Production of Homozygous S1 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

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Production of Homozygous S_1 Seedlings for S Gene in 'Hirado Buntan' Pummelo (*Citrus grandis* Osbeck) and Determination of the S Alleles (S_g and S_{10}) by Pollination with the S_1 Seedlings to *Citrus* Cultivars

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Gametophytic self-incompatibility (SI) in Citrus is known in pummelo, mandarin and many hybrid cultivars with pummelo in their pedigrees. What seems to be lacking, however, is determination of allelic variation in SI gene (S) and the genotypes of self-incompatible and semi self-incompatible cultivars. In previous studies, S genotypes of several Citrus cultivars were estimated on the basis of segregation distortion for Got-3 gene in F_1 progenies between these cultivars. In this study, S_1 seedlings were produced from selfpollinated 'Hirado Buntan' pummelo, and homozygous S_1 seedlings for the S gene were determined by the pollination with 'Hirado Buntan'. Then, the homozygous S_1 seedlings were pollinated with the homozygous S_1 seedling $(S_a S_a)$ each other and classified them into two genotype groups of $S_a S_a$ and $S_b S_b$. The ten cultivars, whose S genotypes were predicted on the basis of joint segregation analysis with Got-3 gene in their progenies, were pollinated with the homozygous S_1 seedlings $(S_aS_a \text{ or } S_aS_b)$. Pollen tube behavior in the style of the ten Citrus cultivars after pollination with the S_a or S_b pollen led to the conclusion that 'Hirado Buntan' has S_a and S_{1a} alleles. Furthermore, pollen tube arrest in the style of self-compatible 'Kabusu' sour orange pollinated with S_g pollen suggested that the genotype of 'Kabusu' is $S_i S_g$. Inscrutably, neither S_g nor S_{ig} alleles were found in a group belonging to the Hirado pummelo line, which started from seeds sown by the 11th feudal load Terasu Matsura of the domain of Hirado. This fact supported the two origins of Hirado pummelo plants described in the literature, and indicated the association of multiple seed and/or pollen parents with different S alleles to the original seed production.

INTRODUCTION

Self-incompatibility (SI) was defined as the inability of a fertile hermaphrodite seed plant to produce zygote after self-pollination (de Nettancourt, 1977). After the early studies of SI in *Citrus* by Nagai and Tanikawa (1928) and the other researchers (Miwa, 1951; Nuriyal, 1952; Nishiura and Iwasaki, 1963; Hearn, 1969), Soost (1965, 1969) suggested on the basis of segregation of self-incompatible seedlings in the progenies from several crosses that the SI in *Citrus* was gametophytic. He also estimated a codominant SI gene (*S*) consisting of one self-fertility allele (*S_f*) that resulted in self-compatibility and at least eight self-incompatibility *S* alleles (*S_n*) that resulted in pollen tube growth arrest in the pistil.

SI is one of the important traits for seedless fruit pro-

duction, when the *Citrus* plants have the high degree of parthenocarpy (Iwamasa and Oba, 1980; Vardi et al., 2000; Yamamoto and Yamada, 2002). In Citrus, however, there is no detailed information about the number of S genes, allelic variation, gene frequency and S genotypes of cultivars. Wakana et al. (1998) found that the crosses with self-incompatible cultivars sometimes resulted in the segregation distortion in the progenies for glutamate oxaloacetate transaminate (GOT) isozyme genes Got-1 and Got-3. The segregation distortion in several progenies suggested that the Got-1 and Got-3 genes linked with an S gene, since segregation distortion in the progenies was detected in reciprocal crossings when the pollen parents were heterozygous for the Got-1 gene or Got-3 gene. Based on the presence or absence of the distorted segregation in the progenies derived from more than 30 crosses with many Citrus cultivars, Wakana et al. (1998) and Ngo (2001) estimated five and seven alleles, respectively.

Ngo *et al.* (2010) carried out controlled pollination with restricted number of pollen grains (about 100 pollen grains per stigma) to verify the estimated S genotypes of the *Citrus* cultivars on the basis of pollen tube growth behavior in the pollinated styles, and reported that the observed pollen tube behavior in each cross pollination well coincided with the predicted S genotypes of the cultivars used for this pollination.

The generative cell division normally occurs by three days after pollination in compatible tubes, but only rarely

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in incompatible tubes in Citrus (Kahn and DeMason, 1988) and almost all own pollen tubes were arrested in the styles (Ngo et al., 2001; Wakana et al., 2004; Yamamoto et al., 2006; Kim et al., 2009). In the pistils of self-incompatible Citrus plants pollinated with their own pollen, no pollen tubes reached the ovaries (Ton and Krezdorn, 1967; Ngo, 2001; Wakana et al., 2004; Ngo et al., 2010). Ngo (2001) investigated pollen tube behavior in self-pollination of 72 self-incompatible pummelo plants, 15 pummelo relatives, three mandarin relatives and four yuzu relatives. Of the 94 self-incompatible plants examined, six plants showed pollen tube penetration into the ovaries, but the number of the pollen tubes was less than four per ovary. The data suggested that observation of pollen tube number in the style base or the lower one-third of style 7 days after self-pollination is useful and crucial to identify whether the plants were self-incompatible or not.

Wakana *et al.* (1998) and Ngo (2001) estimated S genotypes on the basis of joint segregation analysis for Got-3 and S genes in *Citrus*. In these studies, however, a large number of zygotic seedlings are necessary for statistical analysis to estimate their parental S genotypes because of the linkage of the two genes with the relatively long distance of 31cM. In addition to this, reciprocal crosses between two cultivars are required to estimate the S genotypes by joint segregation analysis. Because of the barrier for zygotic seedling production such as nucellar embryogenesis, male sterility and homozygosity for Got-3 genotypes in many cultivars and plants, the joint segregation analysis in the progenies from reciprocal crosses was restricted to a few crosses with a few *Citrus* cultivars and plants.

Thus, the usage of pollen of homozygous S_1 hybrids for *S* gene is useful and precise method to determine whether the *S* alleles in a given cultivar are present or not, although some techniques to reduce the long generation time are necessary for the production of the homozygous S_1 plants. Wakana *et al.* (2004) successfully produced S_1 seeds in several SI cultivars. In the present study, homozygous S_1 seedlings from 'Hirado Buntan' were produced and were used to examine the pollen tube behavior in the styles of the cultivars whose S genotypes were predicted by the previous studies (Wakana *et al.*, 1998; Ngo, 2001; Ngo *et al.*, 2010).

MATERIALS AND METHODS

Plant materials

Ten *Citrus* cultivars and one accession were used in this study (Table 1). The *S* genotypes of these cultivars were estimated on the basis of segregation distortion in their progenies for the *Got*-3 gene. These were grown in the Sasaguri orchard of the Experimental Farm of Kyushu University, Fukuoka, and were used for pollination to determine two *S* alleles of 'Hirado Buntan', which were tentatively termed as S_a and S_b .

Eight old Hirado pummelo seedlings were collected from Hirado city, Nagasaki Prefecture, and were grown in Sasaguri orchard of Kyushu University. These cultivars and plants were also used to detect the presence or absence of S alleles of 'Hirado Buntan' that was introduced from National Institute of Fruit Tree Science (NIFTS). The collection site and the other information of the seven Hirado pummelo accessions were listed in Table 2.

Production of self-fertilized (S₁) seedlings of 'Hirado Buntan'

Twenty-five-year-old 'Hirado Buntan' trees were used for pollination to produce self-fertilized (S_1) seedlings homozygous for the *S* gene in 2002 (Wakana *et al.*, 2004). One day before anthesis, the flower buds of the cultivar were bagged. On the day following bagging, the pollen on the dehisced anthers was directly used for self-pollination. The self-pollination was made with young flower buds at the time when the bud length was about a half of that just before anthesis and the stigmatic exudate was not secreted. The self-pollinated flowers were immediately covered to prevent outcross with paraffin paper bags, and the fruits obtained from the selfpollinated flowers were harvested in November. Seed coats were removed and the derived embryos were immediately germinated on the wet filter paper under

Table 1. Citrus cultivars and plants used for S allele determina	ation
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Cultivar name or accession No.	Scientific name	$\begin{array}{c} {\rm Self-} \\ {\rm compatibility}^{\scriptscriptstyle b} \end{array}$	Poly embryony
Amanatsu ^a	C. natsudaidai Hayata	SSC	poly
Anseikan	C. anseikan hort. ex Tanaka	SI	poly
Banpeiyu	C. grandis Osbeck (C. maxima Mer.)	SI	mono
Hassaku	C. hassaku hort. ex Tanaka	SI	mono
Hirado Buntan	C. grandis Osbeck (C. maxima Mer.)	SI	mono
Hyuganatsu	C. tamurana hort. ex Tanaka	SI	mono
Shishiyuzu	C. pseudogulgul hort. ex Shirai	SI	mono
Tachibana No.1°	C. tachibana Tanaka	\mathbf{SC}	poly
Tosa Buntan	C. otachibana hort. ex Y. Tanaka	SI	mono
Yuge-hyokan	C. yuge–hyokan hort. ex Y. Tanaka	SI	mono
Kabusu	C. aurantium L.	\mathbf{SC}	poly

^a Synonym 'Kawano–natsudaidai'. ^b SI: self–incompatible; SC: self–compatible: SSC; semi self–compatible.

[°] Tachibana No.1 was introduced from the Okitsu Branch of National Institute of Fruit Tree Science (NIFTS).

Table 2. Hirado pummelo accessions used for S allele determination

Hirado pummelo name (Accession No.)	Collection site ^a	Estimated tree age ^b	Propagation
Hirado Matsura Buntan	Garden of Matsura mansion	>140	seedling
(Hirado pummelo No.1)	Kagamigawa–cho, Hirado City		
Hirado Matsurahata Buntan	Hirado pummelo Orchard in Matsura mansion	>130	seedling
(Hirado pummelo No.2)	Kagamigawa–cho, Hirado City		
Hirado Murao Buntan	Murao's garden near to Matsura mansion,	≈130	seedling
(Hirado pummelo No.3)	Miyanoura–cho, Hirado City		
Hirado Tabira Buntan	Beef Cow Improvement Center, Kotedamen,	≈70	seedling
(Hirado pummelo No.4)	Tabira–cho. The land was leased by Matsuras		
Kessaku	Garden of Matsura mansion,	≈80	seedling
(Hirado pummelo No.6)	Kagamigawa–cho, Hirado City		
Hirado Suzuki–A pummelo	seedling of Hirado Murao Buntan,	≈ 60	seedling
(Hirado pummelo No.12)	Okubo–cho, Hirado city		
Hirado Suzuki–B pummelo	seedling of Hirado Murao Buntan,	≈40	seedling
(Hirado pummelo No.13)	Okubo–cho, Hirado city		
Hirado Ijima Buntan	garden of Ijima's residence,	≈100	seedling
(Hirado pummelo No.14)	Kagamigawa–cho, Hirado city		
Hirado Buntan	NIFTS (Okitsu); one of the three seedlings	dead	grafting
(Hirado pummelo No.20)	raised by Moriyama in 1845: originated		
	from the seedling raised by Shinozaki, Okubo-		
	cho, Hirado city		

^a The Matsuras was a long line of the loads of a domain locating in northern Kyushu district from 1500's to 1868. ^b The tree age was estimated mainly based on the hearing from old persons who were raising or cultivating the pummelo trees.

25 °C. After germination of the embryos, they were transplanted on pots filled with commercial soil and allowed growth under green house conditions up to March of the next year. The growing self-fertilized seedlings were top-grafted on the adult trees of 'Hashimoto Wase Unshiu' Satsuma mandarin, and allowed further growth for 6 years until their flowers were available for pollination.

Pollination of self-fertilized (S₁) seedlings with 'Hirado Buntan'

To determine the homozygous S_1 seedlings of 'Hirado Buntan' for the *S* gene, all of the S_1 seedlings were pollinated with 'Hirado Buntan'. Flower buds just before anthesis were emasculated and their pistils were sufficiently pollinated by hand with the fresh pollen of 'Hirado Buntan' until the white stigma turned yellow. The fresh pollen was collected according to the procedure mentioned above.

Pollination between S₁ seedlings of 'Hirado Buntan'

Pollination between the homozygous S_1 seedlings of 'Hirado Buntan' was carried out to determine the genotypes of the homozygous S_1 seedlings for the *S* gene. The procedure of pollination was similar to that mentioned above.

Pollination of homozygous S_1 seedlings to cultivars with predicted S genotypes

To determine whether the predicted S genotypes in several self-incompatible *Citrus* cultivars are precise or not, and to determine the *S* genotypes of 'Hirado Buntan', eleven cultivars with the predicted *S* genotypes were pollinated with the homozygous 'Hirado Buntan' S_1 seed-lings (S_aS_a and S_bS_b). The procedure of pollination was similar to that mentioned above. Three flower buds just before anthesis were used in each cross.

Pollination of homozygous S_1 seedlings to Hirado pummelo accessions

To determine the presence or absence of the two S alleles of 'Hirado Buntan' in Hirado pummelo accessions, eight 'Hirado Buntan' relatives collected from Hirado city locating in most northwestern part of Kyushu district (Table 2) were pollinated with the homozygous 'Hirado Buntan' S₁ seedlings (S_aS_a and S_bS_b). The procedure of pollination was similar to that mentioned above.

Pollen tube observation

All of the pollinated pistils were collected seven days after pollination. The pistils were fixed in a solution of acetic acid alcohol (1:3 v/v) for 24 h, and stored in 70% ethanol at 4 °C until use. The fixed pistils were softened in 0.8 N sodium hydroxide for 24 h at room temperature, stained with 0.1% aniline blue dissolved in 0.1 M tribasic potassium phosphate for 24 h at room temperature. Prior to squashing, the pistils were cut into two parts (style with stigma and ovary) and then the styles were further cut longitudinally into four sections (stigma, upper one-third, middle one-third and lower one-third of style) and split each section into two, and squashed on microscope slides. All sections of the pistils were observed by a Nikon E800 epifluorescence microscope, the number of pollen tubes reaching each of the lower one-third of the style (the base of the style) was recorded. When no or a few pollen tubes were observed in the lower one-third of the style, number of pollen tubes in the upper and middle one-third sections of the style was also scored.

RESULTS AND DISCUSSION

When six six–year–old S_1 seedlings of 'Hirado Buntan' were pollinated with 'Hirado Buntan', the mean number of pollen tubes penetrating into the lower part of styles ranged from 7.3 to 184.8 per style (Table 3). Since the mean number of pollen tubes reaching the style base was 5.7 per style in 'Hirado Buntan' self–pollination, one of the six S_1 seedlings containing 7.3 pollen tubes in the style base was determined to be heterozygous (S_aS_b) for S gene. On the contrary, five of the six S_1 seedlings having more than 60.7 pollen tubes in the style base were determined to be homozygous (S_aS_a or S_bS_b) for S gene.

To distinguish the two homozygous S genotypes in the S₁ seedlings of 'Hirado Buntan', six S₁ seedlings were pollinated with a homozygous S₁ seedling HBS₁–3 whose S genotype was defined here as S_aS_a (Table 4). In three of the six S₁ seedlings, a large number of pollen tubes of 82 to 213 were penetrated into the lower styles, indicating that the S genotypes of the three homozygous seedlings were S_bS_b . In another three S₁ seedlings, a few pollen tubes of 1 to 9 per style penetrated into the style base. Thus, the S genotype of each of the three S₁ seedlings was determined to be S_aS_a for HBS₁–4, S_aS_a or S_aS_b for HBS₁–7 and S_aS_b for HBS₁–18. To determine the S genotype of HBS₁–7, pollination experiment with S_aS_b plants is necessary.

Ten *Citrus* cultivars, whose S genotypes were predicted on the basis of segregation distortion for the Got-3 gene in their progenies, were pollinated with each of homozygous S_1 seedlings HBS_1-2 (S_bS_b) and HBS_1-3 $(S_a S_a)$ (Table 5). When ten cultivars were pollinated with HBS1-2 (S_bS_b) , many pollen tubes penetrated into the lower style of them with the range from 49 to 267 per style. When the ten cultivars were pollinated with HBS1-3 (S_aS_a) , pollen tubes penetrated into the lower style of nine cultivars with the range from 67 to 246 per style and were arrested in lower style in one cultivar 'Kabusu'. It was suggested from these results that 'Hirado Buntan' does not have alleles of S_1 to S_2 , and has the same S allele as 'Kabusu'. Thus, it is defined here that 'Hirado Buntan' has $S_{g}S_{10}$ genotype for S gene and 'Kabusu' has $S_t S_g$ genotype.

Eight Hirado pummelo accessions collected from Hirado pummelo orchard of Matura's mansion and citizen's gardens around the mansion in Hirado city were pollinated with homozygous S_1 seedlings for S_g or S_{10} (Table 6). When six Hirado pummelo accessions were pollinated with HBS₁-3 (S_gS_g), pollen tubes penetrated into their lower style with the range from 164 to 378 per style, suggesting they do not have S_g allele. When eight Hirado pummelo accessions were pollinated with HBS₁-1 ($S_{10}S_{10}$) or HBS₁-2 ($S_{10}S_{10}$), pollen tubes penetrated into their lower style with the range from 142 to 304 per style, suggesting they also do not have S_{10} allele. This is the interesting fact that Hirado pummelo plants collected from Hirado city do not have the same S alleles as

Pollination	No. of flowers pollinated	Mean No. of pollen tubes in style base	Estimated S genotype of seed parent
$\text{HBS}_1 - 1 \times \text{Hirado Buntan} (S_a S_b)$	4	184.8	$S_a S_a$ or $S_b S_b$
$\text{HBS}_1 - 2 \times \text{Hirado Buntan} (S_a S_b)$	3	168.3	$S_a S_a$ or $S_b S_b$
$\text{HBS}_1 - 3 \times \text{Hirado Buntan} (S_a S_b)$	3	131.0	$S_a S_a$ or $S_b S_b$
$\text{HBS}_1 - 4 \times \text{Hirado Buntan} (S_a S_b)$	3	127.0	$S_a S_a$ or $S_b S_b$
$\text{HBS}_1-11 \times \text{Hirado Buntan} (S_a S_b)$	3	60.7	$S_a S_a$ or $S_b S_b$
$\text{HBS}_1-18 \times \text{Hirado Buntan} (S_a S_b)$	3	7.3	$S_a S_b$
Hirado Buntan $(S_a S_b) \times$ Hirado Buntan $(S_a S_b)$	3	5.7	$S_{a}S_{b}$

Table 3. Determination of S genotypes of 'Hirado Buntan' S_1 seedlings by pollination with 'Hirado Buntan' $(S_a S_b)$

Table 4. Determination of *S* genotypes of 'Hirado Buntan' S_1 seedlings by pollen tube behavior in their styles pollinated with a homozygous S_1 seedling HBS₁-3 for *S* gene. The genotype of homozygous S_1 seedling HBS₁-3 was defined as S_aS_a

Pollination	No. of flowers pollinated	Mean No. of pollen tubes in style base	Determined S genotype of seed parent
$HBS_{i}-1 (S_{a}S_{a} \text{ or } S_{b}S_{b}) \times HBS_{i}-3 (S_{a}S_{a})$	1	213	$S_b S_b$
$\text{HBS}_1-2 (S_a S_a \text{ or } S_b S_b) \times \text{HBS}_1-3 (S_a S_a)$	2	83.5	$S_{b}S_{b}$
HBS_1 -4 ($S_a S_a$ or $S_b S_b$) × HBS_1 -3 ($S_a S_a$)	2	8.5	$S_a S_a$
HBS_1 -7 ($S_a S_a$ or $S_a S_b$) × HBS_1 -3 ($S_a S_a$)	3	0.7	$S_a S_a$ or $S_a S_b$
$\text{HBS}_1-11 \ (S_a S_a \text{ or } S_b S_b) \times \text{HBS}_1-3 \ (S_a S_a)$	1	82	$S_{b}S_{b}$
$HBS_{1}-18 (S_{a}S_{b}) \times HBS_{1}-3 (S_{a}S_{a})$	2	5.0	$S_a S_b$

'Hirado Buntan' originated in the same city.

According to the Nagasaki Prefectural Agricultural Experiment Station (NPAES) Report (1917), Hirado pummelo plants were classified into two lines, the eleventh domanial load Terasu Matsura (1812–1858) line and his vassal Senkichi Moriyama (?–1846) line, as follows. When the load of northwestern Kyushu district Terasu Matsura stayed in Nagasaki in 1845, there was a person offering the load several pummelo fruits shipped from Jakarta. Since the taste of the pummelo fruits was unusu-

Table 5. Determination of Citrus cultivars with or without S_a or S_b alleles by pollen tube behavior in their styles
pollinated with homozygous 'Hirado Buntan' S_i seedlings for the S gene

Pistillate parent (Predicted S	Pollen parent (Determined S	No. of flowers	Mean No. of pollen tubes in each part of style	
genotype) ^a	genotype)	pollinated	Upper	Lower
Banpeiyu $(S_{I}S_{2})$	\times HBS ₁ -2 (S _b S _b)	3	— ^b	170.3
Banpeiyu (S_1S_2)	\times HBS ₁ -3 (S_aS_a)	3	-	120.7
Amanatsu $(S_{g}S_{f})$	\times HBS ₁ -2 (S _b S _b)	1	_	132
Amanatsu $(S_{\mathcal{E}}S_{f})$	\times HBS ₁ -3 (S_aS_a)	3	-	162.0
Tosa Buntan (S_1S_3)	\times HBS ₁ -2 (S _b S _b)	3	-	213.3
Tosa Buntan (S_1S_3)	\times HBS ₁ -3 (S_aS_a)	3	-	177.0
Hassaku (S_4S_5)	\times HBS ₁ -2 (S _b S _b)	3	-	141.7
Hassaku (S_4S_5)	\times HBS ₁ -3 (S_aS_a)	3	_	154.3
Yuge-hyokan $(S_{\theta}S_{7})$	\times HBS ₁ -2 (S _b S _b)	3	-	58.3
Yuge-hyokan $(S_{\theta}S_{7})$	\times HBS ₁ -3 (S_aS_a)	3	-	147.7
Tachibana No.1 $(S_{\beta}S_{\ast})$	\times HBS ₁ -2 (S _b S _b)	3	-	82.7
Tachibana No.1 $(S_{f}S_{4})$	\times HBS ₁ -3 (S _a S _a)	3	_	66.7
Anseikan (S_1S_2)	\times HBS ₁ -2 (S _b S _b)	2	_	49.0
Anseikan (S_1S_2)	\times HBS ₁ -3 (S_aS_a)	3	_	213.0
Hyuganatsu $(S_i S_i)$	\times HBS ₁ -2 (S _b S _b)	3	_	148.0
Hyuganatsu $(S_i S_i)$	\times HBS ₁ -3 (S_aS_a)	3	_	155.7
Shishiyuzu $(S_{I}S_{6})$	\times HBS ₁ -2 (S _b S _b)	3	_	266.7
Shishiyuzu $(S_{I}S_{6})$	\times HBS ₁ -3 (S _a S _a)	3	_	245.7
Kabusu $(S_{j}S_{j})$	\times HBS ₁ -2 (S _b S _b)	3	_	99.7
Kabusu $(S_{S_{\gamma}})$	\times HBS ₁ -3 (S_aS_a)	2	72.0	0

^a Predicted by Wakana *et al.* (1998) and Ngo (2001) on the basis of joint segregation analysis for *Got–3* in many progenies from crosses between *Citrus* cultivars, and partiallyrdetermined by Mori *et al.* (2009) and Kim *et al.* (2009) from the results of pollination with either S_i or S_a pollen. ^b: not examined.

Table 6. Hirado pummelo accessions with or without S_g or S_{10} allele determined by pollen tube behavior in the crosses with homozygous 'Hirado Buntan' S_1 seedlings ($S_g S_g$ and $S_{10} S_{10}$)

Pistillate parent	Pollen parent (Determined <i>S</i> genotype)	No. of flowers pollinated	Mean No. of pollen tubes in lower one third of style ^a
Hirado pummelo No.1	\times HBS ₁ -1 (S ₁₀ S ₁₀)	3	164.3
Hirado pummelo No.1	\times HBS ₁ -2 ($S_{10}S_{10}$)	3	178.7
Hirado pummelo No.2	\times HBS ₁ -2 ($S_{10}S_{10}$)	3	192.3
Hirado pummelo No.3	\times HBS ₁ -2 ($S_{10}S_{10}$)	3	214.7
Hirado pummelo No.3	\times HBS ₁ -3 (S_gS_g)	3	186.3
Hirado pummelo No.4	\times HBS ₁ -1 (S ₁₀ S ₁₀)	3	228.7
Hirado pummelo No.4	\times HBS ₁ -2 ($S_{10}S_{10}$)	3	247.3
Hirado pummelo No.4	\times HBS ₁ -3 (S _g S _g)	3	214.0
Hirado pummelo No.6	\times HBS ₁ -2 ($S_{10}S_{10}$)	3	141.7
Hirado pummelo No.6	\times HBS ₁ -3 (S _g S _g)	3	113.0
Hirado pummelo No.12	\times HBS -1 (S ₁₀ S ₁₀)	2	303.5
Hirado pummelo No.12	\times HBS ₁ -2 ($S_{10}S_{10}$)	3	235.3
Hirado pummelo No.12	\times HBS ₁ -3 (S _g S _g)	3	317.7
Hirado pummelo No.13	\times HBS ₁ -2 ($S_{10}S_{10}$)	3	299.0
Hirado pummelo No.13	\times HBS -3 (S_gS_g)	3	377.7
Hirado pummelo No.14	\times HBS ₁ -2 ($S_{10}S_{10}$)	3	209.7
Hirado pummelo No.14	\times HBS ₁ -3 (S _g S _g)	3	201.7

^a Pollen tubes were counted in the lower one-third of the style.

ally excellent, the load distributed one of the fruits to his vassals including Senkichi Moriyama, who got three seeds from one and half segments of the pummelo fruit. He sowed the three seeds in Hirado and three relative families, Moriyama, Nomoto and Shinozaki, grew one seedling respectively. The seedling grown in Shinozaki's garden was excellent in the fruit quality. It was propagated as 'Hirado Buntan' and was spread around 1910. The original tree died after the naming. Thus, 'Hirado Buntan' introduced from NIFTS Okitsu Branch which used for the present study belongs to this Moriyama– Shinozuka line.

In Hirado, on the other hand, the domanial load Terasu Matsura also sowed the seeds derived from different fruits from the fruit that he distributed to his vassals. The seedlings were grown in the garden of his large mansion in Hirado, although there was no description about the number of the planted seedlings and size of the orchard in his garden. According to the explanation by the present head of the Matsura family Mr. Akira Matsura (personal communication, 2001), the Hirado pummelo orchard succeeded by now but many Hirado pummelo trees (probably seedlings) planted in the orchard were cut down to build a new residence in 1954, when the Matsura family presented their mansions including a guest house "Senzaikaku" to Hirado city as a Matsura Historical Museum in 1955. In 1998 and 2001, about ten Hirado pummelo trees were cultivated in the orchard and two Hirado pummelo plants were grown in the garden of the new house. Most of these Hirado pummelo plants were appeared to be over 100-year-old seedlings and their characteristics resemble each other except one plant called 'Kessaku' which was considered to be a hybrid between Hirado pummelo and citrus plant like tangelo.

Ngo et al. (2008) reported that the GOT isozyme genotypes of 'Hirado Buntan' were cg for Got-1 and fffor Got-3 whereas those of Hirado pummelo plants cultivated in Matsura's Hirado pummelo orchard were gg for Got-1 and either ee or ef for Got-3, suggesting their different origin. The present result that all Hirado pummelo plants have neither S_g nor S_{10} allele confirms this suggestion. It is considered some possibility that the several pummelo fruits that the domanial load Terasu Matsura got in Nagasaki were genetically different from each other or the contributed pollen for the production of fruits was from multiple pummelo parents with different S alleles. Because of the relatively uniformity in their fruit characteristics, 'Hirado Buntan' and Hirado pummelo plants were seemed to originate from one pummelo tree. The NPAES Report (1917) described, however, the variations in fruit characteristics such as fruit shape, morphology of oil gland, flesh color and smoothness of rind, and defined that Hirado pummelo individuals with excellent white-flesh and spheroidal fruit shape was the same pummelo as 'Hirado Buntan'. The facts obtained from this study and GOT isozyme analysis (Ngo et al., 2001), namely, genetic analysis with S, Got-1 and Got-3 genes, indicated that Hirado pummelo plants belonging to Matsura line and Shinozaki line ('Hirado Buntan') were distinguished each other, but their characteristics were very resemble (data was not presented).

Around the Matsura mansion there were several Hirado pummelo seedlings growing in the personal gardens. Among them, three Hirado pummelo seedling trees were over 100-year-old. One of the three trees was certified to be the seedling of Hirado pummelo cultivated in the Matsura' orchard. The NPAES Report (1917) suggested the expansion of the 'Hirado Buntan' originating from the seedling raised by Moriyama. The present result of S gene analysis suggests that the Hirado pummelo trees expanded in Hirado city are not 'Hirado Buntan' originating from the Shinozaki seedling but 'Hirado Buntan' originating from the Shinozaki seedling but 'Hirado Buntan' –like Hirado pummelos originating from the seedlings raised by Terasu Matsura.

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