar derived from 'Osa-Nijisseiki' ($S_2S_4^{sm}$). As the self-compatibility of 'Osa-Nijisseiki' is due to lack of the S_4-RNase gene in the S_4-locus (S_4^{sm}-locus) (Sassa et al. 1997), we concluded that 'Akibae'

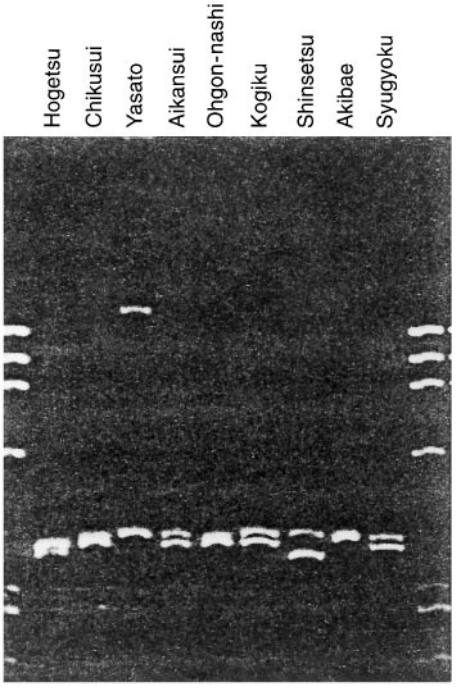

Fig. 3 Analysis of PCR fragments from nine Japanese pear cultivars whose *S*-genotypes are unknown: 'Hogetsu', 'Chikusui', 'Yasato', 'Aikansui', 'Ohgon-nashi', 'Kogiku', 'Shinsetsu', 'Akibae' and 'Syugyoku'. The band sizes of the commercial molecular marker on both sides are, from top to bottom, 1353, 1078, 872, 603, 314, 281 and 234 bp. Fragments were electrophoresed in a 6% polyacrylamide gel

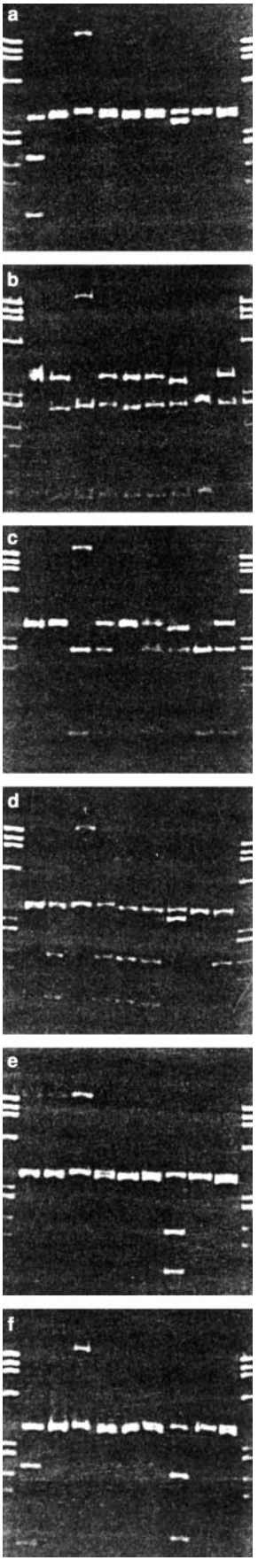

Fig. 4a–f Analysis of the PCR fragment digests with restriction endonucleases. The restriction endonucleases employed were (**a**) *Sfc*I, (**b**) *Ppu*MI, (**c**) *Alw*NI, (**d**) *Nde*I, (**e**) *Hin*cII, (**f**) *Acc*II. In each gel the lanes, from left to right, show the commercial molecular marker, 'Hogetsu', 'Chikusui', 'Yasato', 'Aikansui', 'Ohgon-nashi', 'Kogiku', 'Shinsetsu', 'Akibae', 'Syugyoku', and the commercial molecular marker. The band sizes of the molecular marker are, from top to bottom, 1353, 1078, 872, 603, 314, 281, 234, 194 and 118 bp. Fragments were electrophoresed on 7% polyacrylamide gels

Table 2 *S*-genotypes of nine *S*-allele-unknown cultivars of Japanese pear determined by the PCR-based method

Cultivar	S_2[a]	*Sfc*I[b] (S_1 specific)	*Ppu*MI (S_3, S_5 specific)	*Nde*I (S_4 specific)	*Alw*NI (S_5 specific)	*Hinc*II (S_6 specific)	*Acc*II (S_6, S_7 specific)	*S*-genotype
Hogetsu	−	+	−	−	−	−	+	S_1S_7
Chikusui	−	−	+	+	−	−	−	S_3S_4
Yasato	+	−	+	−	+	−	−	S_2S_5
Aikansui	−	−	+	+	+	−	−	S_4S_5
Ohgon-nashi	−	−	+	+	−	−	−	S_3S_4
Kogiku	−	−	+	+	+	−	−	S_4S_5
Shinsetsu	−	−	+	−	+	+	+	S_5S_6
Akibae	−	−	+	−	+	−	−	$S_4^{sm}S_5$[c]
Syugyoku	−	−	+	+	+	−	−	S_4S_5

[a] Cultivars that produce an S_2-allele-specific fragment by PCR are denoted by +
[b] Cultivars that produce the PCR fragment when digested with each restriction endonuclease are denoted by +
[c] One of the two *S*-alleles of 'Akibae' was assigned to the S_4^{sm}-allele because it was a self-compatible cultivar derived from 'Osa-Nijisseiki' ($S_2S_4^{sm}$)

has the S_4^{sm}-allele, from which no fragment was amplified by PCR, and that the S-genotype of 'Akibae' is $S_4^{sm}S_5$. None of the nine cultivars examined are native varieties, and the S-genotypes of their parents (except 'Ohgon-nashi') were identified by crossing experiments. The *S*-genotypes determined in this study do not conflict with those deduced from the parents' *S*-genotypes.

Selection of self-compatible varieties from the offspring of the self-compatible cultivar 'Osa-Nijisseiki'

Because the self-compatible cultivar 'Osa-Nijisseiki' often is used as a parent to breed a new self-compatible cultivar that has excellent fruit, we used the PCR-based method to screen the self-compatible varieties of the 'Osa-Nijisseiki' offspring.

As the *S*-allele is inherited in a Mendelian manner, the S_2- and S_4^{sm}-alleles of 'Osa-Nijisseiki' separate in a ratio of 1 : 1 in the F_1 progeny. Because PCR amplified no fragment from the S_4^{sm}-allele, self-compatible varieties of the F_1 progeny can be selected by the absence of a PCR fragment specific to the S_2-allele (1347 bp). If, however, 'Osa-Nijisseiki' is crossed with a cultivar with the S_2-allele, self-compatible varieties cannot be screened using the absence of the S_2-fragment. When a cultivar with S_2S_4 is the female parent and 'Osa-Nijisseiki' the male parent, no fertilization occurs because the pollen part of the S_4^{sm}-locus functions normally. When a cultivar with S_2S_x ($x \neq 4$) is the female parent, all the F_1 progeny varieties are self-compatible with the $S_xS_4^{sm}$-allele. When a cultivar with S_yS_4 ($y \neq 2$) is the female parent, all the progeny varieties are self-incompatible with the S_yS_2-allele. When 'Osa-Nijisseiki' self-hybridizes, all the progeny varieties are self-compatible with the *S*-genotype $S_2S_4^{sm}$ or $S_4^{sm}S_4^{sm}$ (1 : 1). Except in these four cases, self-compatible offspring must be identified by the absence of the S_2-allele-specific fragment. We used the PCR-based method to select the self-compatible varieties of the offspring of 'Osa-Nijisseiki'.

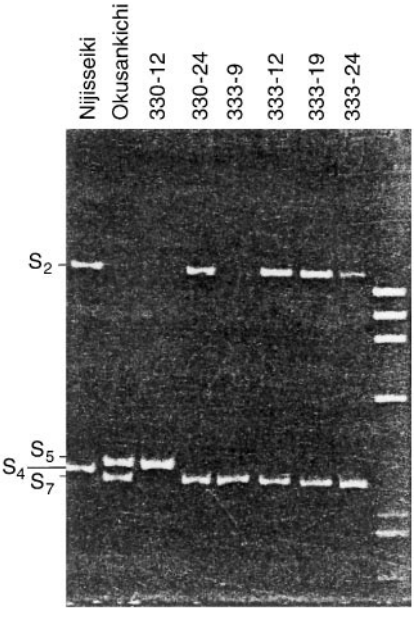

Fig. 5 Analysis of PCR fragments from the offspring of 'Osa-Nijisseiki'. Varieties '330-12' and '330-24' are offspring of a cross between 'Osa-Nijisseiki' (♀) × 'Okusankichi' (♂). Varieties '333-9', '333-12', '333-19', and '333-24' are offspring of a cross between 'Okusankichi' (♀) × 'Osa-Nijisseiki' (♂). PCR fragments from 'Nijisseiki' and 'Okusankichi' were analyzed as the control. The band sizes of the molecular marker in the lane on the right side are, from top to bottom, 1353, 1078, 872, 603, 314, 281, 234, 194 and 118 bp. Fragments were electrophoresed in a 6% polyacrylamide gel

The cultivars '330-12' and '330-24', offspring of a cross between 'Osa-Nijisseiki' ($S_2S_4^{sm}$) (♀) × 'Okusankichi' (S_5S_7) (♂), and '333-9', '333-12', '333-19' and '333-39', offspring of a cross between 'Okusankichi' (♀) × 'Osa-Nijisseiki' (♂), were tested. Fragments of about 1400 bp derived from the S_2-allele were present in '330-24', '333-12', '333-19' and '333-39', but were not detected in '330-12' and '333-9' (Fig. 5 and Table 3). These findings indicate that the first four varieties are

Table 3 Selection of self-compatible varieties from the offspring of 'Osa-Nijisseiki' ($S_2S_4^{sm}$) and 'Okusankichi' (S_5S_7). The *S*-genotypes determined and the expected character [self-incompatible (SI) or self-compatible (SC)] of the offspring are shown

Variety	S_2[a]	S_4	S_5	S_7	*S*-genotype	SI/SC
Nijisseiki	+	+	−	−	S_2S_4	SI
Osa-Nijisseiki	+	−	−	−	$S_2S_4^{sm}$	SC
Okusankichi	−	−	+	+	S_5S_7	SI
330-12	−	−	+	−	$S_4^{sm}S_5$	SC
330-24	+	−	−	+	S_2S_7	SI
333-9	−	−	−	+	$S_4^{sm}S_7$	SC
333-12	+	−	−	+	S_2S_7	SI
333-19	+	−	−	+	S_2S_7	SI
333-39	+	−	−	+	S_2S_7	SI

[a] Cultivars that produce the PCR fragment specific to each *S*-allele are denoted by +

self-incompatible, and the last two self-compatible. Because fragments derived from the S_2-, S_4-, S_5-, and S_7-alleles could be distinguished from one another by polyacrylamide-gel electrophoresis (Fig. 5), the *S*-genotypes of these six offspring were determined (Table 3).

Discussion

We developed a PCR-based method for identifying the *S*-alleles in Japanese pear. The PCR-based method is preferable to 2D-PAGE (Ishimizu et al. 1998 a) because it is more rapid (results obtained within 1 day) and uses only a small amount of young leaves rather than mature styles. The principle of this method is almost the same as that of the system used to identify the *Brassica oleracea* *S*-alleles (Brace at al. 1993, 1994). In the system for identifying the *Brassica* *S*-alleles, the findings were sometimes very complicated because amplification of extra fragments, or predominant amplification of a fragment from one of the two *S*-alleles, may occur (Brace et al. 1993). These phenomena are probably due to the presence of several genes similar to the *S*-allele gene in *Brassica* (Brace et al. 1994). On the other hand, neither an extra fragment nor a predominant fragment from one of the two *S*-alleles was present in the cultivars of Japanese pear examined by our method. Because there is probably no S-RNase-like gene in Japanese pear we carefully chose primers specific to the *S*-RNase gene and common to the 14 rosaceous S-RNases, including the seven S-RNases of Japanese pear.

The system for identifying *Malus* × *domestica* (apple) *S*-alleles was good for only several *S*-alleles because primers with *S*-allele-specific nucleotide sequences were used for the PCR amplification (Janssens et al. 1995; Verdoodt et al. 1998). In contrast, our method can be used, in principle, with all varieties with two *S*-alleles, including a new *S*-allele, because the nucleotide sequences conserved among rosaceous S-RNases were used as the primers. But, when a new *S*-allele is detected by our method, nucleotide-sequence analysis of a PCR fragment should be done to confirm the new *S*-allele, because discrimination of *S*-alleles by our method is based mainly on the presence of the restriction sites.

As an application of this method, the selection of self-compatible varieties from the offspring of 'Osa-Nijisseiki' was examined. Usually, when pear breeders want to develop a new high quality cultivar, about 100 crossed offspring are planted for screening. If half of them can be screened out by our method, then the orchard space allotted for planting the offspring can be halved. There have been attempts to breed a self-compatible, disease-resistant hybrid cultivar between a disease-resistant apple cultivar and a self-compatible Japanese pear cultivar (Banno et al. 1993). Our method can also be used to select this type of hybrid because it uses primers that have the conserved amino-acid sequences of the 14 rosaceous S-RNases.

Because the nucleotide sequences of the primers are also conserved in apple, and probably in other maloideous (of the Rosaceae) species (Chinese pear, quince, and so forth), this method should be useful for identifying the *S*-alleles in various maloideous species. In fact, two *S*-allelic PCR fragments were amplified by this system from a cultivar of Chinese pear (data not shown). These fragments were not digested by any of the six restriction endonucleases used in our study, indicating that they are distinct from the seven *S*-alleles of Japanese pear identified so far. This suggests that our system can be applied to other maloideous species. Japanese pear (*P. pyrifolia*) is thought to have originated in southeast China (Kajiura 1988), and is morphorogically similar to the Chinese pears (*P. ussuriensis*, *P. bretschneideri*, *P. pashia*, *P. hondoensis*, and *P. aromatica*). To clarify the origin of the Japanese pear, the *S*-alleles of native varieties of Japanese and Chinese pears must be identified, and the distribution of the *S*-alleles of these pears in Japan and China analyzed. Our selection method should make a positive contribution to such a project.

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