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https://doi.org/10.5109/18836

出版情報：九州大学大学院農学研究院紀要. 55 (2), pp.239-245, 2010-10-29. Faculty of Agriculture, Kyushu University
バージョン：
権利関係：

J. Fac. Agr., Kyushu Univ., **55** (2), 239–245 (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

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(*Received June 30, 2010 and accepted July 9, 2010*)

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 self–pollinated '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_aS_a) each other and classified them into two genotype groups of S_aS_a and S_bS_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 or S_bS_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_9 and S_{10} alleles. Furthermore, pollen tube arrest in the style of self–compatible 'Kabusu' sour orange pollinated with S_9 pollen suggested that the genotype of 'Kabusu' is S_fS_9. Inscrutably, neither S_9 nor S_{10} 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 production, 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 (S_1) 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 self–pollinated 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 determination

Cultivar name or accession No.	Scientific name	Self–compatibility[b]	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]	*C. tachibana* Tanaka	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.	SC	poly

[a] Synonym 'Kawano–natsudaidai'. [b] SI: self–incompatible; SC: self–compatible: SSC; semi self–compatible.
[c] 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 (Hirado pummelo No.1)	Garden of Matsura mansion Kagamigawa–cho, Hirado City	>140	seedling
Hirado Matsurahata Buntan (Hirado pummelo No.2)	Hirado pummelo Orchard in Matsura mansion Kagamigawa–cho, Hirado City	>130	seedling
Hirado Murao Buntan (Hirado pummelo No.3)	Murao's garden near to Matsura mansion, Miyanoura–cho, Hirado City	≈130	seedling
Hirado Tabira Buntan (Hirado pummelo No.4)	Beef Cow Improvement Center, Kotedamen, Tabira–cho. The land was leased by Matsuras	≈70	seedling
Kessaku (Hirado pummelo No.6)	Garden of Matsura mansion, Kagamigawa–cho, Hirado City	≈80	seedling
Hirado Suzuki–A pummelo (Hirado pummelo No.12)	seedling of Hirado Murao Buntan, Okubo–cho, Hirado city	≈60	seedling
Hirado Suzuki–B pummelo (Hirado pummelo No.13)	seedling of Hirado Murao Buntan, Okubo–cho, Hirado city	≈40	seedling
Hirado Ijima Buntan (Hirado pummelo No.14)	garden of Ijima's residence, Kagamigawa–cho, Hirado city	≈100	seedling
Hirado Buntan (Hirado pummelo No.20)	NIFTS (Okitsu); one of the three seedlings raised by Moriyama in 1845: originated from the seedling raised by Shinozaki, Okubo–cho, Hirado city	dead	grafting

[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 (S_1) 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 S_1 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 seedlings (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' S_1 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 S_1 seedlings of 'Hirado Buntan', six S_1 seedlings were pollinated with a homozygous S_1 seedling HBS_1–3 whose *S* genotype was defined here as S_aS_a (Table 4). In three of the six S_1 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 S_1 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 S_1 seedlings was determined to be S_aS_a for HBS_1–4, S_aS_a or S_aS_b for HBS_1–7 and S_aS_b for HBS_1–18. To determine the *S* genotype of HBS_1–7, 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_aS_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_7, and has the same *S* allele as 'Kabusu'. Thus, it is defined here that 'Hirado Buntan' has S_9S_{10} genotype for *S* gene and 'Kabusu' has S_7S_9 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_9 or S_{10} (Table 6). When six Hirado pummelo accessions were pollinated with HBS_1–3 (S_9S_9), pollen tubes penetrated into their lower style with the range from 164 to 378 per style, suggesting they do not have S_9 allele. When eight Hirado pummelo accessions were pollinated with HBS_1–1 ($S_{10}S_{10}$) or HBS_1–2 ($S_{10}S_{10}$), 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

Table 3. Determination of *S* genotypes of 'Hirado Buntan' S_1 seedlings by pollination with 'Hirado Buntan' (S_aS_b)

Pollination	No. of flowers pollinated	Mean No. of pollen tubes in style base	Estimated *S* genotype of seed parent
HBS_1–1 × Hirado Buntan (S_aS_b)	4	184.8	S_aS_a or S_bS_b
HBS_1–2 × Hirado Buntan (S_aS_b)	3	168.3	S_aS_a or S_bS_b
HBS_1–3 × Hirado Buntan (S_aS_b)	3	131.0	S_aS_a or S_bS_b
HBS_1–4 × Hirado Buntan (S_aS_b)	3	127.0	S_aS_a or S_bS_b
HBS_1–11 × Hirado Buntan (S_aS_b)	3	60.7	S_aS_a or S_bS_b
HBS_1–18 × Hirado Buntan (S_aS_b)	3	7.3	S_aS_b
Hirado Buntan (S_aS_b) × Hirado Buntan (S_aS_b)	3	5.7	S_aS_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 HBS_1–3 for *S* gene. The genotype of homozygous S_1 seedling HBS_1–3 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_1–1 (S_aS_a or S_bS_b) × HBS_1–3 (S_aS_a)	1	213	S_bS_b
HBS_1–2 (S_aS_a or S_bS_b) × HBS_1–3 (S_aS_a)	2	83.5	S_bS_b
HBS_1–4 (S_aS_a or S_bS_b) × HBS_1–3 (S_aS_a)	2	8.5	S_aS_a
HBS_1–7 (S_aS_a or S_aS_b) × HBS_1–3 (S_aS_a)	3	0.7	S_aS_a or S_aS_b
HBS_1–11 (S_aS_a or S_bS_b) × HBS_1–3 (S_aS_a)	1	82	S_bS_b
HBS_1–18 (S_aS_b) × HBS_1–3 (S_aS_a)	2	5.0	S_aS_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_1 seedlings for the *S* gene

Pistillate parent (Predicted *S* genotype) [a]	Pollen parent (Determined *S* genotype)	No. of flowers pollinated	Mean No. of pollen tubes in each part of style	
			Upper	Lower
Banpeiyu (S_1S_2)	× HBS$_1$–2 (S_bS_b)	3	– [b]	170.3
Banpeiyu (S_1S_2)	× HBS$_1$–3 (S_aS_a)	3	–	120.7
Amanatsu (S_2S_7)	× HBS$_1$–2 (S_bS_b)	1	–	132
Amanatsu (S_2S_7)	× HBS$_1$–3 (S_aS_a)	3	–	162.0
Tosa Buntan (S_1S_3)	× HBS$_1$–2 (S_bS_b)	3	–	213.3
Tosa Buntan (S_1S_3)	× HBS$_1$–3 (S_aS_a)	3	–	177.0
Hassaku (S_4S_5)	× HBS$_1$–2 (S_bS_b)	3	–	141.7
Hassaku (S_4S_5)	× HBS$_1$–3 (S_aS_a)	3	–	154.3
Yuge–hyokan (S_6S_7)	× HBS$_1$–2 (S_bS_b)	3	–	58.3
Yuge–hyokan (S_6S_7)	× HBS$_1$–3 (S_aS_a)	3	–	147.7
Tachibana No.1 (S_7S_4)	× HBS$_1$–2 (S_bS_b)	3	–	82.7
Tachibana No.1 (S_7S_4)	× HBS$_1$–3 (S_aS_a)	3	–	66.7
Anseikan (S_1S_7)	× HBS$_1$–2 (S_bS_b)	2	–	49.0
Anseikan (S_1S_7)	× HBS$_1$–3 (S_aS_a)	3	–	213.0
Hyuganatsu (S_1S_4)	× HBS$_1$–2 (S_bS_b)	3	–	148.0
Hyuganatsu (S_1S_4)	× HBS$_1$–3 (S_aS_a)	3	–	155.7
Shishiyuzu (S_1S_6)	× HBS$_1$–2 (S_bS_b)	3	–	266.7
Shishiyuzu (S_1S_6)	× HBS$_1$–3 (S_aS_a)	3	–	245.7
Kabusu (S_7S_7)	× HBS$_1$–2 (S_bS_b)	3	–	99.7
Kabusu (S_7S_7)	× 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_1 or S_2 pollen. [b]: not examined.

Table 6. Hirado pummelo accessions with or without S_9 or S_{10} allele determined by pollen tube behavior in the crosses with homozygous 'Hirado Buntan' S_1 seedlings (S_9S_9 and $S_{10}S_{10}$)

Pistillate parent	Pollen parent (Determined *S* genotype)	No. of flowers pollinated	Mean No. of pollen tubes in lower one third of style [a]
Hirado pummelo No.1	× HBS$_1$–1 ($S_{10}S_{10}$)	3	164.3
Hirado pummelo No.1	× HBS$_1$–2 ($S_{10}S_{10}$)	3	178.7
Hirado pummelo No.2	× HBS$_1$–2 ($S_{10}S_{10}$)	3	192.3
Hirado pummelo No.3	× HBS$_1$–2 ($S_{10}S_{10}$)	3	214.7
Hirado pummelo No.3	× HBS$_1$–3 (S_9S_9)	3	186.3
Hirado pummelo No.4	× HBS$_1$–1 ($S_{10}S_{10}$)	3	228.7
Hirado pummelo No.4	× HBS$_1$–2 ($S_{10}S_{10}$)	3	247.3
Hirado pummelo No.4	× HBS$_1$–3 (S_9S_9)	3	214.0
Hirado pummelo No.6	× HBS$_1$–2 ($S_{10}S_{10}$)	3	141.7
Hirado pummelo No.6	× HBS$_1$–3 (S_9S_9)	3	113.0
Hirado pummelo No.12	× HBS$_1$–1 ($S_{10}S_{10}$)	2	303.5
Hirado pummelo No.12	× HBS$_1$–2 ($S_{10}S_{10}$)	3	235.3
Hirado pummelo No.12	× HBS$_1$–3 (S_9S_9)	3	317.7
Hirado pummelo No.13	× HBS$_1$–2 ($S_{10}S_{10}$)	3	299.0
Hirado pummelo No.13	× HBS$_1$–3 (S_9S_9)	3	377.7
Hirado pummelo No.14	× HBS$_1$–2 ($S_{10}S_{10}$)	3	209.7
Hirado pummelo No.14	× HBS$_1$–3 (S_9S_9)	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 *ff* for *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_9 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' –like Hirado pummelos originating from the seedlings raised by Terasu Matsura.

ACKNOWLEDGEMENTS

This study was partially supported by the Grand in Aid from the Japan Society for the Promotion of Science (JSPS) (No. 21380027). The authors are grateful to NIFTS, Mr. Akira Matsura the forty–second in a line of Matsura family and Hirado citizens in kagamigawa–cho, Okubo–cho and Sinnomiya–cho, since they kindly provided scions from old Hirado pummelo trees from which adult trees used in this experiment were raised.

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