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Original Paper

Rapid identification of apple (*Malus*×*domestica* Borkh.) *S* alleles using sequencing-based DNA marker *APPLid*

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Abstract All apple cultivars harbor the trait called self-incompatibility. Self-incompatibility represents that the pistils of the flowers are not successfully fertilized with own, the same cultivar's pollens. Compatibility or incompatibility of apple flowers are determined by *S* alleles. For example, the most popular apple cultivar 'Fuji' possesses the S_1 and S_9 alleles (S_1S_9). Thus, 'Fuji' is incompatible with S_1S_9 cultivars, but is compatible with the cultivars possessing different combinations of *S* alleles such as S_2S_7 and S_1S_7. Apple *S* alleles have been identified by performing a series of allele-specific PCR amplifications, to detect more than ten different *S* alleles separately. Here, we developed a new type of sequencing-based DNA marker of the apple *S-RNase* gene, which identifies *S* alleles. This DNA marker was named *APPLid* (apple *S*-allele identifier). A 53-base region in the first coding sequence of *S-RNase* is the target of *APPLid* sequencing. Variation in nucleotide sequences in this *APPLid* sequence enables allele identifications. This region is amplified from apple genomic DNA by using a pair of degenerate primers. The forward primer is attached with 'DS5 adaptor.' After PCR amplification, electrophoresis and gel extraction of 177-bp DNA fragments, *APPLid* sequence is determined by direct sequencing with a sequencing primer. The *APPLid* sequences of 20 apple cultivars completely matched their *S* alleles, which include triploid cultivars. In conclusion, *APPLid* identifies apple *S* alleles (S_1, S_2, S_3, S_4, S_5, S_7, S_9, S_{10}, S_{20}, S_{24}, S_{25}, S_{26}, S_{27} and S_{28}, so far) just by a single sequencing analysis.

Key words: apple, degenerate primer, DNA marker, DNA sequencing, *S* allele.

Introduction

Self-incompatibility of apple (*Malus*×*domestica* Borkh.) plants is an agriculturally important trait. All known apple cultivars are self-incompatible, then farmers grow different apple cultivars in their orchard. In spring, bloomed flowers are crossed with each other to fertilize. Relatively large number of apple *S* alleles (more than ten) results in different combinations of *S* alleles between most apple cultivars. As many as 57 different combinations of *S* alleles are reported (Hegedűs 2006). The cultivars with the same *S* alleles are incompatible with each other. Such examples are 'Golden Delicious'/'Rubin' (S_2S_3), 'Gala'/'Topaz' (S_2S_5) and 'Fuji'/'Shinko' (S_1S_9). *S* alleles must be determined when new apple cultivars are released, to know compatibility with the other cultivars. *S* alleles are also used for deducing parental cultivars, and will also help protection of new cultivars.

Determination of apple and pear *S* alleles are performed by separate amplification of each *S* allele. PCR (polymerase chain reaction) markers such as CAPS (Cleaved Amplified Polymorphic Sequence), SSR (Simple Sequence Repeats), STS (Sequence-Tagged Sites), or allele-specific primers are developed and used to detect each allele (Kudo et al. 2002; Larsen et al. 2016; Nashima et al. 2015). Nevertheless, 20 primers must be prepared to amplify 10 different markers, and PCR conditions should be re-confirmed for all primer sets in each laboratory. It is also noteworthy that the protocols followed to determine *S* alleles are not necessarily available in technical reports by the developer of apple cultivars. These layers of practical issues may make *S*-allele identification a relatively restricted technique to the specialists, make its interpretation difficult, and limit its use in wider range of experiments.

Self-incompatibility of apple plants is caused by *S-RNase* gene expressed in pistils (Broothaerts et al.

Abbreviations: A, adenine; *APPLid*, apple *S*-allele identifier; C, cytosine; CAPS, Cleaved Amplified Polymorphic Sequence; CDS, coding sequence; CTAB, cetyltrimethylammonium bromide; DS4, dissimilar synthetic DNA sequence 4; DS5, dissimilar synthetic DNA sequence 5; EDTA, ethylenediaminetetraacetic acid; G, guanine; INSDC, International Nucleotide Sequence Database Collaboration; NCBI, National Center for Biotechnology Information; PCR, polymerase chain reaction; PVPP, polyvinylpolypyrrolidone; SLF, S-locus F-box protein; SSR, Simple Sequence Repeats; STS, Sequence-Tagged Sites; T, thymine; Tris, tris(hydroxymethyl) aminomethane; UTR, untranslated region.

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2004). Considering the report in petunia (Kubo et al. 2015), a series of *SLF* (S-locus F-box protein) genes are aligned at the same chromosomal locus with *S-RNase*, which were presumably obtained by gene duplication and exchange. These *SLF* genes detoxify non-self alleles of S-RNase proteins, but do not detoxify self allele of S-RNase protein. Thus, non-self pollens expressing non-self alleles of *SLF* genes can fertilize the pistil. *S* alleles could be identified by DNA sequencing of the *S* locus including *S-RNase* and *SLF* genes. Genetic structures of apple *SLF* genes are not clear yet. On the contrary, genetic structure of the apple *S-RNase* gene is simple. Genomic sequence of apple cultivar 'Golden Delicious' (Velasco et al. 2010; Genome Database for Rosaceae, https://www.rosaceae.org/) contained genomic sequence of both S_2 and S_3 alleles (denoted *MdS2* and *MdS3* genes in this report). Compared with corresponding mRNA sequences in INSDC (International Nucleotide Sequence Database Collaboration), genomic sequence of apple *S-RNase* consists of 5′-UTR (untranslated region), 1st CDS (coding sequence), intron, 2nd CDS, and 3′-UTR (Kasajima et al., unpublished data). These knowledges may be beneficial for high-throughput identification of apple *S* alleles.

We previously isolated promoter sequences of eight genes expressed in the petals of cyclamen (*Cyclamen persicum* Mill.; Kasajima et al. 2017). From the viewpoint of PCR techniques, this experience may also benefit identification of apple *S* alleles. First, genomic DNA can be efficiently extracted from any horticultural plants, tested up to date, with the modified 'alkaline PVPP buffer' (Kasajima et al. 2013; Kasajima 2017). Second, a new 79-base adaptor sequence called DS4 (dissimilar synthetic DNA sequence 4) was developed, which efficiently amplified promoter fragments in adaptor-PCR analysis (Kasajima et al. 2014). Compared with the popular adaptor sequence, DS4 enjoys lower GC content, longer and preferable sequence for primer design. What is more, DS4 sequence is not homologous to any nucleotide sequence of any organisms or synthetic sequences searched on the NCBI website (National Center for Biotechnology Information; https://www.ncbi.nlm.nih.gov/).

Here we amplified a partial 53-bp sequence of any allele of *S-RNase*, using apple genomic DNA as the template of PCR reaction. PCR was performed using only a single pair of degenerate primers. Sequencing of the whole 53-bp region was enabled by attachment of a DS4-derived sequence to the forward primer. Direct sequencing of the PCR product enabled identification of *S* alleles in all 20 apple cultivars examined.

In this study, 23 apple cultivars were used (Table 1). These include popular cultivars in the world (e.g. 'Fuji' and 'Golden Delicious'), popular cultivars in Japan (e.g. 'Orin' and 'Shinano Gold'), and popular cultivars in Iwate prefecture of Japan (e.g. 'Haruka' and 'Beniiwate'). *S* alleles of all these cultivars are reported as written in Table 1. Many of the apple cultivars are diploid, so that they have two *S* alleles such as S_1S_9. Among the cultivars analyzed in this study, 'Jonagold,' 'Aori-9' and 'Santarou' are triploid cultivars, then they have three *S* alleles such as $S_2S_3S_9$. Genomic DNA was extracted from leaves of these cultivars and used for PCR analysis.

Table 1. Apple cultivars used in this study and their *S* genotypes.

Cultivar	*S* genotype	Reference
Fuji	S_1S_9	Broothaerts et al. 2004; Hegedűs 2006
Orin	S_2S_7	Broothaerts et al. 2004; Hegedűs 2006
Haruka	S_2S_9	Miyagi Prefecture 2013
Sansa	S_5S_7	Broothaerts et al. 2004; Hegedűs 2006
Tsugaru	S_3S_7	Broothaerts et al. 2004; Hegedűs 2006
Jonagold	$S_2S_3S_9$	Broothaerts et al. 2004; Hegedűs 2006
Akane	S_7S_{24}	Broothaerts et al. 2004; Hegedűs 2006
Aori-9	$S_2S_7S_{24}$	Kudo et al. 2002
Kitarou	S_3S_9	Broothaerts et al. 2004
Santarou	$S_3S_9S_{28}$	Soejima et al. 2013
Shinano Sweet	S_1S_7	Harada Seeds 2017a
Shinano Gold	S_1S_3	Miyagi Prefecture 2013
Jonathan (Kougyoku)*	S_7S_9	Broothaerts et al. 2004; Hegedűs 2006
Beniiwate	S_3S_9	Everyone's Agriculture 2012
Aikanokaori	S_3S_9	Harada Seeds 2017b
Golden Delicious	S_2S_3	Broothaerts et al. 2004; Hegedűs 2006
Indo	S_7S_{20}	Broothaerts et al. 2004; Hegedűs 2006
Starking Delicious	S_9S_{28}	Broothaerts et al. 2004
Ralls Janet (Kokkou)*	S_1S_2	Broothaerts et al. 2004; Hegedűs 2006
Delicious	S_9S_{28}	Broothaerts et al. 2004; Hegedűs 2006
Senshu	S_1S_7	Broothaerts et al. 2004; Hegedűs 2006
Prima	S_2S_{10}	Broothaerts et al. 2004; Hegedűs 2006
McIntosh (Asahi)*	$S_{10}S_{25}$	Broothaerts et al. 2004; Hegedűs 2006

*Japanese names of 'Jonathan,' 'Ralls Janet,' and 'McIntosh' are written in parentheses.

Materials and methods

Plant materials

Young expanding leaves of 23 apple cultivars (listed in Table 1) were sampled from apple trees in spring 2016. These apple trees were purchased from farmers, preserved in the orchard of Iwate University (Morioka city, Iwate, Japan), or preserved in Iwate Agricultural Research Center (Kitakami city, Iwate, Japan). Immediately after sampling, approximately 50 mg of leaves were placed in plastic tubes with two metal balls and kept in deep freezer (–80°C).

DNA extraction

Genomic DNA was extracted from apple leaves using the alkaline PVPP buffer (Kasajima et al. 2013), which was modified recently (Kasajima 2017). Briefly, apple leaves in plastic tubes were crushed in Micro Smash MS-100R (Tomy, Tokyo, Japan) at 2500 rpm for 30 s. Tubes were centrifuged briefly and 500 μl of the extraction buffer (Tris-HCl, pH 9.5, 50 mM; EDTA, 10 mM; NaCl, 4 M; CTAB, 1%; PVPP, 0.5%) was added. Here, Tris represents tris(hydroxymethyl)

aminomethane, EDTA represents ethylenediaminetetraacetic acid, CTAB represents cetyltrimethylammonium bromide, and PVPP represents polyvinylpolypyrrolidone. β-mercaptoethanol (1%) was also added to the extraction buffer immediately before use. Tubes were mixed again in Micro Smash at 3000 rpm for 30 s, heated at 60°C for 30 min, centrifuged, and the green supernatant was recovered to new plastic tubes. After treatment with phenol-chloroform-isoamylalcohol twice, water phase was precipitated/purified with isopropanol and treated with RNase A. Solution was treated with phenol-chloroform-isoamylalcohol again, and precipitated with ethanol. DNA was dissolved in 50 μl of sterilized ion-exchanged water and kept at –20°C. Aliquots of these DNA solutions were 100 times diluted with sterilized ion-exchanged water and used as the templates of PCR.

PCR amplification of APPLid *sequence*

PCR was performed with KOD Plus Neo DNA polymerase (Toyobo, Osaka, Japan) following instructions of the manufacturer. Ex Taq (Takara, Kusatsu, Japan) was also tested, but did not successfully amplify *APPLid* sequence. Amplification was performed in GeneAtlas G thermal cycler (Astec, Shime, Fukuoka, Japan). Aliquots of 2.5 μl of diluted DNA solutions were added to 50-μl reactions. Primers were DS5-S-seq-F (5′-ATC CGA AGT CAC GAT GAT TCA GCG GCC TAT AGC ACA TCG GCG CAT AGT TAC GCG GTT AGT CGG ATC CCA CGG ATT ATT WTC AAT TTA CGC AKC ART ATC A-3′) and S-seq-R (5′-GCC ACA AMC CRT GAA CVG TAA ACA-3′). Primer concentrations were 10 times higher than the standard concentrations for KOD Plus Neo polymerase, thus 1 μl each of the 100-μM stocks of both primers were added to 50-μl reactions. Annealing temperature was 52°C unless otherwise described. Number of thermal cycles was 40, and the time of extension was 1 min. A forward primer without attachment of the DS5 adaptor sequence (S-seq-F; 5′-GAT TAT TWT CAA TTT ACG CAK CAR TAT CA-3′) was also used for setting PCR conditions. Degenerate nucleotide signs are as follows: W, A or T; R, A or G; M, A or C; K, T or G; Y, T or C; S, G or C; H, A or C or T; B, G or C or T; V, A or G or C; D, A or G or T; N, A or G or C or T. After electrophoresis in 2% agarose gel, 177-bp *APPLid* DNA fragments were extracted from gel by using MonoFas (GL Sciences, Tokyo, Japan).

DNA sequencing

DNA sequencing was performed by Sigma Genosys (Sigma-Aldrich, Tokyo, Japan). DS4-AP1 primer (5′-GAA GTC ACG ATG ATT CAG CGG CCT ATA GCA-3′) was used for *APPLid* sequencing, together with gel-extracted DNA as the template. DS4-AP2 primer (5′-GGC CTA TAG CAC ATC GGC GCA TAG TTA CG-3′) may be also used for sequencing.

DNA alignment

Partial mRNA sequences of apple *S-RNase* gene corresponding to coding sequences (1st and 2nd CDSs) were aligned by ClustalW (Thompson et al. 1994; http://www.genome.jp/tools/clustalw/). Aligned sequences were then contrasted by BoxShade (http://www.ch.embnet.org/software/BOX_form.html) with default settings (e.g. Output format, Postscript-portrait; Input sequence format, MSF).

Results

Alignment of S-RNase *sequences*

S-RNase sequences were searched on NCBI. Among the apple nucleotide sequences homologous to *S-RNase*, we picked up 13 different mRNA sequences (Table 2). These sequences (genes) were assigned with different *S* alleles. These genes are referred to as *MdS1*, *MdS2*, *MdS3*, *MdS4*, *MdS7*, *MdS9*, *MdS10*, *MdS20*, *MdS24*, *MdS25*, *MdS26*, *MdS27*, and *MdSe*, respectively, in this report. There are two different labelling of *S* alleles: European labelling uses figures like S_1 and S_2, and Japanese labelling uses characters like S_a and S_b. S_a, S_b, S_c, S_d, S_f, S_g, S_i, S_h, and S_z are identical with S_2, S_3, S_9, S_7, S_1, S_{20}, S_{24}, S_{10}, and S_{25}, respectively, but the identity of S_e is not clear (Hegedűs 2006).

Whole coding sequences were available in the 13 mRNA sequences. Coding sequences were aligned as shown in Figure 1. Considering the genetic structure of apple *S-RNase*, these mRNA sequences are the same as genomic sequences of apple *S-RNase*, except that an intron sequence is inserted between the 1st and the 2nd CDSs in the genomic sequence.

Primer design

To design universal primers which can amplify any allele of apple *S-RNase*, we focused on the central part of the 1st CDS. There are two highly conserved sequences in this part of *S-RNase*. In addition, there is no insertion or deletion of nucleotide sequences in this part, in any allele. Forward and reverse sequences were set at the conserved sequences for primer design. Degenerate primers which fit any allelic sequence were designed at these sites. A 53-base region on the 1st CDS is amplified

Table 2. Accession numbers of apple *S-RNase* genes.

Gene	*S* allele	Nucleotide type	Accession*
MdS1	S_1	mRNA	D50837.1
MdS2	S_2	mRNA	U12199.1
MdS3	S_3	mRNA	MDU12200
MdS4	S_4	mRNA	AF327223.1
MdS7	S_7	mRNA	AB032246.1
MdS9	S_9	mRNA	D50836.1
MdS10	S_{10}	mRNA	AB052683.1
MdS20	S_{20}	mRNA	AB019184.1
MdS24	S_{24}	mRNA	AB032247.1
MdS25	S_{25}	mRNA	AB062100.1
MdS26	S_{26}	mRNA	AF016918.1
MdS27	S_{27}	mRNA	AF016919.1
MdSe	S_e	mRNA	AB035273.1

*INSDC (http://www.insdc.org/) accession numbers.

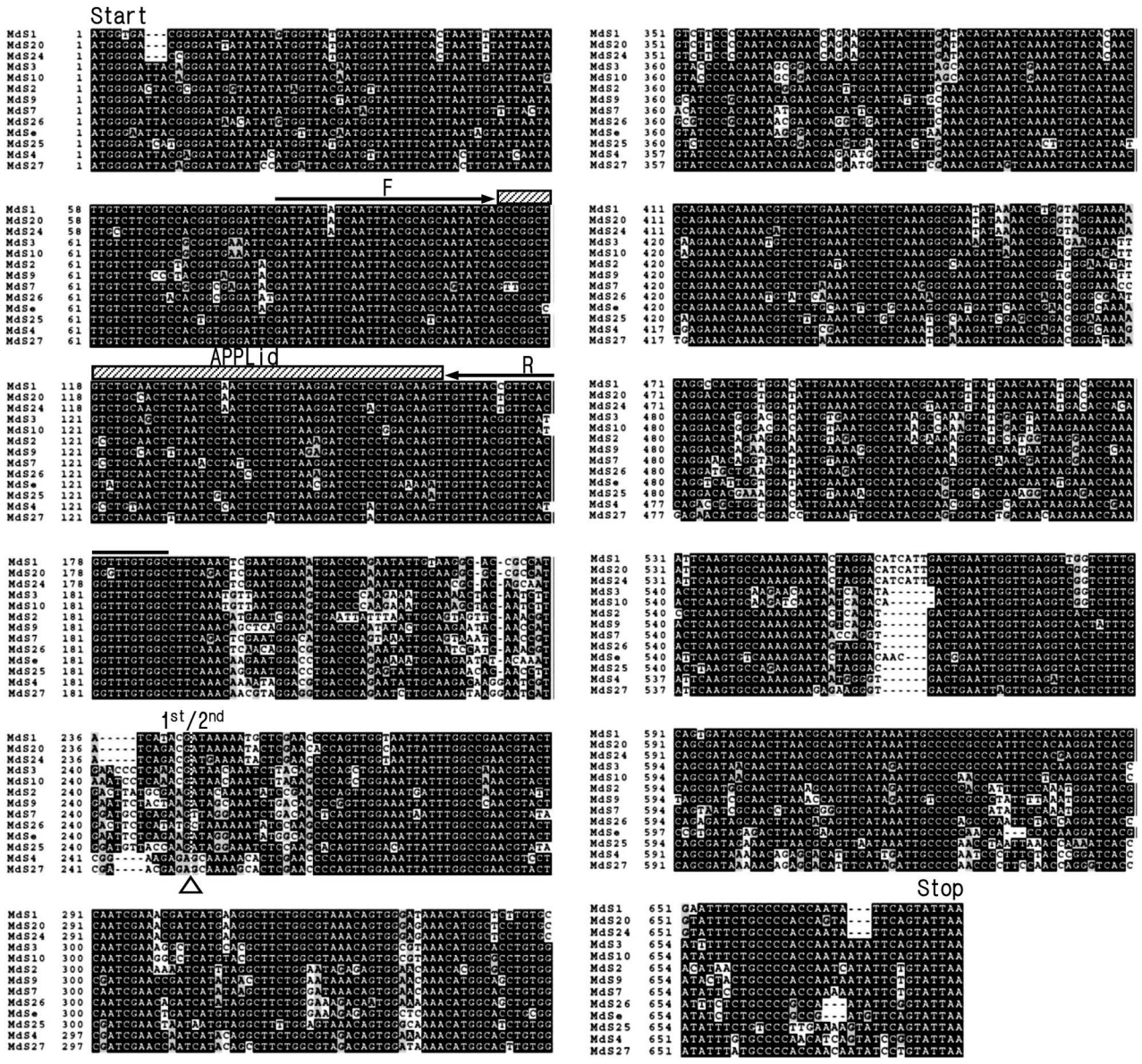

Figure 1. Coding sequences of the alleles of apple *S-RNase*. 'Start' and 'Stop' indicate positions of the start codons and stop codons, respectively. '1st/2nd' and open arrowhead indicate the margin between the 1st and the 2nd CDSs (coding sequences). 'F' and 'R' represent binding sites of the forward and the reverse primers. *APPLid* sequence is between F and R sequences.

with these primers. Judging from the aligned sequences, this 53-base sequence is enough to discriminate between different *S* alleles. This possible DNA marker of apple *S* alleles was named *APPLid* (apple *S*-allele identifier).

Primer structure is illustrated in Figure 2A. The forward primer (DS5-S-seq-F) is attached with a 71-base adaptor sequence (DS5; dissimilar synthetic DNA sequence 5) at the 5′ end. DS5 is a shorter version of DS4. DS5 was attached to the forward primer to obtain complete data of *APPLid* sequence: without interval between the binding site of sequencing primer and the target sequence, clear fluorescent signals are not obtained for the target sequence. Primers such as DS4-AP1 and DS4-AP2 (Kasajima et al. 2014) are available for sequencing.

PCR amplification of the APPLid *fragment*

The initial setting of PCR conditions was performed with primers without attachment of the adaptor sequence (S-seq-F and S-seq-R)(Figure 2A), using 'Fuji' DNA as the template. The successful PCR condition was different from the standard PCR protocol, in that the primer concentration was 10 times higher (2 μM each) than the default concentrations for KOD Plus Neo polymerase (0.2 μM each). *APPLid* DNA fragment was only weakly amplified at the standard primer concentrations.

Clear *APPLid* fragments were obtained when using primers without the adaptor sequence. On the other hand, some level of smear DNA appeared after electrophoresis when the forward primer attached with the DS5 adaptor (DS5-S-seq-F) was used, together with

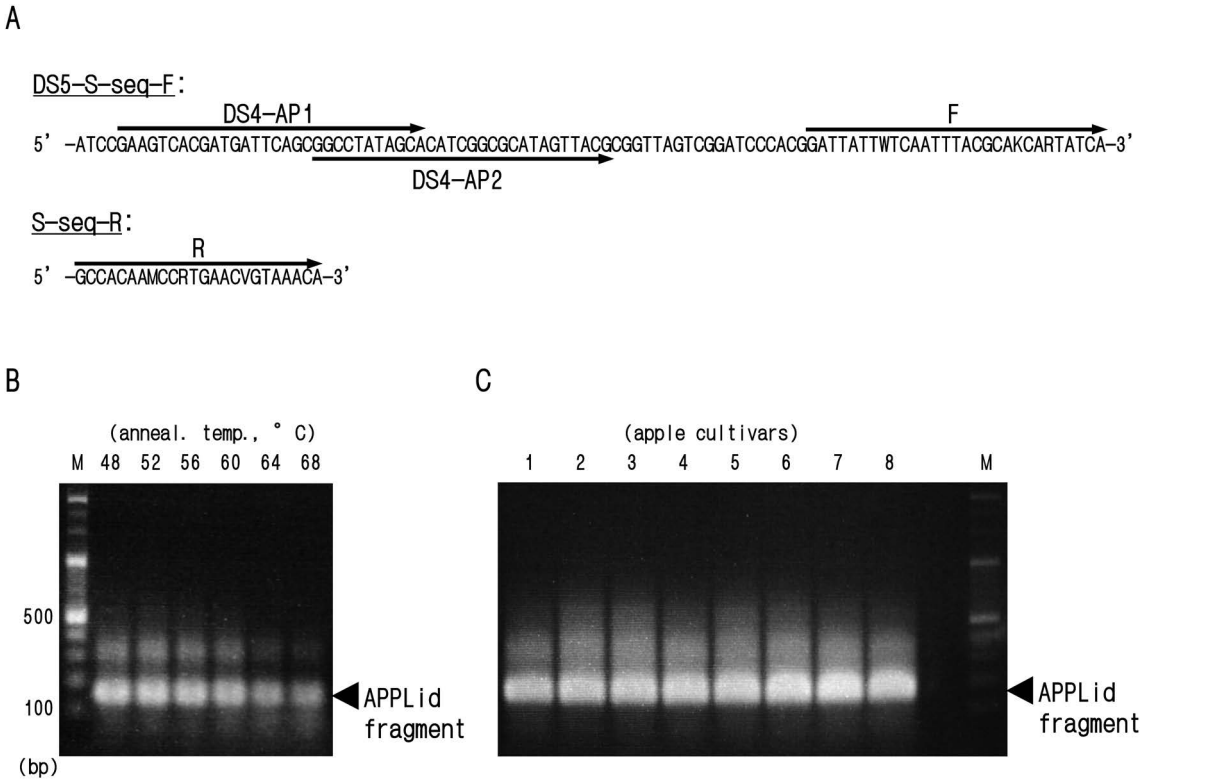

Figure 2. Primer structures and *APPLid* fragments. (A) Structure of the forward primer (DS5-S-seq-F) and the reverse primer (S-seq-R) used in PCR amplification. 'F' and 'R' correspond to the same positions on *S-RNase* sequence as Figure 1. 'DS4-AP1' and 'DS4-AP2' indicate the binding sites of these primers on the DS5 adaptor. (B) Amplification of the *APPLid* fragments from 'Fuji' genomic DNA. (C) Amplification of the *APPLid* fragments from eight cultivars (1, Golden Delicious; 2, Indo; 3, Starking Delicious; 4, Ralls Janet; 5, Delicious; 6, Senshu; 7, Prima; 8, McIntosh). M, molecular weight marker BRG-100-02 (Watson, Tokyo, Japan).

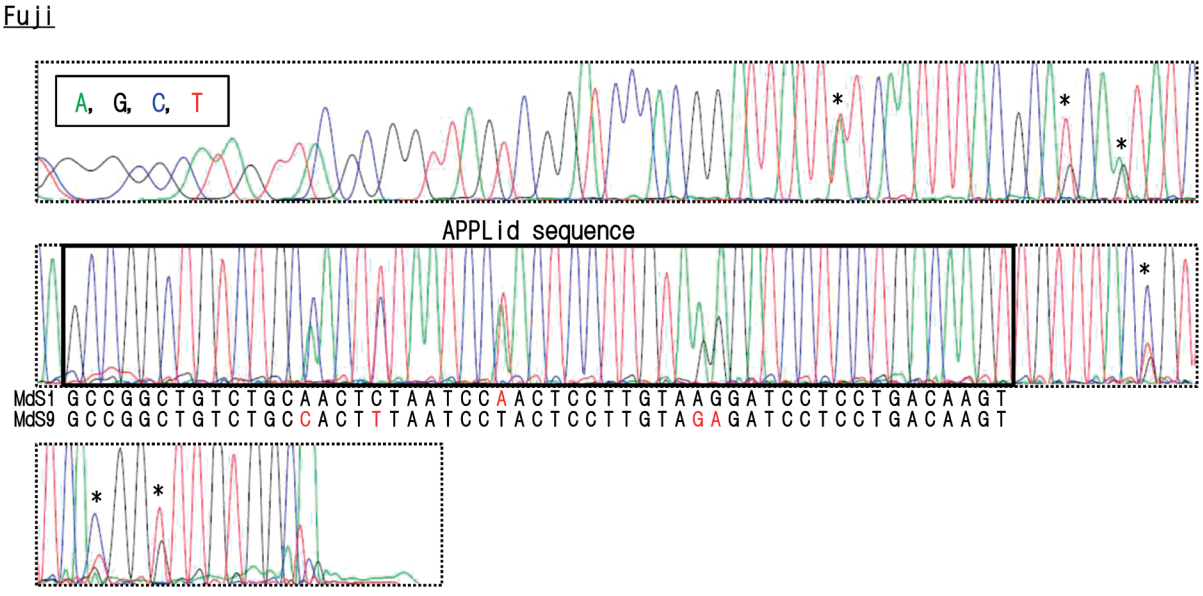

Figure 3. Fluorescence peaks in *APPLid* sequencing of 'Fuji.' *APPLid* fragment of 'Fuji' was sequenced by DS4-AP1 primer. Green, black, blue and red fluorescence signals indicate adenine (A), guanine (G), cytosine (C) and thymine (T) bases of the nucleotides. These bases are also indicated by the same colors in the other figures. Asterisks indicate the positions of degenerate nucleotides in primers. *APPLid* sequences of *MdS1* and *MdS9* are shown below the *APPLid* peaks. Characters are red where *MdS1* or *MdS9* possess unique nucleotides.

the reverse primer (S-seq-R) for PCR amplification (Figure 2B). The amount of *APPLid* fragments was greater at lower annealing temperatures, such as 52°C. The 177-bp *APPLid* fragments were also amplified from all apple cultivars (Figure 2C). To eliminate possible effects of smear DNA on the following analysis, *APPLid*

fragments were isolated from the gel, as is often the case in such direct sequencing analysis of the PCR products.

APPLid *sequencing*

APPLid fragment amplified from 'Fuji' genomic DNA was test sequenced by DS4-AP1 primer or DS4-AP2 primer. DS4-AP1 gave somewhat clearer peak patterns than DS4-AP2, then DS4-AP1 was used in the following experiments. Figure 3 shows the *APPLid* sequence of 'Fuji.' The 53-base target sequence of *APPLid* was completely consistent with the one expected for S_1S_9 alleles of 'Fuji.' Namely, one adenine unique to S_1 allele and four nucleotides unique to S_9 allele were all detected as heterozygous peaks, simultaneously with the consensus nucleotides. The 53-base target sequence is surrounded by the forward and the reverse primer sequences, including six degenerate nucleotides.

List of APPLid *sequences of S alleles*

APPLid sequencing was also tested in the other 22 cultivars, 19 of which gave complete sequences. Incomplete sequences of the rest three cultivars are shown in the Supplementary Figure S1. Among the successfully sequenced cultivars, 'Sansa' has S_5S_7 alleles and 'Delicious' has S_9S_{28} alleles. Because *MdS5* and *MdS28* sequences were lacking in the initial dataset (Table 2), their *APPLid* sequences were back calculated from *APPLid* sequences of 'Sansa' and 'Delicious.' Figure 4A shows *APPLid* sequences of 'Sansa' and 'Delicious.' The peak pattern of 'Sansa' was consistent with the sequence of *MdS7*. From this peak pattern, *APPLid* sequence of *MdS5* was also calculated so that the whole peak pattern is consistent with S_5S_7 alleles. Thus, *APPLid* sequence of *MdS5* has six unique nucleotides as shown in the Figure 4B. The same calculation was performed on the *MdS28* sequence of 'Delicious,' determining its

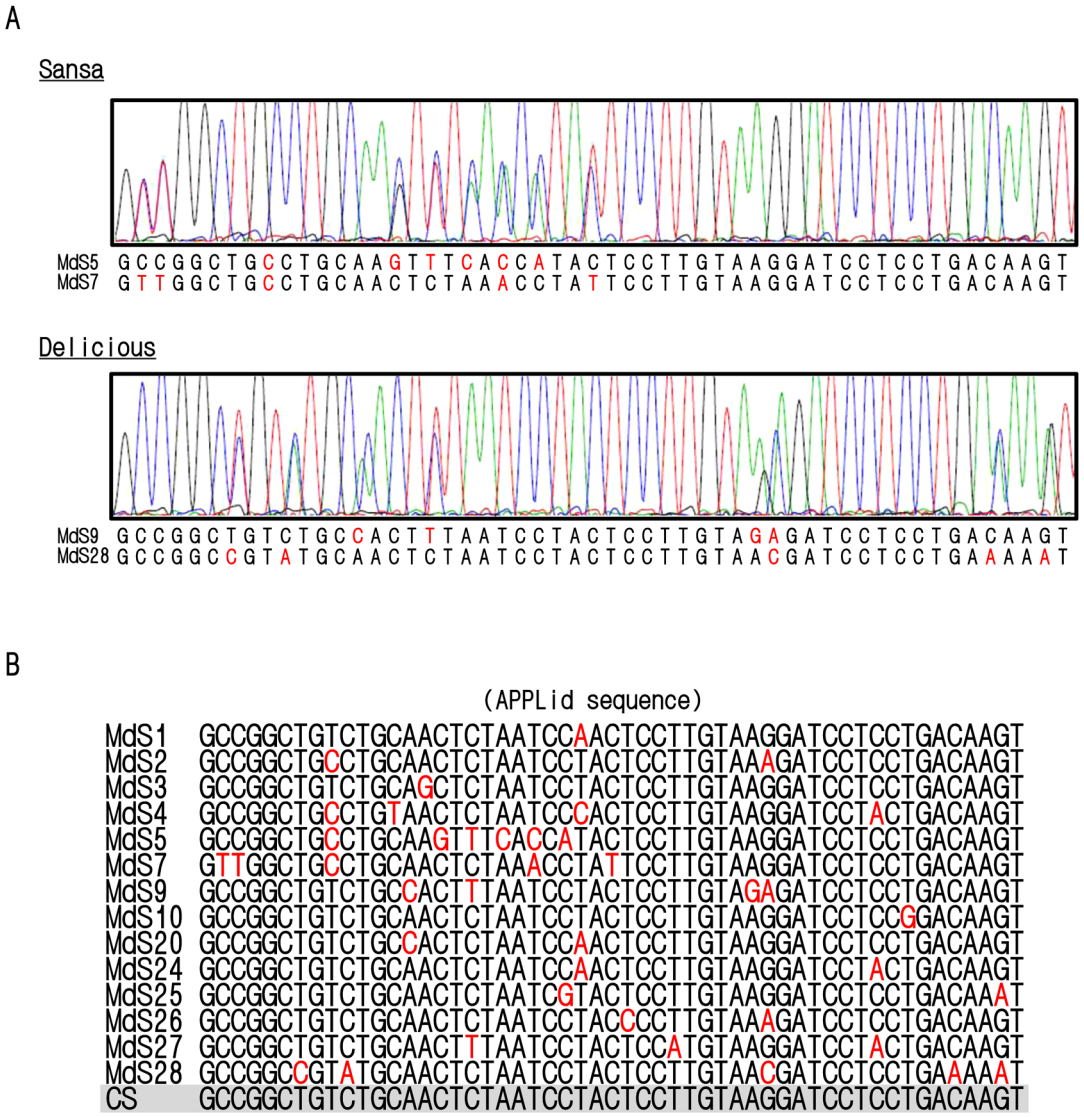

Figure 4. List of *APPLid* sequences. (A) *APPLid* sequences of 'Sansa' and 'Delicious.' (B) List of *APPLid* sequences of 14 *S-RNase* alleles. 'CS' represents the consensus sequence. Unique nucleotides in *S-RNase* alleles, which differ from the consensus sequence, are red.

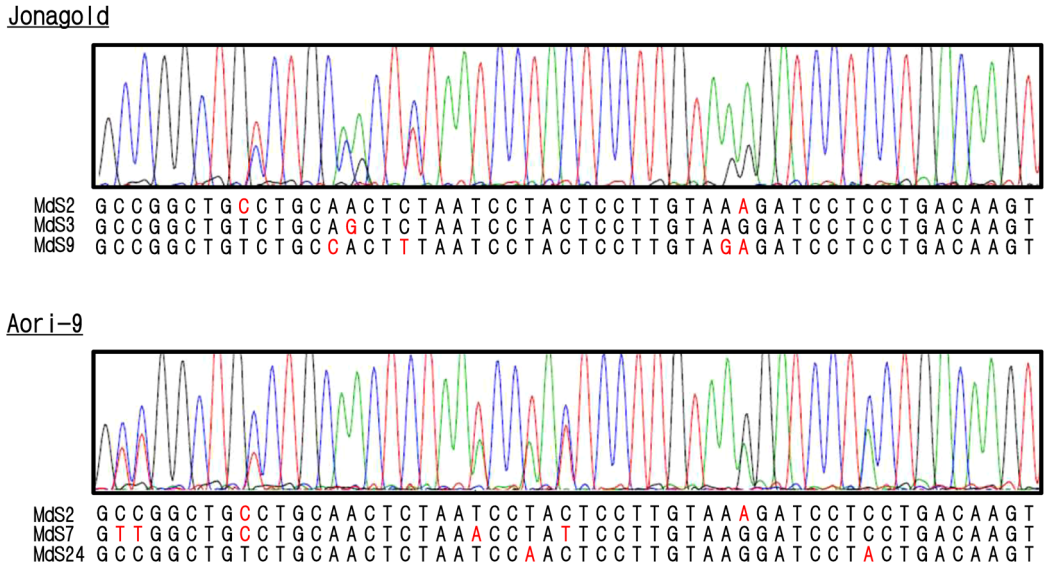

Figure 5. *APPLid* sequences of two triploid cultivars.

APPLid sequence with five unique nucleotides. The *APPLid* sequence of *MdS28* was identical with that of *MdSe*, suggesting that S_{28} allele corresponds to S_e allele.

Including S_5 and S_{28} alleles, *APPLid* sequences of 14 *S* alleles are listed (Figure 4B). All alleles possess at least one different (unique) nucleotide from the consensus sequence. Different combinations and positions of these polymorphisms make it possible to identify both *S* alleles of diploid cultivars.

Comparison of APPLid *sequences*

The list of *APPLid* sequences (Figure 4B) was compared with peak patterns obtained from the other apple cultivars. Figure 5 shows *APPLid* sequences of two triploid cultivars ('Jonagold' and 'Aori-9'). These cultivars are annotated to have $S_2S_3S_9$ and $S_2S_7S_{24}$ alleles respectively. In the case of triploid cultivars, heterozygous nucleotides were always detected as major peaks corresponding to major nucleotides and minor peaks corresponding to minor nucleotides. The whole peak patterns were completely consistent with the annotated *S* alleles. Figure 6 shows *APPLid* sequences of 15 diploid cultivars. All these peak patterns were also completely consistent with annotated *S* alleles.

Discussion

Tips on apple DNA preparation

In this report, we amplified a 53-base target sequence of apple *S-RNase* alleles. The *APPLid* DNA fragments were successfully amplified from genomic DNA of all 23 apple cultivars tested. This result demonstrates general applicability of *APPLid* primers to a broad range of apple cultivars. On the other hand, it should be remembered that the experimental conditions were carefully determined and performed in this report, to minimize the possible risks of the failures in PCR and sequencing. For example, apple DNA was extracted with a highly efficient extraction buffer and partly purified with isopropanol. DNA concentration in the final solution was also checked by electrophoresis and compared with control λ-DNA samples. Judging from band intensities in agarose gel, DNA yield was approximately 2 μg from 50 mg of apple leaf. DNA extraction from apple leaves seems relatively easy compared with the other fruit trees, but the other extraction buffer may yield lower concentrations of apple genomic DNA. In our experience, calculation of DNA concentration from UV absorbance is never reliable in the case of genomic DNA samples from plant tissues. *APPLid* analysis seems to be successful with rougher and simpler protocols, but the experimental conditions had better be optimized to minimize the risk of failures. This matter about optimization of experimental procedures is a general issue, which applies to any PCR analysis. There are many other general tips on DNA preparations and PCR amplifications from plant tissues (Kasajima 2016).

APPLid analysis needs some levels of skills in general DNA experiments, but a clear advantage of the *APPLid* analysis over the other protocols on *S*-allele identification will be that the analysis is completed by just one sequencing analysis. Whole *APPLid* analysis can be also performed with ordinary equipment and standard protocols in the laboratory, without fragment analysis. Users who are not familiar with DNA protocols are welcome to freely make an inquiry at the first author of this report for precise instructions (IK, kasajima@iwate-u.ac.jp/kasajima2008@live.jp).

Effectiveness of the DS5 adaptor

We did not test *APPLid* sequencing without DS5 adaptor attached to the forward primer, or attachment of other adaptor sequence to the forward primer, but DS5 adaptor enabled clear sequencing of *APPLid*. In the previous study (Kasajima et al. 2014), we ligated DS4 adaptor to genomic DNA which had been digested with restriction enzymes. The ligated DNA samples were amplified with adaptor primers such as DS4-AP1 and DS4-AP2. In the present report, we used the DS5-attached forward primer in PCR reaction. Although weak smear DNA was generated with this primer, the target sequence (*APPLid* fragment) was successfully amplified from apple genomic DNA. Thus, DS4/DS5 sequence is applicable not only to adaptor-PCR analysis but also to direct PCR. The forward primer of *APPLid* is 100 bases in length. This size of primer can be ordered with ordinary procedures: we order 100-μM primer solutions in Tris-EDTA butter to FASMAC corporation (Atsugi, Japan).

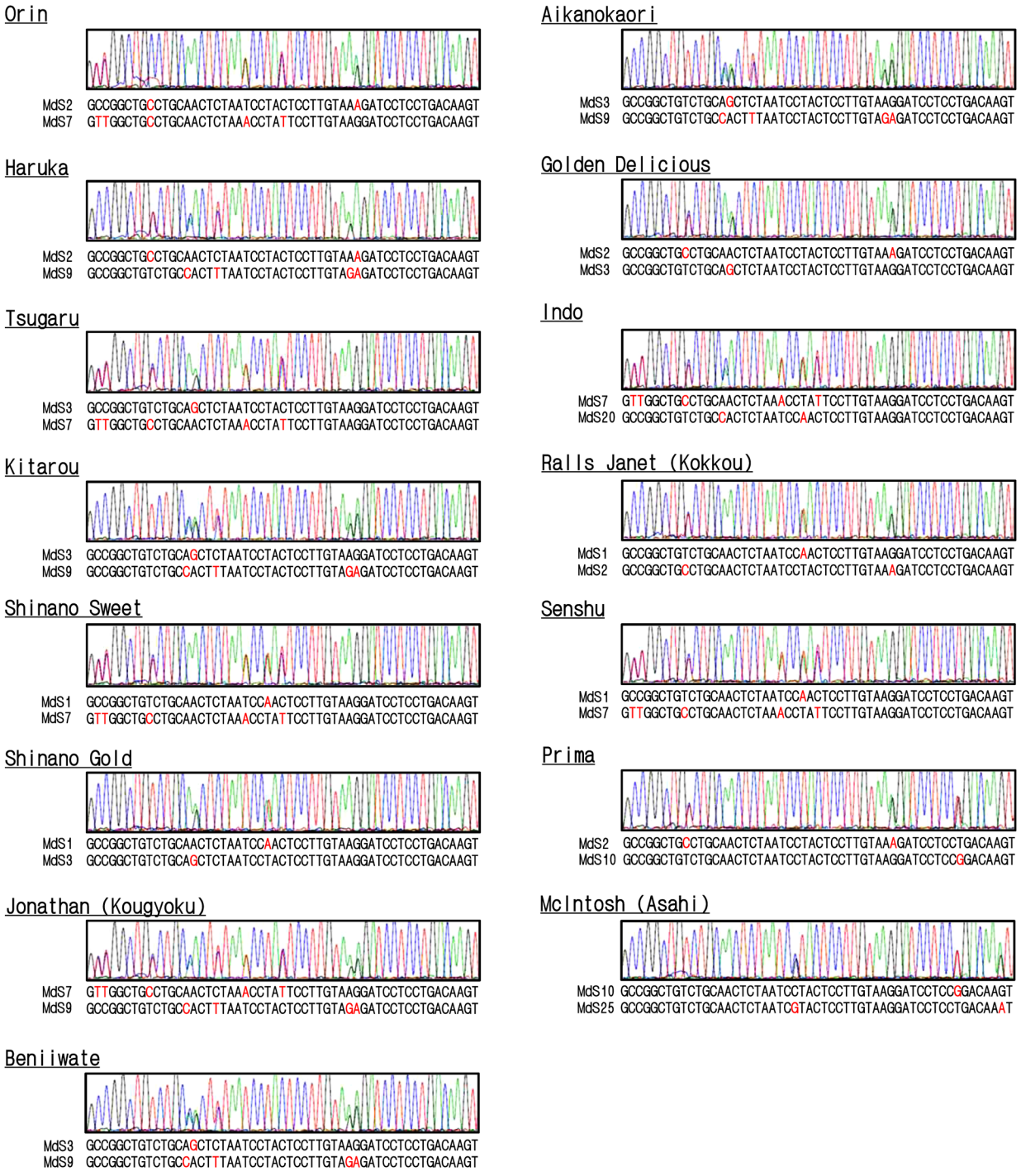

Figure 6. *APPLid* sequences of 15 diploid cultivars.

Design of APPLid *sequencing analysis*

In the *APPLid* sequencing analysis (Figure 3), sharp fluorescence peaks (CCC ACG G...) were obtained at approximately 30 bases and beyond, from the primer-binding site. As anticipated before the beginning of the experiments, there should be at least 50-base separation between the primer-binding site and the target sequence in the sequencing analysis. Clear and complete *APPLid* sequences were obtained for 20 apple cultivars out of 23 cultivars tested (87%). Incomplete but recognizable fluorescence peaks were also obtained for the rest 3 cultivars. The success rate of sequencing reaction is relatively high, considering that the reactions were performed only once for each cultivar. It will be possible to obtain complete *APPLid* sequences of all samples, if the sequencing reactions are repeated for unsuccessful samples.

Capability of APPLid *sequencing and discrimination of triploid cultivars*

The *APPLid* sequences of triploid cultivars were also consistent with their triplet *S* alleles (Figure 5). Interpretation of *APPLid* sequences of unknown triploid apple samples is a bit complicated, but it will be possible to detect triploid cultivars and judge their *S* alleles by *APPLid*. When candidate triploid samples are detected by *APPLid*, it is recommended to confirm ploidy of the samples directly by cell-ploidy analysis or some alternative method. Such combinations of *S* alleles may not be found in existing apple cultivars (Hegedűs 2006), but $S_1S_9S_{27}$, $S_9S_{20}S_{27}$, and $S_9S_{24}S_{27}$ genotypes have the same *APPLid* sequences, except that the ratios of unique nucleotides are different in part (Supplementary Figure S2). Such similar *APPLid* sequences can be observed when S_1, S_2, S_{20} or S_{24} alleles are included in specific combinations of *S* alleles in triploid genome. This is because these four *S* alleles share unique nucleotides in their *APPLid* sequences, with part of the other *S* alleles. Theoretically, *S* alleles of such confusing combinations of unique nucleotides can be confirmed by cloning of *APPLid* fragments into plasmid vectors, followed by sequencing of each clone.

Necessity for the APPLid *sequences of the other S alleles*

In this report, we determined or reconfirmed 53-base *APPLid* sequences of 14 *S* alleles (Figure 4B). These include all major *S* alleles (S_1, S_2, S_3, S_5, S_7, S_9, S_{10}, S_{20}, S_{24} and S_{28}) and four minor *S* alleles (S_4, S_{25}, S_{26} and S_{27}; Broothaerts et al. 2004). This information is sufficient to identify *S* alleles of 158 cultivars out of 171 cultivars (92%) listed in a previous report (Hegedűs 2006). Determination of the *APPLid* sequences of the other minor alleles such as S_6, S_8, S_{16}, S_{22} and S_{23} is necessary to become able to identify *S* alleles of the apple cultivars such as 'Oberrieder,' 'Stafnel Rosen,' 'Wellington,' 'Lobo,' 'Pink Lady,' and 'Granny Smith.'

Further possible applications

As demonstrated in this report, sequencing-based DNA marker using adaptor-attached primer is effective on allele determinations. Sequencing-based DNA marker is advantageous when there are many alleles of the target gene. All allelic gene sequences should be determined before designing primers. Among thousands of apple genes, *S-RNase* is special in that mRNA sequences of nearly all principal alleles are already reported. Many more sequencing-based DNA markers will be designed, after genomic/transcriptomic sequences of crop cultivars become available in the future.

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Conflicts of interest

The authors have no conflicts of interest to declare.

Supplementary information

Supplementary Figure S1. Incomplete fluorescence peaks of three cultivars.

Supplementary Figure S2. Three hypothetical triploid combinations of *S* alleles.

Supplementary figures are available in journal website (http://www.jspcmb.jp/).

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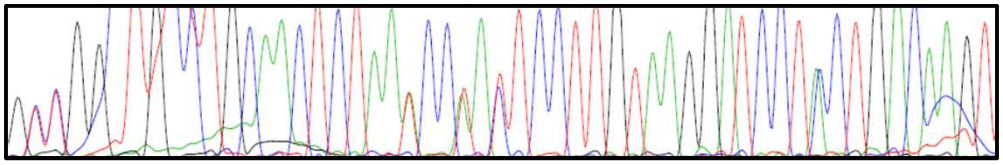
Supplementary Figure S1. Incomplete fluorescence peaks of three cultivars. Fluorescence peaks of Akane, Santarou, and Starking Delicious are shown, together with their *APPLid* sequences.

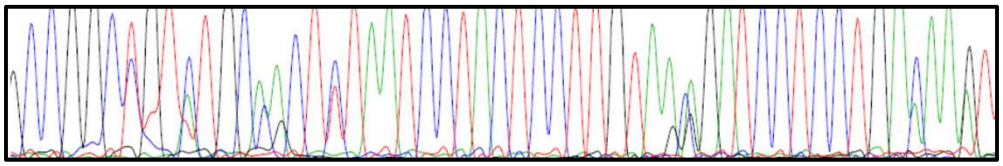
Supplementary Figure S2. Three hypothetical triploid combinations of *S* alleles. *APPLid* sequences of three hypothetical combinations of triplet *S* alleles are shown. Unique nucleotides to each *S* allele are red.

Akane



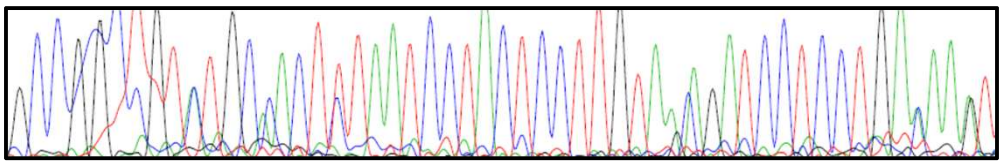
MdS7 GTTGGCTGCCTGCAACTCTAAACCTATTCCTTGTAAGGATCCTCCTGACAAGT
MdS24 GCCGGCTGTCTGCAACTCTAATCCAACTCCTTGTAAGGATCCTACTGACAAGT

Santarou



MdS3 GCCGGCTGTCTGCAGCTCTAATCCTACTCCTTGTAAGGATCCTCCTGACAAGT
MdS9 GCCGGCTGTCTGCCACTTTAATCCTACTCCTTGTAGAGATCCTCCTGACAAGT
MdS28 GCCGGCCGTATGCAACTCTAATCCTACTCCTTGTAACGATCCTCCTGAAAAAT

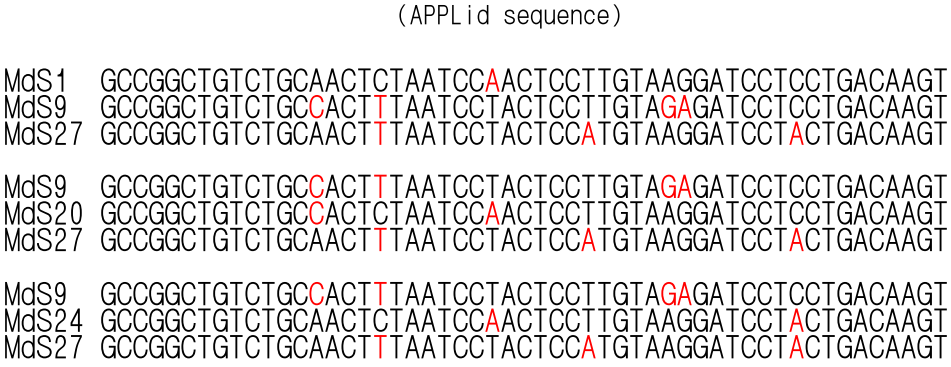
Starking Delicious



MdS9 GCCGGCTGTCTGCCACTTTAATCCTACTCCTTGTAGAGATCCTCCTGACAAGT
MdS28 GCCGGCCGTATGCAACTCTAATCCTACTCCTTGTAACGATCCTCCTGAAAAAT

Supplementary Figure 1

(Kasajima et al.)



Supplementary Figure 2

(Kasajima et al.)