Molecular Characterization of Apple Cultivars in Japan by S-RNase Analysis and SSR Markers

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ABSTRACT. We examined the genetic diversity and relatedness among apple (*Malus ×domestica* Borkh.) cultivars in Japan. The 42 apple cultivars, including major cultivars in Japan, were divided into five groups based on SSR genotypes. Most economically important cultivars belong in three groups: Fuji–Delicious, Golden Delicious, and Jonathan groups, and their genetic backgrounds seemed to be narrow. We also investigated the parent–offspring relationships of nine apple cultivars. ‘Jonathan’, ‘Fuji’, and ‘Rero 11’ were identified as the respective paternal parents of three cultivars described as having unknown paternal parents (i.e., ‘Akagi’, ‘Ambitious’, and ‘Hokuto’). ‘Starking Delicious’, ‘Senshu’, and ‘Golden Delicious’, rather than ‘Ralls Janet’, ‘Hatsuaki’, and ‘Indo’, seemed to be the paternal parents of ‘Kinsei’, ‘Kiou’, and ‘Mellow’, respectively. ‘Carolina Red June’ was excluded as a paternal parent of ‘Ranzan’. Both attributed parents of ‘Scarlet’ (‘Akane’ and ‘Starking Delicious’) were excluded, and it was suggested that ‘Fuji’ was used as either a maternal or a paternal parent of ‘Scarlet’. ‘Jonathan’ rather than ‘McIntosh’ seems to be a maternal parent of ‘Yukari’.

The apple industry in Japan developed following the importation of 75 cultivars from the United States in 1871. The major cultivars in Japan evolved from ‘Ralls Janet’ and ‘Jonathan’ in the 1960s to early 1970s to ‘Starking Delicious’ in the 1970s. Then, ‘Fuji’ replaced them, and is today the leading cultivar in Japan (Soejima et al., 2000). ‘Fuji’ was produced by ‘Ralls Janet’ × ‘Delicious’. Other main cultivars are ‘Tsugaru’ for the early season (produced by ‘Golden Delicious’ × ‘Jonathan’), ‘Jonagold’ for the mid-season (produced by ‘Golden Delicious’ × ‘Jonathan’), and ‘Orin’ for the late season (produced by ‘Golden Delicious’ × ‘Indo’). As a limited number of cultivars were used reproducibly as parents for the breeding of major cultivars in Japan, those cultivars might be characterized by a narrow genetic origin with limited genetic diversity.

Apples have a gametophytic self-incompatibility (GSI) that is due to the failure of pollen tube growth in the style when the pollen shares the same S-haplotype with the pistil (de Nettancourt, 1977). GSI enforces outbreeding and results in heterozygosity. To date, we have investigated the S-genotypes of more than 350 apple cultivars, lineages, and species by the PCR-digestion

method developed based on the polymorphism of an S-locus gene in the pistil (Broothaerts et al., 1995; Janssens et al., 1995; Kitahara and Matsumoto, 2002a, 2002b; Kitahara et al., 2000; Matsumoto and Kitahara, 2000; Matsumoto et al., 1999a, 1999b; Verdoodt et al., 1998). The gene encoding ribonuclease is called S-RNase. Having found that the pedigrees of some cultivars were uncertain due to discrepancies in the inheritance of an S-RNase allele, we established or corrected those pedigrees by the analysis of simple sequence repeats [SSRs (also called microsatellites)] (Kitahara et al., 2005).

SSRs have become the accepted markers of plant species, and have been found suitable for the assessment of genetic diversity within species and of genetic relationships among species (Gianfranceschi et al., 1998; Gupta et al., 1996; Martinez-Gomez et al., 2003). SSRs have also been used as genetic markers for parentage analyses in grapes (*Vitis vinifera* L.) (Bowers and Meredith, 1997; Bowers et al., 1999; Sefc et al., 1997, 1998), peaches [*Prunus persica* (L.) Batsch.] (Testolin et al., 2000; Yamamoto et al., 2003), and pears [*Pyrus pyrifolia* (Burm.) Nakai] (Kimura et al. 2003). In apples, SSR markers have been used for the molecular characterization and identification of cultivars and for genetic mapping. For instance, Guilford et al. (1997) used three SSR markers for differentiation among 21 cultivars. Gianfranceschi et al. (1998) and Hokanson et al. (1998, 2001) developed 16 and eight SSRs, respectively, to distinguish cultivars, identify cultivar synonyms and misidentified accessions, estimate genetic diversity, and identify genetic relationships among cultivars. The genetic linkage maps of the apple have

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been constructed using SSR markers (Gianfranceschi et al., 1998; Hemmat et al., 2003; Liebhard et al., 2002, 2003; Maliepaard et al., 1998). Previously, we analyzed 19 SSR markers of 22 apple cultivars for the parent identification of eight apple cultivars (Kitahara et al., 2005). It is important for the development of efficient apple-breeding programs to accurately determine parent-offspring relationships.

In this study, we analyzed 19 SSR markers of another 20 apple cultivars to establish the genetic relatedness among cultivars and for the parent identification of nine apple cultivars. We also analyzed *S*-RNase allele genotypes of some apple cultivars relating to the parent identification and having apetalous flowers.

Materials and Methods

PLANT MATERIAL. We used 42 apple cultivars to analyze genetic relatedness and parent-offspring relationships. All *Malus* Mill. plants used in this study were from collections at the Apple Research Center of the National Institute of Fruit Tree Science, Tsukuba, Japan, or the Nagano Fruit Tree Experiment Station, Nagano, Japan. Young leaves were collected and stored at -80°C until use.

S-ALLELE SPECIFIC PCR-DIGESTION ANALYSIS. Total DNA from the leaves of individual plants was isolated as described by Thomas et al. (1993). The primers and conditions used for the *S*-allele-specific PCR amplification and digestion were essentially those described by Broothaerts et al. (1995) (*S2*- and *S3*-allele), Janssens et al. (1995) (*S7*- and *S9*-allele), Kitahara et al. (2000) (*S24*-allele), Kitahara and Matsumoto (2002a) (*S10*-allele), Kitahara and Matsumoto (2002b) (*S25*-allele), Matsumoto et al. (1999a) (*S5*- and *S7*-allele), Matsumoto et al. (1999b) (*S1*- and *S20*-allele), Matsumoto and Kitahara (2000) (*S28*-allele), and Verdoodt et al. (1998) (*S4*-, *S26*- and *S16*(=27)-allele).

SSR AMPLIFICATION OF 19 SSR PRIMER PAIRS. CH01c06, CH01d08, CH01d09, CH01f03b, CH01f07a, CH01g05, CH02b07, CH02c02b, CH02c09, CH02d08, CH02g04, CH03a04, CH03a09, CH03d07, CH03d12, CH03e03, CH05a04, CH05c04, and CH05g08 were used for the genotyping of apple cultivars (Liebhard et al., 2002). The reaction to DNA amplification was conducted in a 20- μL mixture consisting of 10 ng of genomic DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl_2 , 0.01% gelatin, 200 μM each deoxynucleotides, 0.5 μM each of three forward primers labeled with a fluorescent chemical (FAM, VIC, or NED) in addition to an unlabelled reverse primer, and one unit of *Taq* DNA polymerase (Applied Biosystems, Foster City, Calif.). The analysis was programmed in a thermal cycler (GeneAmp 2700 apparatus; Applied Biosystems) and conducted under the following conditions: 5 min preheating at 94°C , 1 min at 94°C , 1 min at 55°C , and 2 min at 72°C . The PCR products were separated and detected using a PRISM 310 DNA sequencer (Applied Biosystems). The size of the amplified bands was calculated based on an internal standard DNA (GeneScan 400HD Rox; Applied Biosystems) with GeneScan software (Applied Biosystems).

STATISTICAL ANALYSIS. The observed heterozygosity was calculated as the number of heterozygous genotypes at a given locus divided by the total number of genotypes scored at that locus. The number of expected heterozygous genotypes was calculated based on the frequency of each genotype according to the formula: $H_e = 1 - \sum p_i^2$, where p_i is the frequency of the i^{th} allele of each SSR locus. A phenogram of 42 cultivars was constructed using the unweighted pair-group method using

arithmetic averages (UPGMA) based on Nei's genetic identity (Nei, 1972). The program NTSYS-pc, version 2.1 was used to construct the phenogram (Rohlf, 2000).

Results and Discussion

MOLECULAR DIVERSITY OF APPLE CULTIVARS IN JAPAN. The number of alleles per locus ranged from four for CH02c02b and CH05c04 to nine for CH03a04, CH03d07, and CH05a04, giving an average number of 6.6 (Table 1). The observed heterozygosity (H_o) for individual loci ranged from 0.35 for CH05g08 to 0.90 for CH01c06 with an average respective value of 0.72 (Table 1). The expected heterozygosity (H_e) for individual loci ranged from 0.49 for CH05g08 to 0.81 for CH02d08 with an average respective value of 0.71 (Table 1). Genetic relatedness among 42 apple cultivars was examined based on SSR genotypes. The dendrogram generated from the UPGMA cluster analysis enabled us to identify five major groups and one individual cultivar (Fig. 1). The first group, corresponding to 'Fuji' through 'Empire' (Fuji-Delicious group), is comprised of all cultivars with at least one-half 'Fuji' or 'Delicious' in their pedigrees except for 'Ralls Janet'. Since 'Ralls Janet' and 'Delicious' were used as the maternal and the paternal parent of 'Fuji', respectively, both must be close to 'Fuji' genetically. The second group, corresponding to 'Orin' through 'Ranzan' (Golden Delicious group), includes all cultivars that have at least one-quarter 'Golden Delicious' in their pedigrees, except for 'Indo', 'Yellow Newtown Pippin', and 'Ranzan'. 'Indo' and 'Yellow Newtown Pippin' seem to be close to 'Golden Delicious' genetically. We will discuss 'Ranzan' further below. The third group, corresponding to 'Yoko' through 'Jonathan' (Jonathan group), is made up of all cultivars with at least one-half 'Jonathan' in their pedigrees. The fourth group, corresponding to 'Spencer

Table 1. 19 SSR loci used for characterization of 40 apple cultivars as shown in Fig. 1 (we did not use triploid cultivars Hokuto or Mutsu). Observed heterozygosity (H_o) was calculated by division of the individuals showing heterozygous type by total number of individuals. Expected heterozygosity (H_e) was calculated using the formula $H_e = 1 - \sum p_i^2$, where p_i is the frequency of the i^{th} allele of each SSR locus.

SSR locus	Range of product size (bp)	Putative alleles (no.)	Observed heterozygosity (H_o)	Expected heterozygosity (H_e)
CH05g08	173–180	5	0.35	0.49
CH03e03	186–206	5	0.78	0.73
CH02c02b	112–126	4	0.41	0.62
CH03a04	90–124	9	0.85	0.73
CH03a09	131–143	6	0.73	0.72
CH03d07	185–228	9	0.80	0.77
CH03d12	108–146	6	0.55	0.52
CH01c06	156–188	6	0.90	0.78
CH01f03b	139–179	5	0.80	0.70
CH01f07a	178–206	7	0.80	0.77
CH02b07	103–126	7	0.73	0.71
CH02d08	212–254	8	0.88	0.81
CH01d09	130–176	8	0.63	0.80
CH05c04	186–210	4	0.58	0.56
CH01g05	140–174	7	0.73	0.70
CH01d08	238–292	6	0.80	0.77
CH02c09	233–259	7	0.85	0.80
CH05a04	159–212	9	0.85	0.80
CH02g04	187–196	7	0.63	0.64

Fig. 1. Phenogram for the 42 apple cultivars evaluated in this study. The phenogram was produced based on SSR genotypes from 19 SSR loci using the UPGMA method of Nei's genetic identity between the cultivars. Parents of the cultivars are in parentheses; G.D. = 'Golden Delicious'.

Seedless' through 'Noblow', is comprised of all cultivars having apetalous flowers with the development of parthenocarpic fruits. We identified only the *S3*-RNase allele from both 'Wellington Bloomless' and 'Wickson', while the *S*-RNase allele genotypes of 'Spencer Seedless' and 'Noblow' were identified as *S1S9* and *S4S24*, respectively. All 38 alleles from the 19 SSR loci in 'Wellington Bloomless' and 'Wickson' were completely identical, strongly suggesting that both might be either genetically close or the same cultivar. The observed dendrogram based on SSR analysis revealed close genetic relationships among 'Spencer Seedless', 'Noblow', 'Wellington Bloomless', and 'Wickson'. The fifth group is 'Akane' and 'Worcester Pearmain' (Fig. 1). The paternal parent of 'Akane' is 'Worcester Pearmain'.

Since one allele of the parent was transmitted to its progeny, these results were quite reasonable. The dendrogram might make it possible to know which parent is genetically closer to the cultivar. For instance, 'Aika No Kaori' and 'Hida' are closer to the maternal parent 'Fuji' than to the paternal parents 'Tsugaru' and 'Golden Delicious' (Fig. 1). Also, 'Yoko', 'Hatsuaki', and 'Tsugaru' are closer to 'Jonathan' than to 'Golden Delicious', while 'Akane' is closer to 'Worcester Pearmain' than to 'Jonathan' (Fig. 1). The

similarity will be relative to the loci that we test. Almost all SSR alleles among apple cultivars in Japan were not unique. Since only limited numbers of cultivars have been used as progenitors in breeding programs, we ought to be using different cultivars for an expansion of the genetic base.

PARENTAGE ANALYSIS OF 'AKAGI', 'AMBITIOUS', 'HOKUTO', 'KINSEI', 'KIOU', 'MELLOW', 'RANZAN', 'SCARLET', AND 'YUKARI'. 'Akagi' and 'Ambitious' are chance seedlings of 'Golden Delicious' x an unknown paternal parent, and 'Toko' x an unknown paternal parent, respectively. 'Jonathan' (*S7S9*) and 'Fuji' (*S1S9*) have likely been used as paternal parents of 'Akagi' (*S3S7*) and 'Ambitious' (*S2S9*), respectively, based on *S*-RNase content of them and the maternal parents, 'Golden Delicious' (*S2S3*) and 'Toko' (*S2S7*) (Table 2), and fruit and branch characteristics. Thirty-eight alleles in 'Akagi' and 'Ambitious' have been inherited from 'Golden Delicious' and 'Jonathan', and 'Toko' and 'Fuji', respectively, without discrepancy, indicating that 'Akagi' and 'Ambitious' are hybrids of those two cultivars (Tables 3, 4). The fact that 'Jonathan' and 'Fuji' were used as the paternal parents of 'Akagi' and 'Ambitious', respectively, is also supported by the phenogram results (Fig. 1).

Table 2. *S*-RNase allele genotypes and breeding process of 30 apple cultivars. Only the cultivars with parentage questions and having apetalous flowers were selected.

Cultivar	<i>S</i> -RNase allele genotypes	References	Breeding process of the cultivars relating to parental questions
Wellington Bloomless	<i>S3?</i>	This work	
Wickson	<i>S3?</i>	This work	
Spencer Seedless	<i>S1S9</i>	This work	
Noblow	<i>S4S24</i>	This work	
Akagi	<i>S3S7</i>	Matsumoto et al., 1999a	Sown in 1962, selected in 1969, named in 1973
Ambitious	<i>S2S9</i>	Kitahara et al., 2000	Sown and selected in 1971–73, selected in 1981, registered in 1985
Golden Delicious	<i>S2S3</i>	Janssens et al., 1995	Introduced into Japan in 1923
Toko	<i>S2S7</i>	Matsumoto et al., 1999b	‘Golden Delicious’ was crossed with ‘Indo’ in 1930, fruited in 1938, named in 1962
Jonathan	<i>S7S9</i>	Matsumoto et al., 1999a	Introduced into Japan in 1871
Fuji	<i>S1S9</i>	Matsumoto et al., 1999a	‘Ralls Janet’ was crossed with ‘Delicious’ in 1939, selected in 1958, registered in 1962
Hokuto	<i>S1S7S9</i>	Sakurai et al., 2000	Selected in 1980, registered in 1983
Redspur Delicious	<i>S9S28²</i>	Matsumoto and Kitahara, 2000	Introduced into Japan in 1960
Starkrimson Delicious	<i>S9S28²</i>	Matsumoto and Kitahara, 2000	Discovered in 1953, registered in 1957, introduced into Japan in 1960
Aori 3	<i>S2S28</i>	This work	‘Toko’ was crossed with ‘Richared Delicious’ in 1952, selected in 1963, named in 1970
Rero 11	<i>S7S28</i>	This work	‘Toko’ was crossed with ‘Richared Delicious’ in 1952, published in 1967
Kinsei	<i>S2S9</i>	Matsumoto et al., 1999a	Fruited in 1962, named in 1968, registered in 1972
Ralls Janet	<i>S1S2</i>	Matsumoto et al., 1999a	Introduced into Japan in 1871
Kiou	<i>S1S7</i>	Matsumoto et al., 2003a	Sown in 1984, selected in 1988, registered in 1994
Orin	<i>S2S7</i>	Sakurai et al., 1997	‘Golden Delicious’ was crossed with ‘Indo’, named in 1952
Hatsuaki	<i>S3S9</i>	Matsumoto et al., 1999a	‘Jonathan’ was crossed with ‘Golden Delicious’ in 1939, selected in 1959, named and registered in 1976
Senshu	<i>S1S7</i>	Matsumoto et al., 1999b	‘Toko’ was crossed with ‘Fuji’ in 1966, selected in 1974, named in 1978, registered in 1980
Mellow	<i>S2S3</i>	Matsumoto et al., 2003a	Selected in 1973, registered in 1990
Indo	<i>S7S20</i>	Matsumoto et al., 1999b	Sown in 1875, the first cultivar produced in Japan
Ranzan	<i>S2S9</i>	This work	Registered in 1985
Starking Delicious	<i>S9S28</i>	Matsumoto and Kitahara, 2000	Introduced into Japan in 1929
Carolina Red June	<i>S3S10</i>	This work	Introduced into Japan in 1907
Scarlet	<i>S3S9</i>	Matsumoto et al., 2003b	‘Akane’ was crossed with ‘Starking Delicious’ in 1970, selected in 1973, registered in 1984
Akane	<i>S7S24</i>	Kitahara et al., 2000	‘Jonathan’ was crossed with ‘Worcester Pearmain’ in 1939, registered in 1970
Yukari	<i>S2S9</i>	Matsumoto et al., 2003b	‘McIntosh’ was crossed with ‘Golden Delicious’ in 1970, selected in 1975, registered in 1986
McIntosh	<i>S10S25</i>	Kitahara and Matsumoto, 2002a, b	Introduced into Japan in 1890

²*S*-RNase alleles of ‘Redspur Delicious’ and ‘Starkrimson Delicious’ are from that of ‘Starking Delicious’ since both are sports of ‘Starking Delicious’.

Previously, we excluded ‘Mutsu’ as the paternal parent of ‘Hokuto’, but could not find a true paternal parent since the parental candidates ‘Indo’ and ‘Shin Indo’ were excluded as the paternal parent of ‘Hokuto’ by SSR markers (Kitahara et al., 2005). We selected ‘Redspur Delicious’ (a sport of ‘Starking Delicious’), ‘Starkrimson Delicious’ (a sport of ‘Starking Delicious’), ‘Aori 3’, and ‘Rero 11’ as the remaining candidate for the paternal parent of ‘Hokuto’ from the breeding register. From the *S*-RNase content of ‘Hokuto’ (*S1S7S9*) and its maternal parent, ‘Fuji’ (*S1S9*), Rero 11 (*S7S28*) rather than ‘Redspur Delicious’ (a sport of ‘Starking Delicious’ *S9S28*), ‘Starkrimson Delicious’ (a sport of ‘Starking Delicious’ *S9S28*), and ‘Aori 3’ (*S2S28*) seemed to be the paternal parent of ‘Hokuto’ (Table

2). We investigated 19 SSR loci to confirm the paternal parent of ‘Hokuto’. All 57 alleles in ‘Hokuto’ have been inherited from ‘Fuji’ (38 alleles) and ‘Rero 11’ (19 alleles) without discrepancy, confirming that ‘Hokuto’ is a hybrid of both those cultivars (Tables 3, 4). The UPGMA cluster analysis data confirmed that ‘Hokuto’ belonged to the Fuji–Delicious group, while ‘Rero 11’ belonged to the Golden Delicious group (Fig. 1). The dendrogram reveals genetic similarity, but not family pedigree. ‘Hokuto’ is a triploid, and the 2n and n gametes are contributed by the maternal parent ‘Fuji’ and the paternal parent ‘Rero 11’, respectively. Since ‘Hokuto’ seems to be genetically closer to ‘Fuji’ than to ‘Rero 11’, ‘Hokuto’ might belong to the Fuji–Delicious group.

Table 3. SSR genotypes of 22 apple cultivars analyzed using 19 SSR loci. Only the cultivars having parentage questions were selected. SSR genotypes are denoted by a combination of two or three putative alleles indicated by their size (bp).

Cultivar	SSR genotype (bp)				
	CH05g08	CH03e03	CH02c02b	CH03a04	CH03a09
Golden Delicious ^z	175/175	200/204	116/122	124/124	131/135
Akagi	175/175	204/206	112/116	100/124	135/143
Jonathan ^z	175/179	198/206	112/112	100/116	131/143
Toko ^z	175/175	186/204	112/122	120/124	131/139
Ambitious	175/175	186/198	112/116	124/124	131/131
Fuji ^z	175/175	186/198	112/116	94/124	131/131
Hokuto ^z	175/175/175	186/198/204	112/116/	94/124/	131/135/
Rero 11	175/175	204/204	112/112	96/124	135/139
Kinsei	175/175	186/204	116/116	96/124	131/135
Ralls Janet	175/175	186/198	112/122	94/124	131/135
Starking Delicious ^z	175/179	186/204	112/116	96/124	131/135
Orin ^z	175/175	200/200	112/122	124/124	135/139
Kiou	175/175	186/200	112/122	94/124	131/139
Hatsuaki	175/179	200/206	112/122	100/124	135/143
Senshu	175/175	186/204	112/112	94/120	131/139
Mellow	175/175	200/204	112/122	124/124	131/131
Indo ^z	175/179	186/200	112/112	120/124	131/139
Ranzan	179/179	186/204	116/116	96/124	135/135
Carolina Red June	173/179	186/186	116/122	124/124	133/141
Akane	173/175	198/206	112/116	100/116	131/135
Scarlet	175/180	186/186	112/122	94/124	131/141
Yukari	175/175	198/204	112/116	100/124	131/135

Cultivar	SSR genotype (bp)				
	CH03d07	CH03d12	CH01c06	CH01f03b	CH01f07a
Golden Delicious	188/206	124/124	156/162	139/171	178/198
Akagi	188/202	124/124	162/188	171/171	178/194
Jonathan	202/206	124/124	188/188	171/171	194/196
Toko	188/218	124/124	162/188	139/177	178/178
Ambitious	188/226	124/124	160/162	139/179	178/206
Fuji	226/226	116/124	158/160	171/179	178/206
Hokuto	188/226/	116/124/	158/160/162	171/177/179	178/206/
Rero 11	188/206	124/124	160/162	177/179	178/198
Kinsei	188/206	116/124	160/162	139/139	178/206
Ralls Janet	226/226	116/124	156/158	171/171	178/190
Starking Delicious	206/226	116/124	160/160	139/179	198/206
Orin	206/226	124/124	162/188	139/177	196/198
Kiou	188/226	116/124	162/162	139/177	178/196
Hatsuaki	188/202	124/124	162/188	171/171	178/196
Senshu	188/226	116/124	158/162	139/179	178/178
Mellow	188/206	124/124	160/162	171/177	178/198
Indo	218/226	116/124	160/188	177/179	178/196
Ranzan	188/206	124/124	156/160	139/171	178/206
Carolina Red June	206/226	116/122	162/162	171/171	194/196
Akane	186/206	124/146	156/188	139/171	194/194
Scarlet	206/226	122/124	160/162	171/179	178/194
Yukari	188/202	124/124	156/188	171/171	194/198

Table 3 continued on next page.

'Kinsei', 'Kiou', 'Mellow', and 'Ranzan' were produced by 'Golden Delicious' x 'Ralls Janet' in 1954, 'Orin' x 'Hatsuaki' in 1983, '19Gou' ('Golden Delicious' crossed with 'Indo') x 'Indo' in 1958, and 'Starking Delicious' x 'Carolina Red June' in 1955, respectively. However, the *S*-RNase contents of 'Kinsei' (*S2S9*), 'Kiou' (*S1S7*), 'Mellow' (*S2S3*), and 'Ranzan' (*S2S9*) did not match any of the expected *S*-RNase contents from their reported parents, 'Golden Delicious' (*S2S3*) x 'Ralls Janet' (*S1S2*), 'Orin' (*S2S7*) x 'Hatsuaki' (*S3S9*), '19Gou' ['Golden Delicious' (*S2S3*) x 'Indo' (*S7S20*)] x 'Indo' (*S7S20*), and 'Starking Delicious' (*S9S28*) x

'Carolina Red June' (*S3S10*), respectively (Table 2). Previously, we confirmed their *S*-genotypes by pollination tests (Matsumoto et al., 1999a, 2003b, unpublished results), which suggested that some other paternal (or maternal in the case of 'Kinsei') parents possessing the *S9*, *S1*, *S2* (or *S3*), and *S2* might have been used to produce 'Kinsei', 'Kiou', 'Mellow', and 'Ranzan', respectively. We selected 'Starking Delicious', 'Senshu', 'Golden Delicious', and 'Golden Delicious' (or 'Ralls Janet') as the parents of 'Kinsei', 'Kiou', 'Mellow', and 'Ranzan', respectively, from their *S*-RNase

Table 3. Continued from previous page.

Cultivar	SSR genotype (bp)				
	CH02b07	CH02d08	CH01d09	CH05c04	CH01g05
Golden Delicious	103/111	222/224	132/134	186/200	140/146
Akagi	103/126	224/254	134/134	186/186	140/144
Jonathan	105/126	228/254	134/134	186/186	144/144
Toko	103/111	224/228	130/132	186/200	140/157
Ambitious	103/105	212/228	130/148	186/200	140/140
Fuji	105/105	212/212	148/148	186/208	140/144
Hokuto	103/105/	212/212/212	130/148/	186/200/208	140/144/157
Rero 11	103/103	212/224	130/148	200/200	157/157
Kinsei	103/105	212/224	134/172	200/208	140/157
Ralls Janet	105/126	212/250	148/148	186/210	140/140
Starking Delicious	103/105	212/218	148/172	200/208	144/157
Orin	103/103	222/228	130/134	186/186	140/140
Kiou	103/103	222/224	130/134	186/186	140/140
Hatsuaki	103/105	224/228	132/132	186/200	140/144
Senshu	103/105	212/224	130/148	186/186	140/140
Mellow	103/103	222/224	132/134	186/186	140/140
Indo	103/111	212/228	130/148	186/186	140/157
Ranzan	103/103	212/254	134/172	186/208	157/157
Carolina Red June	103/107	252/254	134/154	186/200	144/174
Akane	124/126	228/250	134/148	186/186	144/159
Scarlet	103/105	212/212	148/172	186/200	144/146
Yukari	105/111	222/228	132/132	186/186	144/146
Cultivar	SSR genotype (bp)				
	CH01d08	CH02c09	CH05a04	CH02g04	
Golden Delicious	248/270	243/257	166/195	187/192	
Akagi	270/270	243/259	166/189	187/191	
Jonathan	238/270	249/257	189/189	191/191	
Toko	248/252	243/243	166/195	187/192	
Ambitious	238/248	233/243	195/195	192/192	
Fuji	238/252	233/245	189/195	192/192	
Hokuto	238/252/	233/243/245	189/195/	187/192/	
Rero 11	252/252	243/255	159/195	187/192	
Kinsei	248/252	243/245	159/195	187/192	
Ralls Janet	252/270	233/257	169/195	191/192	
Starking Delicious	238/252	245/255	159/189	192/192	
Orin	248/252	243/243	165/166	192/192	
Kiou	238/248	243/243	166/195	192/192	
Hatsuaki	270/270	257/257	189/195	187/187	
Senshu	238/248	233/243	166/195	192/192	
Mellow	248/270	243/257	195/195	187/192	
Indo	252/252	243/245	165/195	192/192	
Ranzan	248/252	243/255	159/166	187/192	
Carolina Red June	252/268	257/257	169/189	190/192	
Akane	238/292	245/257	181/189	188/191	
Scarlet	252/252	245/257	169/195	190/192	
Yukari	238/270	257/257	166/189	187/191	

^aData are from Kitahara et al. (2005).

contents ['Starking Delicious' (*S9S28*), 'Senshu' (*S1S7*), 'Golden Delicious' (*S2S3*), and 'Ralls Janet' (*S1S2*)] (Table 2), and from their fruit and branch characteristics. All 38 alleles in 'Kinsei', 'Kiou', and 'Mellow' had been inherited from 'Golden Delicious' + 'Starking Delicious', 'Orin' + 'Senshu', and '19Gou' + 'Golden Delicious', respectively, without discrepancy (Table 3), indicating that they are hybrids of those two cultivars (Table 4). These data were supported by the phenogram results (Fig. 1). In the case of 'Mellow', since we had no samples of '19Gou', we investigated 'Indo' and 'Golden Delicious' instead. In the case of 'Ranzan',

two loci in 'Golden Delicious' (222 bp and 224 bp at CH02d08, and 140 bp and 146 bp at Ch01g05) and seven loci in 'Ralls Janet' (112 bp and 122 bp at CH02c02b, 226 bp at CH03d07, 105 bp and 126 bp at CH02b07, 134 bp and 154 bp at CH01d09, 140 bp at CH01g05, 233 bp and 257 bp at Ch02c09, and 169 bp and 195 bp at Ch05a04) were not inherited by 'Ranzan'. Since one allele in each locus of 'Starking Delicious' was inherited by 'Ranzan' without discrepancy, 'Starking Delicious' seems to have been used as a maternal parent of 'Ranzan'. Although 'Golden Delicious' was excluded as the paternal parent of 'Ranzan', it was

Table 4. Analyses of nine parent–offspring relationships in apple by *S*-RNase content and SSR loci.

Cultivar	Reputed parents	Our results
Akagi	Golden Delicious x unknown	Golden Delicious x Jonathan
Ambitious	Toko x unknown	Toko x Fuji
Hokuto	Fuji x unknown	Fuji x Rero 11
Kinsei	Golden Delicious x Ralls Janet	Golden Delicious x Starking Delicious
Kiou	Orin x Hatsuaki	Orin x Senshu
Mellow	19Gou (Golden Delicious x Indo) x Indo	19Gou x Golden Delicious
Ranzan	Starking Delicious x Carolina Red June	Starking Delicious x unknown
Scarlet	Akane x Starkin Delicious	Fuji x unknown, or unknown x Fuji
Yukari	McIntosh x Golden Delicious	Jonathan x Golden Delicious

suggested that a clone having the *S2*-allele and closely related to ‘Golden Delicious’ might have been used as the paternal parent of ‘Ranzan’ based on the phenogram results (Fig. 1).

Previously, we could not find a paternal parent of ‘Kotoku’, since ‘Delicious’ and ‘Starking Delicious’ were excluded by *S*-RNase content or SSR analyses (Kitahara et al., 2005). From the phenogram results (Fig. 1), a clone having the *S28*-allele and closely related to ‘Delicious’ and ‘Starking Delicious’ might have been used as the paternal parent of ‘Kotoku’.

The breeding process of ‘Scarlet’ is as follows: ‘Akane’ was crossed with ‘Starking Delicious’ in 1970, selected in 1973, registered in 1984. The *S*-RNase content of ‘Scarlet’ (*S3S9*) by PCR-digestion analysis did not match any of the expected *S*-RNase contents from its supposed parents ‘Akane’ (*S7S24*) and ‘Starking Delicious’ (*S9S28*) (Table 2). As the *S*-genotype of ‘Scarlet’ was also confirmed by pollination tests (Matsumoto et al., 2003b), ‘Akane’ appeared not to have been used as the maternal parent of ‘Scarlet’. Moreover, two loci within 19 SSR loci in ‘Starking Delicious’ (198 bp and 206 bp) at CH01f07a, and 159 bp and 189 bp at CH05a04) were not inherited by ‘Scarlet’, suggesting that ‘Starking Delicious’ was not the paternal parent of ‘Scarlet’ (Table 3). At least ‘Fuji’ seems to be related to the production of ‘Scarlet’ from its *S*-RNase content (*S1S9*), phenogram results (Fig. 1), and fruit and branch characteristics. Based on the SSR analysis, one allele in each locus of ‘Fuji’ was inherited by ‘Scarlet’ without discrepancy (Table 3), also supporting the likelihood that ‘Fuji’ was used as a maternal or a paternal parent of ‘Scarlet’ (Table 4). Although ‘Starking Delicious’ was excluded as a parent of ‘Scarlet’, a clone possessing the *S28*-allele and closely related to ‘Starking Delicious’ that was unregistered during the breeding process might have been used as another parent of ‘Scarlet’, based on data of the *S*-RNase content (Table 2) and phenogram results (Fig. 1).

‘Yukari’ was produced by ‘McIntosh’ x ‘Golden Delicious’. However, its *S*-RNase content *S2S9* by PCR-digestion analysis and pollination tests (Matsumoto et al., 2003b) did not match any of the expected *S*-RNase contents from its supposed parents, ‘McIntosh’ (*S10S25*) and ‘Golden Delicious’ (*S2S3*) (Table 2). From the 19 SSR loci analysis, one allele in each locus of ‘Golden Delicious’ was inherited by ‘Yukari’ without discrepancy (Table 3), suggesting that ‘Golden Delicious’ was used as a paternal parent of ‘Yukari’. These results suggested that ‘McIntosh’ had not been used as the maternal parent of ‘Yukari’. ‘Fuji’ (*S1S9*) and ‘Jonathan’ (*S7S9*) were selected as the maternal parents of ‘Yukari’ from their *S*-RNase contents (Table 2), and fruit and branch characteristics. Seven loci within the 19 SSR loci in ‘Fuji’ (226 bp at CH03d07, 158 bp and 160 bp at CH01c06, 178 bp and 206 bp at CH01f07a, 212 bp at CH02d08, 148 bp at CH01d09, 233 bp and 245 bp at CH02c09, and 192 bp at CH02g04) were

not inherited by ‘Yukari’, while all 38 alleles except one (134 bp at CH01d09) in ‘Yukari’ had been inherited from ‘Jonathan’ and ‘Golden Delicious’ without discrepancy (Table 3). Homozygous 132 bp and 134 bp at CH01d09 in ‘Yukari’ and ‘Jonathan’, respectively, might be heterozygous, one with a null allele. These results strongly suggested that ‘Yukari’ was a hybrid of ‘Jonathan’ and ‘Golden Delicious’, a probability supported by the phenogram results (Fig. 1).

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