Genotyping of *Citrus* Accessions with S_9 and/or S_{10} Alleles for Self-incompatibility and Their Allelic Distribution

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Abstract

Gametophytic self-incompatibility, one of the key characteristics for breeding seedless *Citrus* cultivars, occurs in pummelo (*Citrus maxima*), mandarin (*Citrus reticulata*), and their hybrid cultivars. Allelic variation in *Citrus* was reported for the self-incompatibility gene (*S*); however, *S* allele frequencies and *S* genotypes of full- and semi-self-incompatible cultivars have been reported for a small number of alleles. To extend our knowledge of *S* alleles, we tested 146 *Citrus* accessions, including 82 pummelo accessions, for S_9 and S_{10} alleles. Each accession was pollinated with homozygous S_1 seedlings of 'Hirado Buntan' pummelo (S_9S_9 and $S_{10}S_{10}$). The pollen tube growth arrest in the lower styles of their pollinated pistils indicated that four accessions, including 'Hirado Buntan [*Citrus maxima* (Burm.) Merr.]', have the S_9 allele and five accessions, including 'Hirado Buntan', have the S_{10} allele. The percentage of accessions with the S_9 allele was 3.2% (4 of 126 accessions examined), and the S_9 allele frequency was 1.8% (4 of 217 alleles, excluding the S_f allele). The percentage of accessions with the S_{10} allele was 3.9% (5 of 127 accessions examined), and the S_{10} allele frequency was 2.3% (5 of 217 alleles, excluding the S_f allele). Japanese mandarin (another sources of *S* alleles) and its relatives had neither the S_9 nor the S_{10} allele. Pummelo accessions had S_9 and S_{10} alleles at higher rates of 2.9% (2 of 70 accessions examined) and 7.0% (5 of 71 accessions examined), respectively. 'Kabusu' sour orange (a pummelo-mandarin hybrid; *Citrus aurantium*) and 'Kikudaidai' (a sour orange relative; *Citrus canaliculata*) had S_9 alleles. These results suggested that the two alleles originated from pummelo (the main sources of *S* alleles). The *S* genotypes with S_9 and/or S_{10} alleles were fully determined in 'Hirado Buntan' (S_9S_{10}), 'Kabusu' sour orange (S_fS_9), the 'Kikudaidai' (S_9S_{11}) sour orange hybrid, and two local pummelo plants. The results of our study suggest that in comparison with the other *S* alleles reported, the pummelo plants with low frequencies of S_9 and/or S_{10} alleles contributed to very low rates of evolution and development of *Citrus* species and cultivars during the long history of citrus cultivation, except for those generating sour oranges (*Citrus auratinum*), which are used as root stocks, and for flesh and rind processing and ornamental plants. Finally, we examined the degree of self-incompatibility between S_9 and S_{10} alleles in the lower part of styles of S_1 seedlings of 'Hirado Buntan' and *Citrus* accessions with S_9 and/or S_{10} alleles. The result indicated no difference in the self-incompatibility reaction between the two alleles.

Introduction

Citrus is cultivated throughout the world, from tropical to temperate climates where there are suitable soils, sufficient moisture, and optimal temperatures (Soost and Roose, 1996). The importance of the citrus industry to agriculture and the economy is demonstrated by this large-scale production. *Citrus* is known to have gametophytic self-incompatibility (SI) governed by a single gene (*S*) in pummelo [*Citrus maxima* (Burm.) Merr.], mandarin (*Citrus* spp.), and their hybrids (Soost, 1987; Yamamoto et al., 2006; Kim et al., 2011; Ngo et al., 2019). SI is an important trait for breeding and seedless fruit production in *Citrus* cultivars with a high degree of parthenocarpy (Iwamasa and Oba, 1980; Vardi et al., 2000). For *Citrus*, however, there is very limited information about the self-incompatibility *S* gene sequence (Honsho et al., 2019; Liang et al., 2020), *S* allele variation, *S* allele frequencies, and the *S* genotypes of cultivars (Kim et al., 2020).

Ngo et al. (2019) studied the patterns and strength of self-incompatibility in 121 *Citrus* accessions, including 77 self-incompatible pummelos. They found that the degree of SI in the stigmas, upper styles, middle styles, and basal styles of the self-incompatible pistils differed and could be divided into three categories: high, moderate, and low. Eventually, there were no or only a few pollen tubes in the base of the self-pollinated styles of these self-incompatible accessions. These results suggested that self-incompatibility in a given *Citrus* accession could be determined only by observation of the pollen tubes in the basal style (Ngo et al., 2019).

To elucidate the self-incompatibility genotypes of *Citrus* cultivars, Ngo et al. (2011) used *Got-3* allozyme markers associated with the self-incompatibility *S* gene and proposed the S_1S_2 genotype for 'Banpeiyu' (*Citrus maxima*), S_1S_3 for 'Tosa Buntan' (*Citrus maxima*), S_4S_5 for 'Hassaku' (*Citrus hassaku*), S_6S_7 for 'Yuge Hyokan' (*Citrus yuge-hyokan*), and S_1S_8 for 'Hyuganatsu' (*Citrus tamurana*). For the S_1 and S_2 alleles proposed by Ngo et al. (2011), Kim et al. (2011) determined the *S* genotypes in seven full- or semi-self-incompatible *Citrus* accessions by pollination with two homozygous S_1 seedlings (S_1S_1 and S_2S_2), each derived from self-pollination of the self-incompatible 'Banpeiyu' (S_1S_2) pummelo. For the S_4 and S_5 alleles proposed by Ngo et al. (2011), Zhou et al. (2018) determined 12 full- and semi-self-incompatibility *S* genotypes and one full-self-compatibility *S* genotype (S_fS_f) in *Citrus* accessions by pollination with two homozygous S_1 seedlings (S_4S_4 and S_5S_5), each from self-pollination of the self-incompatible 'Hassaku' (S_4S_5). For the S_3 allele proposed by Ngo et al. (2011) and the S_{11} allele discovered by Kim et al. (2020), Kim et al. (2020) determined the *S* genotypes in 28 full- or semi-self-incompatible *Citrus* accessions by pollination with two homozygous S_1 seedlings (S_3S_3 and $S_{11}S_{11}$), each from self-pollination of the self-incompatible Clementine (S_3S_{11}). In these *S*-genotyping studies with more than 100 full- and semi-self-incompatible *Citrus* accessions, the *S* allele frequency was calculated for S_1 and S_2 (Kim et al., 2011), S_3 (Kim et al., 2020), S_4 and S_5 (Zhou et al., 2018), and S_{11} (Kim et al., 2020) alleles.

In the previous study for S_9 and S_{10} alleles (Kim et al., 2010), homozygous S_1 seedlings (S_aS_a and S_bS_b) of 'Hirado Buntan' (S_aS_b) were produced through bud self-pollination and allelic symbols of S_9 and S_{10} were determined, respectively, for S_a and S_b, and then using the S_9 and S_{10} alleles, pollen detection of *Citrus* accessions with S_9 and S_{10} alleles was carried out focusing on a small number of pummelos growing on Hirado Island adjacent to the northernmost part of

Kyushu Island, Japan. A total of only 18 *Citrus* accessions was used for the determination and detection of S_9 and S_{10} alleles with the homozygous S_1 seedlings (S_9S_9 and $S_{10}S_{10}$) of 'Hirado Buntan' in the previous study. Because of the small number of accessions, we could not determine the origin of the S_9 and S_{10} alleles, the rates of *Citrus* accessions with S_9 or S_{10} alleles, and S_9 and S_{10} allele frequencies in pummelo (a source of S alleles) and *Citrus* accessions (introgression of S alleles from pummelo). The present study was carried out to clarify the origin of the S_9 and S_{10} alleles and to calculate the rates of *Citrus* accessions and pummelos with S_9 or S_{10} alleles and the S_9 and S_{10} allele frequencies in pummelo and *Citrus* accessions through *S*-genotyping of 146 *Citrus* accessions (not including artificial hybrid cultivars) with S_9 or S_{10} alleles. When comparing the results of *S*-genotyping between pummelo (source of *S* alleles) and *Citrus* accessions (introgression of *S* alleles from pummelo), the genetic diversity in pummelo and its relation to the development and evolution of full-self-incompatible and semi-self-incompatible *Citrus* species and cultivars determined using *S* alleles as markers. Here, we aimed to uncover the genetic diversity in pummelo and the development and evolution of *Citrus* species and cultivars by monitoring their *S* alleles.

Materials and Methods

Plant Materials

We collected 146 *Citrus* accessions, including 82 pummelo accessions, to use for pollination with two homozygous S_1 seedlings (S_9S_9 and $S_{10}S_{10}$) of 'Hirado Buntan' [*Citrus maxima* (Burm.) Merr.]. Most *Citrus* cultivars included in these accessions were provided by the National Institute of Fruit Tree Science (NIFTS), the Kumamoto Prefectural Fruit Tree Experiment Station (KMPFTES), and the Kagoshima Prefectural Fruit Tree Experiment Station (KSPFTES). All *Citrus* accessions and homozygous S_1 seedlings (S_9S_9 and $S_{10}S_{10}$) were trees more than ten years old, grown in the Experimental Farm of Kyushu University, Sasaguri, Fukuoka. Self-incompatibility in these accessions had been determined through the observation of pollen tube arrest in their style base eight days after self-pollination (Ngo et al., 2019).

Production of Homozygous S_1 Seedlings of 'Hirado Buntan' for the *S* Gene

Self-incompatible 'Hirado Buntan' was used to produce S_1 seedlings homozygous for the *S* gene (Wakana et al., 2004). Seeds were collected and planted from fruits obtained by bud pollination on 'Hirado Buntan', and these S_1 seedlings were top-worked on twenty-year-old 'Hashimoto Wase' satsuma mandarin (*Citrus unshiu*) trees and allowed to grow for more than ten years, until their flowers were available for pollination examination. The *S* genotype of the top-worked S_1 seedlings homozygous for the *S* gene (S_9S_9 or $S_{10}S_{10}$) was determined by Kim et al. (2010). Of the S_1 seedlings homozygous for the *S* gene, two S_1 seedlings (HBS3 and HBS4) with the S_9S_9 genotype and two S_1 seedlings (Hirado Buntan Seedling No.2; HBS2 and Hirado Buntan Seedling No.11; HBS11) with the $S_{10}S_{10}$ genotype were used to pollinate *Citrus* accessions. In addition to the four S_1 seedlings, HBS19 and HBS25 seedlings with the S_9S_9 genotype and an HBS20 seedling with the $S_{10}S_{10}$ genotype, which were determined in the present study by a self-incompatibility reaction in the lower styles eight days after pollination with S_9 and S_{10} pollen of HBS3 and HBS2, respectively, were used for the present study because they produced enough pollen to carry out the pollination experiment.

Pollination of *Citrus* Accessions by Homozygous S₁ Seedlings (S_9S_9 or $S_{10}S_{10}$)

To examine whether the 146 *Citrus* accessions share one or both of the *S* alleles (S_9 and/or S_{10}) with 'Hirado Buntan' (S_9S_{10}), they were pollinated with the homozygous S₁ seedlings of 'Hirado Buntan', i.e., HBS3, HBS4, HBS19, and HBS25 seedlings with the S_9S_9 genotype and HBS2, HBS11, and HBS20 seedlings with the $S_{10}S_{10}$ genotype. Normal flowers from which petals and stamens were removed just before flowering were selected, and the stigmas of the normal pistils were pollinated with the S_9 or S_{10} pollen of 'Hirado Buntan' homozygous S₁ seedlings until they showed a yellow color; they were then bagged to prevent further pollination. Five emasculated flowers were pollinated per *Citrus* accession in each year. Eight days after pollination, the styles were collected and fixed with acetic acid:alcohol (1:3 v/v). These were stored in 70% ethanol at 4 ℃ until use and were softened with 0.8 N sodium hydroxide solution for 24 hours when used. After that, the softened styles were stained overnight with a solution of 0.1% aniline blue. The stained pistils were divided into five parts (stigma, upper style, middle style, lower style, and ovary). The lower styles were observed under a fluorescence microscope (Nikon 50i). When no or fewer than ten pollen tubes were observed in the lower styles, their upper styles or stigmas were observed to ascertain the presence of a large number of pollen tubes. The pollen-tube observation and the determination of *S* alleles were carried out according to the report of Zhou et al. (2018). The pollen-tube observation was carried out using three pistils per accession and was repeated for two to four years, especially in the accessions in which the incompatibility reaction is not clear. The mean number of pollen tubes in the lower styles observed in each year was averaged per year in each accession.

In the previous study, we determined the *S* genotype of 'Kabusu' sour orange (S_fS_9) by pollination with a homozygous S₁ seedling (S_9S_9) of 'Hirado Buntan' (Kim et al., 2010). However, further certification of the 'Kabusu' *S* genotype was carried out in the present study, since it was determined using two pistils and since 'Zadaidai' sour orange had a different *S* genotype (S_fS_{11}) (Kim et al., 2020) from 'Kabusu' despite that they are considered as clones (personal communication).

Variation of the Number of Pollen Tubes in Lower Styles of Back-cross-incompatible Pistils (S_9S_{10}) When Pollinated with 'Hirado Buntan' (S_9S_{10})

Five heterozygous S₁ seedlings (HBS5, HBS15, HBS18, HBS25 and HBS27) with the S_9S_{10} genotype were pollinated with 'Hirado Buntan'. The number of pollen tubes was examined in lower styles. It was also examined in their upper styles to check the existence of many pollen tubes. The procedure of pollination and pollen tube observation was similar to that mentioned above.

Variation of the Number of Pollen Tubes in the Lower Styles of Incompatible Pistils of *Citrus* Plants Carrying S_9 and/or S_{10} Alleles When Pollinated with S_9 or S_{10} Pollen

In the present study, when 'Hirado Buntan' was pollinated with S_9 pollen of 'Hirado Buntan' S₁ seedlings, almost no S_9 pollen tubes were observed in the lower styles, whereas when pollinated with S_{10} pollen of another 'Hirado Buntan' S₁ seedling, nine S_9 pollen tubes on average were observed per lower style. To examine the difference in the degree of SI in the lower part of styles between S_9 and S_{10} pollen tubes, seven S₁ seedlings [HBS2 ($S_{10}S_{10}$), HBS3 (S_9S_9), HBS4 (S_9S_9), HBS7 (S_9S_9), HBS18 (S_9S_{10}), HBS19 (S_9S_9) and HBS25 (S_9S_9)] and six *Citrus* accessions ['Hirado Buntan' (S_9S_{10}), 'Kabusu' (S_fS_9), Amakusa No.6 (S_9S_{10}), Yatsushiro No.4 ($S_{10}S_{11}$) , 'Bangkok Buntan' ($S_{10}S_?$) and Kawanabe No.2 (S_5S_{10})]

carrying S_9 and/or S_{10} alleles were used. HBS3 (S_9S_9), HBS4 (S_9S_9) and HBS25 (S_9S_9) were used as pollen parents generating S_9 pollen, while HBS2 ($S_{10}S_{10}$) was used as a pollen parent generating S_{10} pollen. The number of pollen tubes was examined in lower styles, and also in their upper styles to check the existence of many pollen tubes. The procedure of pollination with S_9 or S_{10} pollen and pollen tube observation was similar to that mentioned above.

Results

Determination of *Citrus* Accessions with S_9 Pollen

When the S_9 pollen of 'Hirado Buntan' pummelo homozygous S_1 seedlings (S_9S_9) was pollinated to 126 *Citrus* accessions, many S_9 pollen tubes penetrated into the lower styles of 122 accessions (Table 1 and Fig. 1). The average number of pollen tubes observed in the lower styles of the 122 accessions ranged from 38.0 to 488.7. This result suggested that these accessions show cross compatibility with S_9 pollen and do not have an S_9 allele. In four of the 126 accessions, no or only a few pollen tubes penetrated into the lower styles: 0.5 for the Amakusa No. 6 pummelo, 1.0 for the 'Hirado Buntan' pummelo, 0.0 for the 'Kabusu' sour orange, and 0.0 for the 'Kikudaidai' sour orange relative. This result suggested that these accessions show cross incompatibility with S_9 pollen and have an S_9 allele.

Table 1. Determination of *Citrus* accessions with S_9 and/or S_{10} alleles using pollen of homozygous S_1 seedlings of 'Hirado Buntan' (S_9S_{10}) for the self-incompatibility (*S*) gene

Scientific name, plant No., accession (Tanaka's classification No.; Tanaka, 1969)[z], and origin	S_9 pollen[y]		S_{10} pollen[y]		Presence (+) or absence (−) of S_9 and S_{10} alleles	Determined *S* genotype[x]
	No. of flowers pollinated	Mean No. of pollen tubes in style base	No. of flowers pollinated	Mean No. of pollen tubes in style base		
Lime, citron and relatives						
C. limon (L.) Burm.f. (36)						
1. Allen Eureka*[?]	3	146.0	3	109.0	−S_9 −S_{10}	$S_?S_?$
2. Cook Eureka*[?]	3	74.3	3	179.7	−S_9 −S_{10}	$S_?S_?$
3. Lisbon*[?]	3	228.3	3	224.3	−S_9 −S_{10}	$S_?S_?$
4. Villafranca*[?]	3	213.0	3	177.0	−S_9 −S_{10}	$S_?S_?$
Pummelo and relatives						
C. maxima (Burm.) Merr. (56)						
5. Akune No.1** Japan	3	158.7	ne[w]	-	−S_9 ne	$S_?S_?$
6. Akune No.4** Japan	ne	-	3	266.3	ne −S_{10}	$S_?S_?$
7. Akune No.6** Japan	2	180.5	6	127.7	−S_9 −S_{10}	$S_?S_?$
8. Amakusa No.1** Japan	3	176.3	3	120.7	−S_9 −S_{10}	$S_{11}S_?$
9. Amakusa No.2** Japan	3	161.3	3	98.3	−S_9 −S_{10}	$S_5S_?$
10. Amakusa No.3** Japan	3	337.7	3	186.7	−S_9 −S_{10}	$S_?S_?$
11. Amakusa No.4** Japan	3	145.3	3	254.7	−S_9 −S_{10}	$S_?S_?$
12. Amakusa No.6** Japan	6	0.5	6	0.0	+S_9 +S_{10}	S_9S_{10}
13. Amakusa No.8** Japan	5	117.6	3	382.7	−S_9 −S_{10}	$S_?S_?$
14. Amakusa No.12** Japan	3	239.7	3	239.0	−S_9 −S_{10}	$S_?S_?$
15. Amakusa No.13** Japan	3	122.3	ne	-	−S_9 ne	$S_{11}S_?$

Table 1. Determination of *Citrus* accessions with S_9 and/or S_{10} alleles using pollen of homozygous S_1 seedlings of 'Hirado Buntan' (S_9S_{10}) for the self-incompatibility (*S*) gene (Continued)

Scientific name, plant No., accession (Tanaka's classification No.; Tanaka, 1969)[z], and origin	S_9 pollen[y]		S_{10} pollen[y]		Presence (+) or absence (−) of S_9 and S_{10} alleles	Determined *S* genotype[x]
	No. of flowers pollinated	Mean No. of pollen tubes in style base	No. of flowers pollinated	Mean No. of pollen tubes in style base		
16. Amakusa No.14** Japan	6	294.8	3	298.7	$-S_9 -S_{10}$	$S_?S_?$
17. Amakusa No.15** Japan	6	294.5	3	243.7	$-S_9 -S_{10}$	$S_?S_?$
18. Amakusa No.17** Japan	3	185.0	3	183.3	$-S_9 -S_{10}$	$S_{11}S_?$
19. Amami No.1** Japan	3	223.3	6	208.3	$-S_9 -S_{10}$	$S_?S_?$
20. Amami No.2** Japan	5	176.2	4	128.7	$-S_9 -S_{10}$	$S_?S_?$
21. Amami No.3** Japan	6	238.2	4	136.5	$-S_9 -S_{10}$	S_2S_3
22. Amami No.5** Japan	ne	–	4	149.6	ne $-S_{10}$	$S_?S_?$
23. Amami No.6** Japan	4	159.0	3	264.3	$-S_9 -S_{10}$	$S_?S_?$
24. Amami No.7** Japan	3	292.0	ne	-	$-S_9$ ne	$S_3S_?$
25. Bangladesh No.48** Bangladesh	2	278.0	2	166.0	$-S_9 -S_{10}$	$S_?S_?$
26. Bangladesh No.49** Bangladesh	6	220.2	4	160.5	$-S_9 -S_{10}$	$S_?S_?$
27. Bangladesh No.52** Bangladesh	6	261.0	4	107.2	$-S_9 -S_{10}$	$S_3S_?$
28. Bangkok Buntan (KPFTES)** Thai	3	150.0	3	0.0	$-S_9 +S_{10}$	$S_{10}S_?$
29. Banpeiyu** Vietnam	3	120.7	3	170.3	$-S_9 -S_{10}$	S_1S_2
30. Bansei Siam Buntan** Thai	6	176.9	6	241.0	$-S_9 -S_{10}$	$S_{11}S_?$
31. Chandler** USA	5	367.0	8	229.5	$-S_9 -S_{10}$	S_4S_{11}
32. Hirado Buntan** Japan	6	1.0	3	9.0	$+S_9 +S_{10}$	S_9S_{10}
33. Hirado No.1** Japan	ne	-	6	171.5	$-S_9 -S_{10}$	$S_?S_?$
34. Hirado No.2** Japan	ne	-	3	192.3	$-S_9 -S_{10}$	$S_?S_?$
35. Hirado No.3** Japan	6	188.3	4	225.9	$-S_9 -S_{10}$	$S_?S_?$
36. Hirado No.4** Japan	3	214.0	6	238.0	$-S_9 -S_{10}$	$S_?S_?$
37. Hirado No.6** Japan	3	113.0	3	141.7	$-S_9 -S_{10}$	$S_?S_?$
38. Hirado No.12** Japan	3	317.7	5	262.6	$-S_9 -S_{10}$	$S_?S_?$
39. Hirado No.13** Japan	3	377.7	3	299.0	$-S_9 -S_{10}$	$S_?S_?$
40. Hirado No.14** Japan	3	201.7	3	209.7	$-S_9 -S_{10}$	$S_?S_?$
41. Honda Buntan** Japan	3	229.0	ne	-	$-S_9$ ne	$S_?S_?$
42. Ibusuki Buntan** Japan	ne	-	3	77.3	ne $-S_{10}$	$S_1S_?$
43. Indonesia No.2050** Indonesia	6	305.7	3	202.7	$-S_9 -S_{10}$	$S_?S_?$
44. Indonesia No.2080** Indonesia	3	289.7	3	239.6	$-S_9 -S_{10}$	S_1S_{11}
45. Ipoh No.1** Malaysia	2	241.0	3	181.0	$-S_9 -S_{10}$	$S_2S_?$
46. Ipoh No.3** Malaysia	6	309.7	4	233.7	$-S_9 -S_{10}$	S_2S_{11}
47. Ipoh No.5** Malaysia	4	131.2	6	134.4	$-S_9 -S_{10}$	$S_2S_?$
48. Iriki Buntan** Japan	4	274.3	3	326.3	$-S_9 -S_{10}$	S_1S_2
49. Kaopan** Thai	6	391.5	6	182.0	$-S_9 -S_{10}$	S_1S_2
50. Kaophuang** Thai	3	194.0	3	264.7	$-S_9 -S_{10}$	$S_?S_?$
51. Kawanabe Buntan** Japan	3	299.0	3	357.7	$-S_9 -S_{10}$	$S_?S_?$
52. Kawanabe No.2** Japan	3	153.7	3	1.7	$-S_9 +S_{10}$	S_5S_{10}
53. Kirapeiyu** Taiwan	3	356.3	3	163.3	$-S_9 -S_{10}$	$S_1S_?$

Table 1. Determination of *Citrus* accessions with S_9 and/or S_{10} alleles using pollen of homozygous S_1 seedlings of 'Hirado Buntan' (S_9S_{10}) for the self-incompatibility (*S*) gene (Continued)

Scientific name, plant No., accession (Tanaka's classification No.; Tanaka, 1969)[z], and origin	S_9 pollen[y]		S_{10} pollen[y]		Presence (+) or absence (−) of S_9 and S_{10} alleles	Determined *S* genotype[x]
	No. of flowers pollinated	Mean No. of pollen tubes in style base	No. of flowers pollinated	Mean No. of pollen tubes in style base		
54. Koshiki No.1** Japan	3	141.0	ne	-	−S_9 ne	$S_2S_?$
55. Koshiki No.2** Japan	6	209.2	4	104.0	−S_9 −S_{10}	$S_2S_?$
56. Koshiki No.3** Japan	ne	-	3	261.3	ne −S_{10}	$S_5S_?$
57. Koshiki No.4** Japan	3	99.7	ne	-	−S_9 ne	$S_2S_?$
58. Koshiki No.6** Japan	6	154.2	6	362.3	−S_9 −S_{10}	$S_5S_?$
59. Koshiki No.9** Japan	ne	-	3	143.0	ne −S_{10}	$S_5S_?$
60. Mato Anyu** Taiwan	3	488.7	3	185.0	−S_9 −S_{10}	$S_2S_?$
61. Mato Buntan** Taiwan	6	338.8	6	181.7	−S_9 −S_{10}	S_5S_{11}
62. Mato Peiyu** Taiwan	3	288.3	3	94.0	−S_9 −S_{10}	$S_1S_?$
63. Nagashima No.1** Japan	3	108.0	3	190.0	−S_9 −S_{10}	$S_2S_?$
64. Nagashima No.5** Japan	3	298.3	3	105.0	−S_9 −S_{10}	$S_2S_?$
65. Nagashima No.9** Japan	3	278.7	ne	-	−S_9 ne	$S_5S_?$
66. Nameless** Japan	3	180.3	3	178.7	−S_9 −S_{10}	$S_2S_?$
67. Nejime Buntan** Japan	4	62.5	ne	-	−S_9 ne	$S_1S_?$
68. Omuta No.1** Japan	6	263.3	6	327.5	−S_9 −S_{10}	$S_1S_?$
69. Sekitoyu** Taiwan	6	171.7	6	176.7	−S_9 −S_{10}	$S_2S_?$
70. Shatienyu (NIFTS)** China	2	149.0	3	212.0	−S_9 −S_{10}	$S_2S_?$
71. Shatienyu ZS(KUPFTES)**China	6	147.2	5	175.6	−S_9 −S_{10}	$S_2S_?$
72. Soyu** Taiwan	ne	-	2	149.0	ne −S_{10}	S_1S_2
73. Suanyu seedling** China	3	197.7	ne	-	−S_9 ne	$S_2S_?$
74. Taiwan Buntan** Taiwan	3	176.3	6	105.5	−S_9 −S_{10}	S_5S_{11}
75. Tanegashima No.4** Japan	3	267.0	3	181.3	−S_9 −S_{10}	$S_5S_?$
76. Tanegashima No.9** Japan	3	88.0	3	166.3	−S_9 −S_{10}	$S_2S_?$
77. Tanegashima No.11** Japan	ne	-	3	45.0	ne −S_{10}	$S_2S_?$
78. Tanegashima No.12** Japan	3	175.0	ne	-	−S_9 ne	$S_2S_?$
79. Tanegashima No.13** Japan	4	146.8	6	104.5	−S_9 −S_{10}	$S_2S_?$
80. Tanegashima No.15** Japan	ne	-	3	94.7	ne −S_{10}	$S_2S_?$
81. Tanegashima No.16** Japan	ne	-	3	179.0	ne −S_{10}	$S_2S_?$
82. Tanegashima No.17** Japan	ne	-	3	148.3	ne −S_{10}	$S_2S_?$
83. Vietnam No.2** Japan	3	320.0	2	142.5	−S_9 −S_{10}	$S_2S_?$
84. Yatsushiro No.1** Japan	2	157.5	ne	-	−S_9 ne	$S_1S_?$
85. Yatsushiro No.4** Japan	3	287.3	6	1.5	−S_9 +S_{10}	$S_{10}S_?$
86. Yatsushiro No.8** Japan	3	189.0	3	166.7	−S_9 −S_{10}	$S_2S_?$
C. maxima complex						
87. Bangkok Buntan (NIFTS)**	ne	-	3	172.3	ne −S_{10}	$S_2S_?$
88. Higo Pummelo**	3	244.3	2	150.0	−S_9 −S_{10}	S_4S_{11}
89. Hino Buntan**	3	127.0	3	178.7	−S_9 −S_{10}	$S_1S_?$
90. Itoshima-bankan**	3	213.0	3	136.3	−S_9 −S_{10}	$S_5S_?$

Table 1. Determination of *Citrus* accessions with S_9 and/or S_{10} alleles using pollen of homozygous S_1 seedlings of 'Hirado Buntan' (S_9S_{10}) for the self-incompatibility (*S*) gene (Continued)

Scientific name, plant No., accession (Tanaka's classification No.; Tanaka, 1969)[z], and origin	S_9 pollen[y]		S_{10} pollen[y]		Presence (+) or absence (−) of S_9 and S_{10} alleles	Determined *S* genotype[x]
	No. of flowers pollinated	Mean No. of pollen tubes in style base	No. of flowers pollinated	Mean No. of pollen tubes in style base		
91. Kessaku**	3	113.0	2	141.7	$-S_9$ $-S_{10}$	$S_?S_?$
92. Kochihakuyu**	3	286.3	3	127.1	$-S_9$ $-S_{10}$	$S_?S_?$
93. Natsudai*	6	150.2	ne	-	$-S_9$ ne	$S_?S_2$
94. Kugatsukan**	3	248.0	3	149.7	$-S_9$ $-S_{10}$	$S_?S_?$
95. Morrison*	3	228.7	3	92.7	$-S_9$ $-S_{10}$	$S_?S_?$
96. Tanikawa Buntan**	5	213.2	3	182.0	$-S_9$ $-S_{10}$	$S_?S_?$
C. truncata hort. *ex* Tanaka (57)						
97. Kaikokan*	ne	-	3	275.7	ne $-S_{10}$	$S_?S_4$
C. pseudogulgul hort. *ex* Shirai (59)						
98. Shishiyuzu**	3	245.7	3	266.7	$-S_9$ $-S_{10}$	$S_1S_?$
C. paradisi hort. Macf. (62)						
99. Foster Pink*	3	133.0	3	183.0	$-S_9$ $-S_{10}$	$S_?S_?$
100. Mash*	3	114.0	3	253.3	$-S_9$ $-S_{10}$	$S_?S_?$
101. Triumph*	3	300.0	3	162.7	$-S_9$ $-S_{10}$	$S_?S_?$
C. hassaku hort. Tanaka (74)						
102. Hassaku**	3	154.3	3	118.0	$-S_9$ $-S_{10}$	S_4S_5
C. iwaikan hort. *ex* Y. Tanaka (75)						
103. Iwaikan**	3	161.5	3	211.0	$-S_9$ $-S_{10}$	$S_?S_?$
C. tengu hort. *ex* Tanaka (76)						
104. Tengu*	3	96.0	3	112.3	$-S_9$ $-S_{10}$	$S_?S_4$
C. medioglobosa hort. *ex* Tanaka (77)						
105. Naruto*[?]	3	183.7	3	162.3	$-S_9$ $-S_{10}$	$S_?S_?$
C. natsudaidai Hayata (78)						
106. Kawano Natsudaidai*	3	162.0	ne	-	$-S_9$ ne	$S_?S_2$
C. obovoidea hort. *ex* I. Tanaka (79)						
107. Kinkoji**	2	71.0	ne	-	$-S_9$ ne	$S_4S_?$
C. otachibana hort. *ex* Y. Tanaka (80)						
108. Tosa Buntan**	3	177.0	3	213.3	$-S_9$ $-S_{10}$	S_1S_3
C. ampullaceal hort. *ex* Tanaka (81)						
109. Hyokan**	6	138.1	2	135.5	$-S_9$ $-S_{10}$	$S_?S_?$
C. yuge-hyokan hort. *ex* Tanaka (82)						
110. Yuge-hyokan**	6	135.0	3	58.3	$-S_9$ $-S_{10}$	$S_4S_?$
C. sulcata hort. *ex* Takahashi (84)						
111. Sanbokan*	5	206.2	4	133.7	$-S_9$ $-S_{10}$	$S_?S_?$
C. taiwanica hort. Tanaka *et* Shimada (87)						
112. Nanshodaidai*[?]	1	91.0	ne	-	$-S_9$ ne	$S_?S_?$
C. papillaris hort. Blanco (87)						
113. Chizon*[?]	3	295.3	ne	-	$-S_9$ ne	$S_?S_?$

Table 1. Determination of *Citrus* accessions with S_9 and/or S_{10} alleles using pollen of homozygous S_1 seedlings of 'Hirado Buntan' (S_9S_{10}) for the self-incompatibility (*S*) gene (Continued)

Scientific name, plant No., accession (Tanaka's classification No.; Tanaka, 1969)[z], and origin	S_9 pollen[y]		S_{10} pollen[y]		Presence (+) or absence (−) of S_9 and S_{10} alleles	Determined *S* genotype[x]
	No. of flowers pollinated	Mean No. of pollen tubes in style base	No. of flowers pollinated	Mean No. of pollen tubes in style base		
C. anseikan hort. *ex* Tanaka						
114. Anseikan**	3	213.0	2	49.0	$-S_9 -S_{10}$	$S_1S_?$
Sour orange and relatives						
C. aurantium Linn. (93)						
115. Bonotsu-daidai*	3	329.0	6	184.5	$-S_9 -S_{10}$	$S_?S_?$
116. Bouquet des Fleurs*	3	330.0	3	110.0	$-S_9 -S_{10}$	$S_?S_?$
117. Kabusu*	5	0.0	6	110.5	$+S_9 -S_{10}$	$S_?S_9$
118. Zadaidai*	6	41.3	9	123.4	$-S_9 -S_{10}$	$S_?S_{11}$
C. aurantium × *C. paradisi*?						
119. Smooth Seville*[?]	3	330.0	3	284.7	$-S_9 -S_{10}$	$S_?S_?$
C. canaliculata hort. *ex* Y. Tanaka (99)						
120. Kiku-daidai*	3	0.0	3	106.0	$+S_9 -S_{10}$	S_9S_{11}
Sweet orange and relatives						
C. sinensis Osbeck (100)						
121. Otao orange*	2	132.5	3	257.7	$-S_9 -S_{10}$	$S_?S_3$
122. Person Brown*	6	125.0	2	197.5	$-S_9 -S_{10}$	$S_?S_3$
123. Ruby Blood*	3	291.7	3	146.0	$-S_9 -S_{10}$	$S_?S_{3sm}$
124. Shamouti*	6	233.0	2	113.0	$-S_9 -S_{10}$	$S_?S_3$
125. Trovita*	3	139.3	3	66.7	$-S_9 -S_{10}$	$S_?S_3$
126. Yoshida Navel*	3	240.3	3	132.3	$-S_9 -S_{10}$	$S_?S_3$
Yuzu and relatives						
C. tamurana hort. *ex* Tanaka (107)						
127. Hyuganatsu**	3	155.7	3	148.0	$-S_9 -S_{10}$	$S_1S_?$
128. Orange Hyuga**	3	175.0	ne	-	$-S_9$ ne	$S_1S_?$
C. luteo-turgida Tanaka (109)						
129. Dada**	ne	-	2	93.5	ne $-S_{10}$	$S_?S_?$
C. sudachi hort. *ex* Shirai (115)						
130. Sudachi*[?]	3	72.0	3	141.7	$-S_9 -S_{10}$	$S_?S_?$
C. pseudoaurantium hort. *ex* Y. Tanaka (119)						
131. Henka-mikan*[?]	ne	-	3	115.0	ne $-S_{10}$	$S_?S_?$
C. sphaerocarpa hort. *ex* Tanaka (121)						
132. Kabosu*	3	294.7	3	95.0	$-S_9 -S_{10}$	$S_?S_4$
Mandarin and relatives						
C. nobilis cv. King × *C. deliciosa*						
133. Encore*	ne	-	3	193.3	ne $-S_{10}$	$S_?S_?$
C. nobilis complex						
134. Oshima-mikan Hakunikukei*[?]	ne	-	2	79.5	ne $-S_{10}$	$S_?S_?$
135. Kusakunebu*[?]	3	84.0	3	158.7	$-S_9 -S_{10}$	$S_?S_?$

Table 1. Determination of *Citrus* accessions with S_9 and/or S_{10} alleles using pollen of homozygous S_1 seedlings of 'Hirado Buntan' (S_9S_{10}) for the self-incompatibility (*S*) gene (Continued)

Scientific name, plant No., accession (Tanaka's classification No.; Tanaka, 1969)[z], and origin	S_9 pollen[y]		S_{10} pollen[y]		Presence (+) or absence (−) of S_9 and S_{10} alleles	Determined *S* genotype[x]
	No. of flowers pollinated	Mean No. of pollen tubes in style base	No. of flowers pollinated	Mean No. of pollen tubes in style base		
C. unshiu (L1)* + *C. obovoidea* (L2, L3)**						
136. Kinkoji Unshu*	3	93.3	ne	-	−S_9 ne	$S_?S_4+S_4S_?$
C. genshokan hort. *ex* Tanaka (132)						
137. Genshokan*	3	167.3	3	152.3	−S_9 −S_{10}	$S_?S_?$
C. tangerina hort. *ex* Tanaka (133)						
138. Dancy*	3	110.3	3	147.3	−S_9 −S_{10}	$S_?S_?$
C. tangerina × *C. paradisi*						
139. Seminole*	3	232.7	3	317.3	−S_9 −S_{10}	$S_?S_?$
C. clementina hort. *ex* Tanaka (134)						
140. Clementine**	2	85.0	ne	-	−S_9 ne	S_3S_{11}
C. tachibana Tanaka (143)						
141. Tachibana No.1 (NIFTS)*[?]	3	66.7	3	82.7	−S_9 −S_{10}	$S_?S_?$
142. Tachibana No.2 (KUPFTES)*[?]	3	38.0	3	221.0	−S_9 −S_{10}	$S_?S_?$
C. depressa Hayata (153)						
143. Hijakunibu*[?]	ne	-	3	116.7	ne −S_{10}	$S_?S_?$
144. Kaachi*[?]	2	91.8	2	140.0	−S_9 −S_{10}	$S_?S_?$
145. Ogimi Kugani*[?]	ne	-	2	100.5	ne −S_{10}	$S_?S_?$
C. flaviculpus hort. *ex* Tanaka						
146. Ogonkan (Kimikan)**	2	105.5	2	223.0	−S_9 −S_{10}	$S_4S_?$

[z]Self-incompatibility (**) and semi-self-incompatibility (*) in most accessions are based on the report of Ngo et al. (2019), and the others depend on the following reports: present study, Kim et al. (2010, 2011, 2020), Soost and Roose (1969), Yamamoto et al. (2006), Vardi et al. (2000) and Zhou et al. (2018).

[y]Fresh S_9 pollen was collected from HBS3, HBS4, HBS19 and HBS25 with the S_9S_9 genotype, while S_{10} pollen was collected from HBS2, HBS11 and HBS20 with the $S_{10}S_{10}$ genotype. All the pollinated pistils were collected 8 days after pollination. Data for accession Nos. 29, 33-40, 98, 102, 106, 108, 110, 114, 117, 127 and 143 include those reported by Kim et al. (2010).

[x]Determined *S* genotypes except for those determined in the present study is based on the report of Kim et al. (2010) for S_9 and S_{10}, Kim et al. (2011) for S_1 and S_2, Zhou et al. (2018) for S_4 and S_5, and Kim et al. (2020) for S_3 and S_{11}.

[w]'ne' means no experiment.

Determination of *Citrus* Accessions with S_{10} Pollen

When the S_{10} pollen of 'Hirado Buntan' pummelo homozygous S_1 seedlings (S_9S_9) was pollinated to 127 *Citrus* accessions, many S_9 pollen tubes penetrated into the lower styles of 122 accessions (Table 1 and Fig. 1). The average number of pollen tubes observed in the lower styles of the 122 accessions ranged from 45.0 to 382.7. This result suggested that these accessions show cross compatibility with S_{10} pollen and do not have an S_{10} allele. In five of the 127 accessions, no or only a few pollen tubes were observed in the lower styles: 0.0 for Amakusa No. 6 pummelo (*Citrus maxima*), 9.0 for the 'Hirado Buntan' pummelo, 0.0 for the 'Bangkok Buntan' (KPFTES) pummelo (*Citrus maxima*), 1.7 for the Kawanabe No. 2 pummelo (*Citrus maxima*), and 1.5 for the Yatsushiro No. 4 pummelo (*Citrus maxima*). This result suggested that
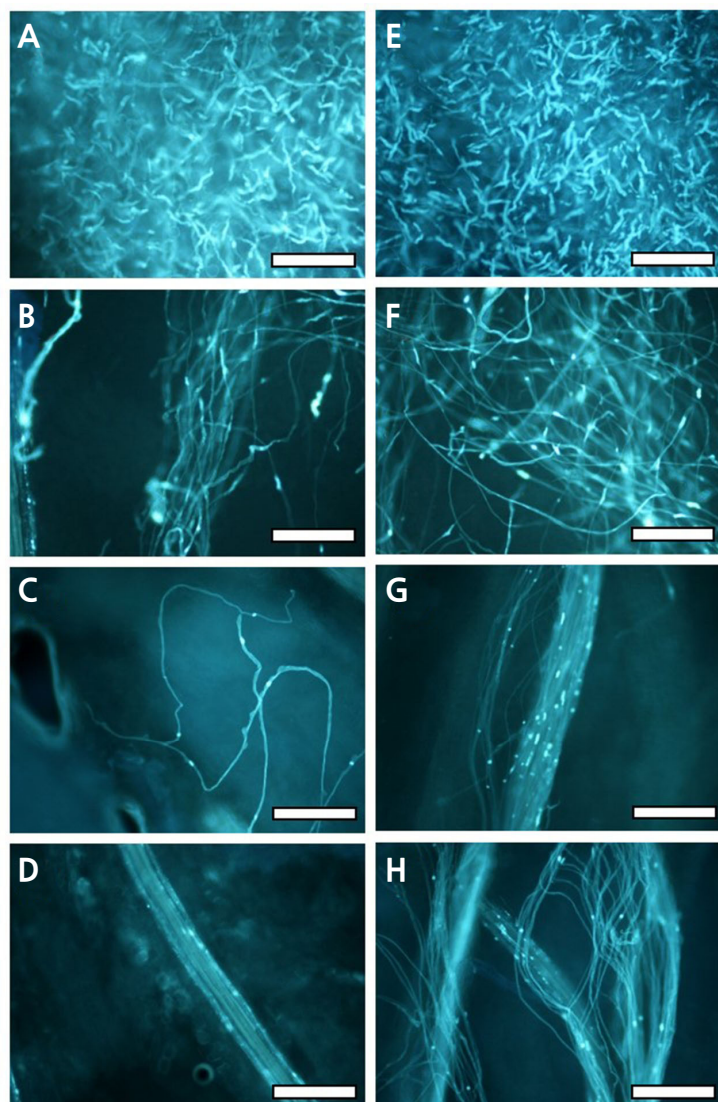
Fig. 1. Pollen tube arrest in the pistil of 'Hirado Buntan' (S_9S_{10}) pollinated with HBS2 ($S_{10}S_{10}$) (A-D), and normal pollen tube growth in the pistil of 'Hassaku' (S_4S_5) pollinated with HBS3 (S_9S_9) (E-H). A and E: Stigma; B and F: upper part of style; C and G: middle part of style; D and H: lower part of style. Note the multiple pollen tubes in each section of the style from the stigma to the style base in right photos and the reduction of the number of pollen tubes from multiple (A) to zero (D) in the left photos. In photo D, no pollen tube is seen; the pollen tube-like structure emitting strong fluorescence is the phloem of conductive tissue in the style base. Bars indicate 0.1 mm.

these pummelo accessions show cross incompatibility with S_{10} pollen and have an S_{10} allele. Except for accessions belonging to the pummelo group, those that have an S_{10} allele were not detected in the other groups of *Citrus* accessions examined.

Variation of the Number of Pollen Tubes in Lower Styles of Back-cross-incompatible Pistils (S_9S_{10}) Pollinated with 'Hirado Buntan' (S_9S_{10})

In the upper styles of back-crossed pistils (S_9S_{10}) with 'Hirado Buntan' (S_9S_{10}), more than 200 pollen tubes per style were observed, while in the lower styles the average number of pollen tubes ranged from 2.5 for HBS27 to 8.0 for HBS5

with the average number of 5.8 per style (Table 2). The average number of 5.8 pollen tubes per style was almost the same as that in self-pollination of 'Hirado Buntan' reported by Ngo et al. (2019) (Table 2).

Variation of the Number of Pollen Tubes in the Lower Styles of Incompatible Pistils of *Citrus* Plants Carrying S_9 and/or S_{10} Alleles When Pollinated with S_9 or S_{10} Pollen

In the upper styles of all cross-incompatible pistils pollinated with S_9 pollen, more than 72 pollen tubes per style were observed (Table 3). In the lower styles of all pistils pollinated with S_9 pollen, the average number of pollen tubes observed ranged from 0 for Kabusu (S_fS_9) × HBS3 (S_9S_9) to 8.5 for HBS4 (S_9S_9) × HBS3 (S_9S_9) with the average of 2.3 per style (Table 3). In the lower styles of all pistils pollinated with S_9 pollen from HBS3 (S_9S_9), the average number of pollen tubes

Table 2. Small variation for SI in lower styles between self- and back-crosses pollinated with 'Hirado Buntan' pummelo

Pistillate parent × pollen parent (*S* genotype)	Pollen genotype	No. of pistils examined	No. of pollen tubes in indicated part of style	
			Upper	Lower
Hirado Buntan (S_9S_{10}) × Hirado Buntan (S_9S_{10})[z]	S_9 and S_{10}	3	>150.0	5.7
HBS5 (S_9S_{10}) × Hirado Buntan (S_9S_{10})	S_9 and S_{10}	2	>300.0	8.0
HBS15 (S_9S_{10}) × Hirado Buntan (S_9S_{10})	S_9 and S_{10}	2	>300.0	7.0
HBS18 (S_9S_{10}) × Hirado Buntan (S_9S_{10})[y]	S_9 and S_{10}	3	>200.0	7.3
HBS25 (S_9S_{10}) × Hirado Buntan (S_9S_{10})	S_9 and S_{10}	2	>300.0	4.0
HBS27 (S_9S_{10}) × Hirado Buntan (S_9S_{10})	S_9 and S_{10}	2	>300.0	2.5
Average number of pollen tubes in lower styles				5.8

[z]Cited from Ngo et al., 2019.
[y]Cited from Kim et al., 2010.

Table 3. Degree of pollen tube arrest for S_9 and S_{10} pollens

Pistillate parent × pollen parent (*S* genotype)	Pollen genotype	No. of pistils examined	No. of pollen tubes in indicated part of style	
			Upper	Lower
HBS4 (S_9S_9) × HBS3 (S_9S_9)[z]	S_9	2	>150.0	8.5
HBS7 (S_9S_9) × HBS3 (S_9S_9)[z]	S_9	3	>150.0	0.7
HBS18 (S_9S10) × HBS3 (S_9S_9)[z]	S_9	2	>150.0	5.0
HBS19 (S_9S_9) × HBS25 (S_9S_9)	S_9	3	>150.0	1.0
Hirado Buntan (S_9S_{10}) × HBS3 (S_9S_9)	S_9	7	>200.0	0.8
Hirado Buntan (S_9S_{10}) × HBS4 (S_9S_9)	S_9	1	>200.0	2.0
Kabusu (S_fS_9) × HBS3 (S_9S_9)	S_9	5	72.0	0.0
Amakusa No.6 (S_9S_{10}) × HBS3 (S_9S_9)	S_9	3	249.3	0.3
Average number of S_9 pollen tubes in lower styles				2.3
Amakusa No.6 (S_9S_{10}) × HBS2 ($S_{10}S_{10}$)	S_{10}	3	180.7	0.0
Yatsushiro No.4 ($S_{10}S_{11}$) × HBS2 ($S_{10}S_{10}$)	S_{10}	3	>200.0	0.0
Bangkok Buntan ($S_{10}S_?$) × HBS2 ($S_{10}S_{10}$)	S_{10}	3	>200.0	0.0
Kawanabe No.2 (S_5S_{10}) × HBS2 ($S_{10}S_{10}$)	S_{10}	3	>200.0	1.7
Hirado Buntan (S_9S_{10}) × HBS2 ($S_{10}S_{10}$)	S_{10}	4	>200.0	9.0
Average number of S_{10} pollen tubes in lower styles				2.1

[z]Cited from Kim et al., 2010.

was 8.5, 0.7 and 5.0 per lower style of HBS4, HBS7 and HBS18, respectively, and 0.8, 0.7 and 0.6 for 'Hirado Buntan', 'Kabusu' and Amakusa No.6 pummelos (Table 3). HBS19 (S_9S_9) × HBS25 (S_9S_9) and Hirado Buntan (S_9S_{10}) × HBS4 (S_9S_9) showed 1.0 and 2.0 pollen tubes per lower style.

In the upper styles of all cross-incompatible pistils pollinated with S_{10} pollen from HBS2 ($S_{10}S_{10}$), more than 180.7 pollen tubes per style were observed (Table 3). In the lower styles of pistils pollinated with S_{10} pollen, the average number of pollen tubes observed was the highest (9.0) in 'Hirado Buntan', followed by 1.7 in Kawanabe No. 2 (S_5S_{10}) and 0 in Amakusa No.6 (S_9S_{10}), Yatsushiro No.4 ($S_{10}S_{11}$) and 'Bangkok Buntan' ($S_{10}S_?$) pummelos with the average number of 2.1 pollen tubes per lower style (Table 3). The average number of 2.1 per lower style was almost the same as that of 2.3 in cross-incompatible pistils pollinated with S_9 pollen.

Discussion

Distribution of *Citrus* Accessions with S_9 and/or S_{10} Alleles

In this study, S gene alleles were found in accessions belonging to pummelo and a pummelo relative for the S_9 allele and those belonging to pummelos for the S_{10} allele. This suggests that S_9 and S_{10} alleles have their origin in pummelo and not in mandarin. In the previous study with S_9 and S_{10} pollens, neither the S_9 nor the S_{10} allele was found in seven Hirado Island pummelo accessions estimated to be relatives of the 'Hirado Buntan' pummelo (Kim et al., 2011). This suggests that the S_9 and S_{10} alleles of 'Hirado Buntan' are unique to the Hirado Island pummelo group. In the present study, we found that S_9 and S_{10} alleles exist in the Amakusa No. 6 pummelo, which was collected from an old tree located at Yunuki in the Amakusa Islands, Kumamoto Prefecture. The Amakusa No. 6 pummelo has a morphology similar to that of 'Hirado Buntan' in the tree, flowers, and fruit. The old tree was seeded and likely was not grafted, and the self-incompatibility reaction in the pistils to S_{10} pollen differed from that observed in 'Hirado Buntan,' i.e., 0.0 for the Amakusa No. 6 pummelo and 9.0 for 'Hirado Buntan' (Table 1). It may be said that the Amakusa No. 6 pummelo and 'Hirado Buntan' are not the same plant but different genotypes.

'Kabusu,' one of four sour orange accessions examined, has an S_9 allele (Table 1) as suggested by Kim et al. (2011), while three of the four accessions have no S_9 allele. Kim et al. (2020) reported that the 'Zadaidai' sour orange, one of the four sour orange accessions examined in this study, has an S_{11} allele. On the other hand, two of the four sour orange accessions examined have neither an S_9 allele nor an S_{10} allele. Although it has been reported that the sour orange originated from an F_1 hybrid between a pure pummelo and mandarin (Wu et al., 2014) or is an offspring of a cross between lemon and the F_1 of a pummelo and mandarin cross (Shimizu et al., 2016), it has also been reported that sour orange accessions show various differences not only in morphology but also in molecular markers (Siragusa et al., 2006). Thus, the S allele difference among 'Kabusu' ($S_?S_9$), 'Zadaidai' ($S_?S_{11}$), and two of the four sour orange accessions ('Bonotsu-daidai' and 'Bouquet des Fleurs') may suggest that they are genetically different cultivars, not clones in which S allele mutation occurred during the long history of cultivation. To verify the genotype of the original or pure sour orange, further assessment of common or standard sour oranges is necessary. In the present study, we demonstrated that 'Kikudaidai,' a very close relative of sour orange, has an S_9 allele, while Kim et al. (2020) reported that 'Kikudaidai' has an S_{11} allele. This suggests the possibility that 'Kikudaidai' (S_9S_{11}) was produced by direct crossing between 'Kabusu' and 'Zadaidai' or by crossing between a plant with 'Kabusu' and one with 'Zadaidai' in their pedigrees.

In addition to Amakusa No. 6 (S_9S_{10}) and 'Hirado Buntan' (S_9S_{10}), 'Bangkok Buntan' (KPFTES), the Kawanabe No. 2 pummelo, and Yatsushiro No. 4 pummelo have an S_{10} allele. Although the S_{10} allele is distributed in pummelo accessions at a higher rate than the S_9 allele, it does not appear in the *Citrus* accessions except for pummelo and 'Kikudaidai'. These results suggest that the distribution of *Citrus* accessions with S_9 and/or S_{10} alleles is limited to pummelo and sour orange, probably because of the low fruit quality of these accessions, resulting in limited expansion of *Citrus* accessions with S_9 and/or S_{10} alleles during the long history of cultivation of pummelo and sour orange—about 500 years (estimation by authors) and about 2000 years in Japan (Tanaka, 1948b) respectively, and for several thousand years in other parts of the world. A 'Hirado Buntan' pummelo with high fruit quality was introduced in Japan in 1846 (Tanaka, 1948a), and thus, the introgression of S_9 and/or S_{10} alleles into the *Citrus* accessions is thought to be very limited in Japan.

Characteristics of the *S* Allele Frequency for S_9 and S_{10} Alleles

The rates of *Citrus* accessions with S_n alleles except for S_f alleles have been reported for the S_1 and S_2 (Kim et al., 2011), S_3 (Kim et al., 2020), S_4 and S_5 (Zhou et al., 2018), and S_{11} (Kim et al., 2020) alleles of pummelo origin. According to these reports, the rates of *Citrus* accessions with S_n alleles except for the S_f allele are 29.9% for S_1, 21.3% for S_2, 17.7% for S_3, 9.4% for S_4, 6.3% for S_5, and 13.2% for S_{11}. The present result for the rates of *Citrus* accessions with S_9 and S_{10} alleles are 3.1% (4 of 132 accessions) and 3.9% (5 of 129 accessions), respectively. When six sweet orange accessions and two Hyuganatsu accessions (Table 1) are counted as one genotype each, the rates of *Citrus* genotypes with S_9 and S_{10} alleles are 3.2% (4 of 126 accessions) and 3.9% (5 of 127 accessions), respectively. The pummelo accessions had S_9 and S_{10} alleles at higher rates of 2.9% (2 of 70 accessions examined) and 7.0% (5 of 71 accessions examined), respectively. The rates for S_9 and S_{10} alleles are the lowest and second lowest among the eight rates determined. The rates of pummelo accessions with S_n alleles except for the S_f allele are 34.4% for S_1, 26.4% for S_2, 12.2% for S_3, 1.4% for S_4, 11.8% for S_5, and 33.3% for S_{11}. These S allele frequencies are 17.2% for S_1, 13.2% for S_2, 6.1% for S_3, 0.7% for S_4, 5.9% for S_5, and 16.7% for S_{11}. The rates of pummelo accessions with S_9 and S_{10} alleles are 2.9% and 7.0%, respectively. Therefore, the S_9 and S_{10} allele frequencies are 1.8% and 2.3%, respectively. The S_9 and S_{10} allele frequencies are second and third lowest among the eight S allele frequencies in pummelo. The total of the eight S allele frequencies in pummelo is 64.6%, from which it is roughly estimated that more than twelve S alleles are estimated to exist in pummelo.

Differences in the Self-incompatibility Reaction in Lower Styles between S_9 and S_{10} Alleles

Ngo et al. (2019) classified the self-incompatibility reaction into several types by the degree of pollen tube arrest in the upper, middle, and lower parts of the styles. They classified self-incompatibility in 'Hirado Buntan' into an MMH type (Moderate in stigma, Moderate in upper style, High in middle style). It exhibited, however, a relatively slow or mild self-incompatibility reaction in the lower styles with an average penetration of 5.7 pollen tubes per style and in ovaries with an average penetration of 3.7 pollen tubes per ovary, although the self-pollinated fruit is seedless (Ngo et al., 2019). Yamamoto et al. (2006) observed a similar phenomenon in the self-pollination of 'Hirado Buntan' carrying S_9 and S_{10} pollen. The present data for back-cross-incompatibility in pollinations with 'Hirado Buntan' pummelo showed a slow self-incompatibility reaction in the lower part of styles with an average penetration of 5.8 pollen tubes per lower style, similar to the value of self-pollination of 'Hirado Buntan' (Table 2). The result suggests that the same extent of mild SI

reaction in the lower part of styles occurs in all five back-crosses. This also suggests the possibility that the mild SI reaction in the lower part of styles of 'Hirado Buntan' is an inherited trait.

On the other hand, we postulated that the extent of mild SI reaction in the lower part of styles will be different between pollinations with S_9 pollen and S_{10} pollen, since when 'Hirado Buntan' (S_9S_{10}) was pollinated with S_9 pollen of 'Hirado Buntan' S_1 seedlings (S_9S_9), 1.0 S_9 pollen tube on average per lower style was observed, whereas when pollinated with S_{10} pollen of another 'Hirado Buntan' S_1 seedling ($S_{10}S_{10}$), 9.0 S_{10} pollen tubes on average per lower style were observed (Table 1). The average number of S_9 pollen tubes of HBS3 and HBS4 penetrating into the lower style of 'Hirado Buntan' was 1.1 and 2.0, respectively, while the average number of S_{10} pollen tubes of HBS2 penetrating into the lower style of 'Hirado Buntan' was 9.0 (Table 3). The number of S_9 and S_{10} pollen tubes may suggest that in the lower style of 'Hirado Buntan', pollen tubes carrying the S_{10} allele show a slower incompatibility reaction than those carrying the S_9 allele. Hiratsuka et al. (2012) reported that in the Japanese pear [*Pyrus pyrifolia* (Burm.f.) Nakai], the degree of self-incompatibility is different between the pollen grains carrying different S alleles. In all crosses with S_9 or S_{10} pollen, however, no difference was detected in the number of pollen tubes in the lower styles between S_9 and S_{10} pollen with the average of 1.4 S_9 pollen tubes per lower style and 2.2 S_{10} pollen tubes per lower style (Table 3). The slow or mild self-incompatibility reaction in the lower styles of self-pollinated 'Hirado Buntan' and the back-crossed S_1 seems to be specific to the pollination with 'Hirado Buntan'. On the other hand, it may be said generally that there is no clear difference in the mild self-incompatibility reaction in the lower styles between S_9 and S_{10} pollens; the extent of the mild self-incompatibility reaction in the lower styles will be different in pollinations with S_9 or S_{10} pollen.

Conclusion

Of the 146 *Citrus* accessions including 82 pummelo accessions examined, self-incompatibility genotypes have been completely ascertained or determined in 'Kabusu' sour orange (S_fS_9), a 'Kikudaidai' sour orange relative (S_9S_{11}) and two local pummelo plants. The S allele frequencies for S_9 and S_{10} alleles of pummelo origin are very low in pummelo accessions and eventually in *Citrus* accessions compared to those of the other S alleles of pummelo origin reported, suggesting pummelo plants with S_9 and S_{10} alleles did not contribute to the evolution and development of pummelos and eventually *Citrus* cultivars, except for 'Hirado Buntan' pummelo and sour oranges that are used in the commercial production of fresh fruit and root stocks. On the other hand, the S_9 allele may be a good marker to detect cultivars and hybrids with 'Hirado Buntan' and sour oranges in their pedigrees. The mild self-incompatibility reaction seen in the lower style of self-pollinated 'Hirado Buntan' was also confirmed in the lower styles of the S_1 plants pollinated with 'Hirado Buntan'. However, there is no clear difference in the mild self-incompatibility reaction in the lower styles between S_9 and S_{10} pollen. Finally, we examined the degree of self-incompatibility between S_9 and S_{10} alleles in the lower part of styles of S_1 seedlings of 'Hirado Buntan' and *Citrus* accessions with S_9 and/or S_{10} alleles. The result indicated no difference in the reaction between the two alleles.

Literature Cited

Hiratsuka S, Fujimura M, Hayashida T, Nishikawa Y, Nada K (2012) Pollen factors controlling self-incompatibility strength in Japanese pear. Sex Plant Reprod 25:347-352. doi:10.1007/s00497-012-0202-7

Honsho C, Umegatani S, Furukawa D, Ishimura S, Tetsumura T (2019) Isolation and characterization of *S*-RNase-homologous genes expressed in styles in 'Hyuganatsu' (*Citrus tamurana* hort. ex Tanaka). Hort J 88:338-346. doi:10.2503/hortj.UTD-032

Iwamasa M, Oba Y (1980) Seedlessness due to self-incompatibility in Egami-Buntan, a Japanese pummelo cultivar. Bull Fac Agr Saga Univ 49:39-45

Kim JH, Handayani E, Wakana A, Sato M, Miyamoto M, Miyazaki R, Zhou X, Sakai K, Mizunoe Y, et al (2020) Distribution and evolution of *Citrus* with S_3 and/or S_{11} alleles for self-incompatibility with an emphasis on sweet orange [*Citrus sinensis* (L.) Osbeck; S_fS_3 or S_fS_{3sm}]. Genet Resour Crop Evol 67:2101-2117. doi:10.1007/s10722-020-00964-x

Kim JH, Mori T, Wakana A, Ngo BX, Masuda J, Sakai K, Kajiwara K (2010) Production of homozygous S_1 seedlings for *S* gene in 'Hirado Buntan' pummelo (*Citrus grandis* Osbeck) and determination of the *S* alleles (S_9 and S_{10}) by pollination with the S_1 seedlings to *Citrus* cultivars. J Fac Agr Kyushu Univ 55:239-245. doi:10.5109/18836

Kim JH, Mori T, Wakana A, Ngo BX, Sakai K, Kajiwara K (2011) Determination of self-incompatible *Citrus* cultivars with S_1 and/or S_2 alleles by pollination with homozygous S_1 seedlings(S_1S_1 or S_2S_2) of 'Banpeiyu' pummelo. J Jpn Soc Hortic Sci 80:404-413. doi:10.2503/jjshs 1.80.404

Liang M, Cao Z, Zhu A, Liu Y, Tao M, Yang H, Xu Jr Q, Wang S, Liu J, et al (2020) Evolution of self-compatibility by a mutant S_m-RNase in citrus. Nat Plants 6:131-142. doi:10.1038/s41477-020-0597-3

Ngo BX, Chu TD, Wakana A, Bui TT, Nguyen TD, Nguyen TT, Nguyen TH, Kim JH, Sakai K (2019) Patterns and strength of pollen tube arrest in self-incompatible *Citrus* accessions (*Rutaceae*). J Fac Agr Kyushu Univ 64:225-236. doi:10.5109/2339113

Ngo BX, Kim JH, Wakana A, Isshiki S, Mori T (2011) Estimation of self-incompatibility genotypes of *Citrus* cultivars with *Got-3* allozyme markers. J Jpn Soc Hortic Sci 80:284-294. doi:10.2503/jjshs1.80.284

Shimizu T, Kitajima A, Nonaka K, Yoshioka T, Ohta S, Goto S, Toyoda A, Fujiyama A, Mochizuki T, et al (2016) Hybrid origins of *Citrus* varieties inferred from DNA marker analysis of nuclear and organelle genomes. *PLoS ONE* 11:e0166969 doi:10.1371/journal.pone.0 166969. doi:10.1371/journal.pone.0166969

Siragusa M, De Pasquale F, Abbate L, Tusa N (2006) Identification of sour orange accessions and evaluation of their genetic variability by molecular marker analyses. HortScience 41:84-89. doi:10.21273/HORTSCI.41.1.84

Soost RK (1987) Breeding, citrus-genetics and nucellar embryony p. 83-110 In: Abbott AJ, Atkin RK (eds.) Improving vegetatively propagated plants. Academic Press, San Diego

Soost RK, Roose ML (1996) Citrus. pp 257-323 In: Janick J, Moore JN (eds.) Fruit breeding, Volume I, Tree and tropical fruits. Willey & Sons, Inc

Tanaka T (1969) Misunderstanding with regards citrus classification and nomenclature. Bull Univ Osaka Prefecture Series B 21:139-145

Tanaka Y (1948a) Iconograph of Japanese citrus fruits Vol. 1. Yokendo, Tokyo

Tanaka Y (1948b) Iconograph of Japanese citrus fruits Vol. 2. Yokendo, Tokyo

Vardi A, Neumann H, Frydman-Shani A, Yaniv Y, Spiegel-Roy P (2000) Tentative model on the inheritance of juvenility, self-incompatibility and parthenocarpy. Acta Hort 535:199-205. doi:10.17660/ActaHortic.2000.535.24

Wakana A, Ngo BX, Fukudome I, Kajiwara K (2004) Estimation of the degree of self-incompatibility reaction during flower bud development and production of self-fertilized seeds by bud pollination in self-incompatible *Citrus* cultivars. J Fac Agr Kyushu Univ 49:307-320. doi:10.5109/4591

Wu GA, Prochnik S, Jenkins J, Salse J, Hellste U, Murat F, Perrier X, Ruiz M, Scalabrin S, et al (2014) Sequencing of diverse mandarin, pummelo and orange genomes reveals complex history of admixture during citrus domestication. Nat Biotechnol 32:656-662. doi:10.1038/nbt.2906

Yamamoto M, Kubo T, Tominaga S (2006) Self- and cross-incompatibility of various *Citrus* accessions. J Jpn Soc Hortic Sci 75:372-378. doi:10.2503/jjshs.75.372

Zhou X, Kim JH, Wakana A, Sakai K, Kajiwara K, Mizunoe Y (2018) Distribution and evolution of citrus with S_4 and/or S_5 gene alleles for self-incompatibility with special focus on the origin of satsuma mandarin (*Citrus unshiu* Marc.; S_fS_4). Genet Resour Crop Evol 65:1013-1033. doi:10.1007/s10722-017-0592-3