

Citrus Breeding 2.0 in Japan

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Developing varieties with diverse features that satisfy varied commercial needs, improving overall fruit quality, and quickly releasing them, are prerequisites in citrus breeding. However, these three goals require trade-offs in conventional breeding, even with the application of the marker-assisted selection technique. Conventional breeding cannot achieve these three goals simultaneously and it has been regarded as a breeding trilemma. Integrating a genomics-assisted breeding (GAB) approach that relies on quantitative trait locus detection by genome-wide association study and genome-wide prediction of a trait by genomic selection using enriched marker genotypes enhances breeding efficiency and contributes to eliminating the trilemma. Besides these efforts, the analysis of the genealogy of indigenous citrus varieties revealed that many high-quality indigenous varieties were selected within a few generations. It suggested that selecting a new premium quality hybrid is possible by integrating it with the GAB technique and helps avoid the trilemma. Researchers describe an ongoing comprehensive approach for integrating genomic-assisted breeding (GAB) with citrus genealogy on citrus breeding, called Citrus Breeding 2.0. This method can develop new cultivars with premium quality in a short period.

citrus breeding

marker-assisted selection

genomics-assisted breeding

genome-wide association study

genomic selection

genealogy

1. Introduction

Major citrus cultivars such as sweet orange, Clementine, lemon, grapefruit, and Satsuma have been produced worldwide for over 100 years, and are still considered economically important crops ^{[1][2]}. However, the demand has increased for high-quality new cultivars easy-to-peel and which meet consumers' needs in a short period ^[3]. Tremendous efforts have been devoted to mutation breeding in citrus to select a cultivar from sport, nucellar seedling ^{[4][5][6][7]}, or after irradiation ^[8]. Though mutation breeding has successfully selected spontaneous or induced mutants in various varieties based on color break, rind or flesh color, fruit shape, acidity, or sugar content, mutation occurs at a low frequency ^[9], and the mutation spectrum depends on the varieties used for the method ^{[3][5][6][7]}. These limitations hamper the use of mutation breeding as a ubiquitous method for the development of new varieties. In contrast, crossbreeding is beneficial for developing novel, high-quality cultivars; however, in conventional breeding, it requires more time than mutation breeding, and the possibility to obtain a new elite hybrid depends on both the breeder's experience and chance.

Crossbreeding is a systematic approach for developing new cultivars with novel traits using hybridization ^{[3][10][11]}. However, it requires maintaining many seedlings in the orchard for an extended period and repeatedly evaluating

their characteristics, and consequently this lengthy process is time-consuming and expensive. Therefore, in conventional breeding, satisfying fast breeding of a new hybrid, increasing fruit trait variation, and improving overall fruit quality are trade-offs, and it is difficult to achieve them simultaneously [12]. This constraint is regarded as a 'breeding trilemma' that is difficult to avoid in conventional breeding [13]. To solve these constraints in conventional breeding, the marker-assisted selection (MAS) technique has been introduced in citrus breeding [12]. However, conventional MAS is limited to specific traits controlled by a few genes, and it is difficult to efficiently select various fruit traits that involve multiple genes in complex ways.

2. Citrus Crossbreeding Effort Aims to Improve Fruit Quality

2.1. Past Efforts of the Citrus Breeding Program in NARO

The fruit tree breeding program aims to develop new and unique high-quality cultivars. The citrus crossbreeding program of the Institute of Fruit Tree and Tea Science, NARO (NIFTS) started in 1946 and has produced hybrid cultivars intended for the domestic market [14]. To date, the program has developed 39 hybrid cultivars with eight intermediary mother selections by repeatedly selecting a superior hybrid from a single cross. When the breeding program started, Satsuma, sweet orange, ponkan mandarin, and pummelo were typical in Japan, but each cultivar had drawbacks. For instance, although Satsuma is easy-to-peel, seedless, and productive, it has less flavor and a lower sugar content than oranges and exhibits alternate bearing. Sweet orange sets fruits with high sugar content and a strong and excellent aroma that is suitable for direct consumption and juice production. However, the rind of the sweet orange is hard to peel and requires a knife to eat. Ponkan mandarin has a preferred flavor and higher sugar content than Satsuma, but it has a puffy rind and contains many seeds that hamper eating. Pummelo is the generic name of a set of cultivars that produce large fruit with a thick yellow rind. These pummelo sets fruits with a high soluble solid content (SSC) and pleasant aroma. But they are difficult to peel, set many seeds, and the segment membrane is difficult to chew. The initial goal of the program was to develop a new cultivar with peelability comparable with Satsuma and a preferable aroma similar to sweet orange. 'Kiyomi' is a tangor selected from a cross between Satsuma and a sweet orange cross [15]. 'Kiyomi' had better peelability than sweet orange, but not comparable to Satsuma, and while its aroma was superior to that of Satsuma, it was not comparable to sweet orange.

Besides its fruits, 'Kiyomi' has played a pivotal role in the breeding program, and it has been used as a mother plant because of its monoembryony (**Figure 1**). 'Kiyomi' has contributed to the development of ten hybrid cultivars ('Seihou', 'Tsunokaori', 'Youkou', 'Harumi', 'Akemi', 'Amaka', 'Nishinokaori', 'Tamami', 'Tsunonozomi', and 'Shiranuhi'), and six cultivars ('Amakusa', 'Setoka', 'Harehime', 'Reikou', 'Tsunokagayaki', and 'Asumi') are its progeny (**Figure 1** and **Table 1**). These cultivars expanded the harvest season for five months (August to April) at open culture, enlarged fruit size (150–280 g), and improved SSC (>15). All of these cultivars except 'Amakusa', 'Tamami', and 'Tsunonozomi' set seedless fruit in open fields. Most of them are comparable to Satsuma in that they are easy to peel, and the segment membrane is easy to chew (**Table 1**). Many of these cultivars have "good" flavor; among which, 'Harehime', 'Tamami' and 'Asumi' have a strong and excellent aroma similar to that of sweet orange. In addition, some of these cultivars have unique features for less rind puffing ('Tsunokaori', 'Tsunonozomi'),

less alternate bearing ('Amakusa'), grenadine to red rind ('Akemi', 'Reikou'), rich in beta-cryptoxanthin ('Tsunokagayaki', 'Asumi') and unique fruit shape ('Shiranuhi'). All these cultivars are protected under the Plant Variety Protection and Seed Act, Japan except 'Shiranuhi' [16], which is now produced in the USA ('Sumo citrus'), Korea ('Hallabong'), and Brazil ('Kinsei'). Thus, starting from 'Kiyomi', a series of cultivars that have diverse and improved traits have been bred through several crossings. It demonstrates the performance of crossbreeding that enables the expansion of diversity and improvement of fruit quality.

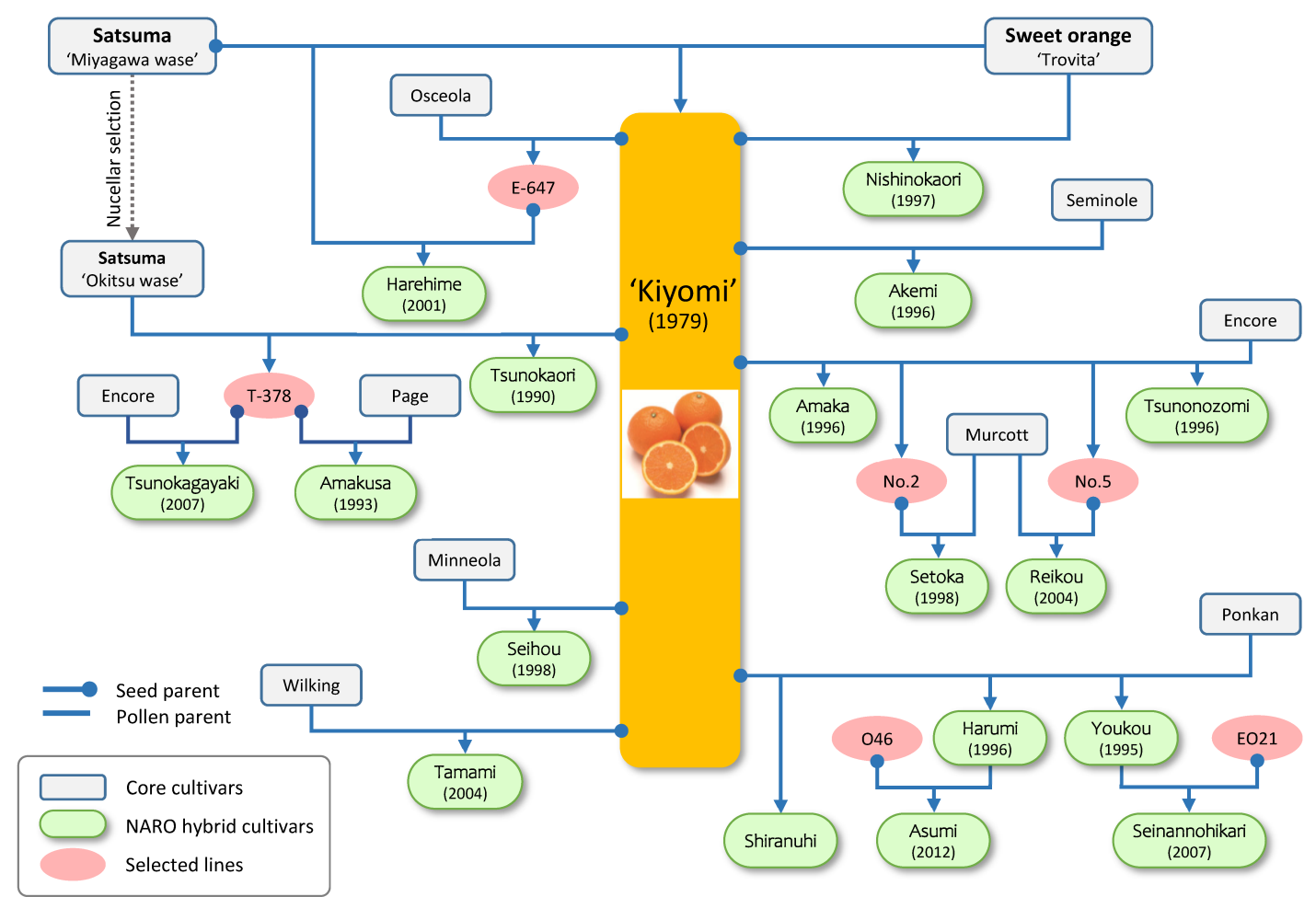


Figure 1. Pedigree tree of 'Kiyomi' tangor with its offspring cultivars. Connector line terminating in a circle denotes seed parent, straight end denotes pollen parent.

Table 1. List of the offspring cultivars of 'Kiyomi'. YTD: Years to development, SL: seedless (<5 seeds per fruit), BCP: beta-cryptoxanthin, SSC: soluble solid content.

Cultivar	Year of Release	YTD	Harvest	Fruit Size (g)	Peelability	Seeds	Features
Kiyomi	1979	30	Late March	~200	Moderate	SL	A tangor (mandarin x orange). Orange-like aroma and juicy

Cultivar	Year of Release	YTD	Harvest	Fruit Size (g)	Peelability	Seeds	Features
Seihou	1988	17	Late January	~200	Hard	SL	Large fruit with orange-like good flavor and soft flesh
Tshunokaori	1990	18	March–April	~160	Easy	SL	High SSC (>13), and no rind-puffing
Amakusa	1993	11	August–January	~200	Hard	--	High SSC (>12), and less alternate bearing
Youkou	1995	23	January–February	250–300	Easy	SL	Large fruit with good flavor and soft flesh
Harumi	1996	17	January	180–200	Easy	SL	Large fruit with good flavor and soft flesh
Akemi	1996	21	March	160–180	Moderate	SL	High SSC (>12), grenadine rind, and soft flesh
Amaka	1996	22	August–January	200–250	Easy	SL	Soft fruit with good orange-like aroma
Nishinokaori	1997	31	August–January	100–180	Easy	SL	High SSC (>12), orange-like flavor, and soft flesh
Setoka	1998	14	February	200–280	Easy	SL	Large fruit with good flavor, high SSC (>12), and soft flesh
Harehime	2001	11	August	180	Easy	SL	Early type with good flavor, juicy, and soft flesh
Tamami	2004	24	January	150	Easy	--	High SSC (>12) and good orange-like flavor
Reikou	2004	20	January	210	Moderate	SL	High SSC (>12), good flavor, and red-skin
Tsunokagayaki	2008	24	January–February	180–250	Easy	SL	High SSC (>13), soft fruit, BCP rich, and suitable for greenhouse production
Tsunonozomi	2011	37	August	190	Easy	--	Early type with good flavor, high SSC (>12), juicy, and less rind puffing
Asumi	2013	21	February	150	Moderate	SL	Very high SSC (>15), orange-like flavor, and BCP rich

methods maintain many seedlings in the orchard for an extended period to evaluate their traits for several years to minimize the influence of potential variations. Alternate bearing is another drawback of some citrus fruits. Evaluating fruit traits at least twice is essential for reliable performance evaluation. However, it requires significant effort to maintain thousands of seedlings for the selection of just one elite hybrid. The NARO citrus breeding program took

Cultivar	Year of Release	YTD	Harvest	Fruit Size (g)	Peelability	Seeds	^[13] Features	
Shiranuhi *			February–March	230	Easy	SL	High SSC (>14), soft fruit, good flavor, and unique fruit shape	g program 20 years; that even valuation in p is time-

consuming and incurs high costs over a long period to maintain the seedlings. Therefore, the cost of breeding a new cultivar can be reduced by increasing the number of promising hybrids acquired per seedling evaluated.

*. Not patented

In the conventional breeding approach in NARO, the germinated seeds are nurtured for one to two years, after which a portion of them will be selected empirically for grafting at the orchard. The fruit traits of the seedlings selected for grafting are then evaluated for several years, and if any of them show promise, they will be subjected to further evaluation. Developing a high-quality new, unique hybrid is required to adjust for the broad array of consumer preferences ^{[3][11]}. In addition, there is now a need to breed new cultivars in the shortest possible period to adjust for rapid changes in the market ^[12]. The citrus breeding program of NARO aims to achieve for satisfying three goals simultaneously: releasing new cultivars quickly, developing new cultivars that satisfy varied commercial needs, and improving overall fruit quality. However, satisfying these three objectives necessitates trade-offs—prioritizing one makes it difficult to achieve the others. Among those goals, the low probability of obtaining a promising hybrid from a set of the provided seedlings forms the background of the breeding trilemma. The insufficient number of candidate hybrids obtained from a single cross that causes generations needing to be repeated, prolonging the duration for breeding.

3. Suggestions from the Citrus Genealogy for Breeding

3.1. Genomic Breeding Aims to Avoid the Breeding Trilemma

In citrus breeding, the longest part of the breeding period consists of the long juvenile period until first blooming (6–7 years) and the period required for trait evaluation in the field. GAB is an emerging technology to improve breeding efficiency by rapidly selecting promising individuals of high quality ^{[17][18]}. This approach aims to avoid the breeding trilemma, but selection by MAS and GS alone is not sufficient to shorten the time required for breeding. ‘Fast breeding’ that intends to develop unique, unconventional cultivars in the shortest possible time is another constraint to the breeding trilemma ^[13]. Minimizing the time from crossing to the selection of an elite hybrid in each generation would be effective in achieving fast breeding. However, most citrus cultivars have long juvenile periods, and alternate bearing that hampers the trait evaluation in the short term is severe in young citrus trees. It extends the period for repeating the trait evaluation to maintain accuracy, making the whole breeding process long. The citrus breeding program in NARO evaluates over 20 traits. The current prediction accuracy in citrus by GS is up to 80% for particular traits, but other traits do not reach this level ^[19]. That means traits that MAS or GS cannot select or are less predictable need to be evaluated in the field, along with confirming the traits selected by MAS or GS. Therefore, even if using GAB, a drastic reduction in the breeding period cannot be expected because both MAS and GS are the selection method, not the methods that shorten the juvenile period. Furthermore, considering the period required for a single generation (over 20 years), achieving a breeding process that had required several

generations by the conventional methods within a single generation will drastically shorten the time required for developing a cultivar.

The conventional breeding program often uses existing superior cultivars as parents. Although this approach ensures that breeders develop a new hybrid of high quality in a short period, crosses between similar cultivars lower the novelty of the hybrid breed. Using cultivars not used as breeding parents to cross may contribute to enlarging the diversity of hybrids and selecting a novel individual. While providing these unfamiliar cultivars for breeding is expected to contribute to developing individuals with novel traits, the multiple unwanted traits of the parent may decrease the ratio of individuals that exceed the selection threshold. Although few studies have examined the inheritance of quantitative fruit traits in citrus, Combrink et al. showed the continuous distribution of the height and width of fruit in six offspring populations developed from different cultivars, using Kiyomi as the common female plant [20]. They used six cultivars whose fruit sizes are smaller than Kiyomi for the male plants. The study showed that the medians of fruit widths and height of the six populations were close to that of another parent that set fruit smaller than Kiyomi. Their results strongly suggested that more seedlings could be required to select individuals of larger fruit sizes than Kiyomi when crossing Kiyomi with a small fruit cultivar. In contrast, these parental cultivars' fruit shape indexes (ratio of fruit width per fruit height) showed no considerable differences and a narrow distribution. Although medians of the fruit shape indexes of these populations were close to those of parental cultivars, a few individuals showed substantial differences from their parents for the fruit shape index [20]. They also revealed that rind color factors (L^* and a^*) of the same six populations of Kiyomi showed similar distributions in their offspring populations as observed in fruit size in another study [21].

Fruit size is one of the primary traits for selection in the NARO citrus breeding program that uses Kiyomi to cross frequently, as demonstrated in **Figure 1**. Many cultivars that have not been used for breeding set small fruits in the program. Thus, as demonstrated by Combrink et al. [20], the average fruit size of the population is smaller than Kiyomi and is an obstacle when using a small fruit cultivar for crossing to expand the diversity. These results were examined for five fruit traits in populations of Kiyomi as the common parent, and great care must be taken to expand the finding to all crosses and fruit traits. However, these results strongly suggest that it is challenging in conventional breeding to develop new hybrid cultivars that exceed the threshold on the broad traits in a single generation because using non-elite cultivars would suppress the occurrence of promising individuals in a population.

For example, NARO recently released the 'Aurastar' [22], 'Nou No. 7', and 'Nou No. 8' cultivars [23]. These are second-generation trifoliate oranges (TF) obtained by crossing Hassaku × TF 'Flying dragon' followed by crossing with Banpeiyu pummelo or Kiyomi to introduce resistance to the citrus tristeza virus of TF into the commercial cultivars [22][23]. Although these have improved fruit traits compared to TF, they retain the unpleasant odor of TF and are inedible. Thus, they require further introgression of genomes of edible citrus cultivars. This is a typical example of the breeding trilemma. Serra et al. proposed an approach (Marker-Assisted Introgression; MAI) to shorten the generation required for the introgression of a distant cross with MAS in peach [24]. Despite the use of the MAI approach, it requires several generations and avoiding a lengthy breeding period is difficult in citrus. Therefore, a different approach is needed to resolve the trilemma.

3.2. Lessons from the Revealed Genealogy of Citrus Cultivars

Understanding how existing indigenous citrus cultivars have been selected would provide clues to improve breeding efficiency and resolve the trilemma. A recent study revealed that modern citrus species emerged from iterated hybridization of ancestral citrus cultivars between China and India approximately 9 million years ago [25][26]. Although the process by which current indigenous citrus cultivars were established had long been unknown, Shimizu et al. revealed the pedigree of over 60 of these cultivars [27]. Tanaka classified more than 160 indigenous citrus ‘species’ [28][29]. However, these results confirmed that most of these ‘species’ are natural hybrids and thus cannot be classified as individual species [27].

The study of citrus genealogy provided important suggestions: (1) Repeated crossing of several key cultivars, such as Kishu, Kunenbo, Yuzu, and pummelo, has led to the current diverse group of cultivars within a few generations. This indicates that the genetic diversity of those indigenous cultivars has decreased, and Shimizu et al. [27] confirmed this using SSR marker analysis. (2) Fruit traits such as size, shape, color, rind thickness, SSC, acidity, and peelability had changed significantly in just one generation. This suggests that repeated generations are not always essential to produce an elite hybrid. (3) The elucidated genealogies indicate that cultivars not previously used in breeding were the parents of several unique cultivars. Using these unfamiliar exotic cultivars as breeding parents would be helpful in developing new cultivars with novel traits.

3.3. Hybrids Selected from Kishu and Kunenbo

Kishu (*C. kinokuni* hort. ex Tanaka) is an ancient cultivar that has been known since at least 740 A.D. in Japan [30]. Shimizu et al. demonstrated that Kishu served as the direct parent of 12 cultivars (Kunenbo, Satsuma, Yatsushiro, Naruto, Oukan, Natsudaikai, Nidonari mikan, Fukure mikan, Suruga Yuko, Sokitsu, Sanbokan, and Andokan) and as the indirect parent of 24 cultivars (Jabara, Mochiyu, Kabosu, Henka mikan, Kizu, Konkoji, Hyoukan, Hassaku, Asahikan, Kaikoukan, Yamabuki, Iyo, USSR tangelo, Shunkokan, Kawabata, Unzoki, Kabuchi, Keraji, Yuge hyoukan, Ujutkitsu, Kawachi bankan, Sanbokan, Andokan, and Yuukunibu) through Naruto (*C. medioglobosa* hort. ex Tanaka) or Kunenbo (*C. nobilis* Lour. var. kunep Tanaka) [27]. Kunenbo is an offspring of Kishu, crossed with an unidentified seed parent [27]. This was confirmed using whole-genome sequencing analysis [31].

Comparing the parental Kishu and Kunenbo with their offspring cultivars shows the changes in fruit size, rind color, and rind smoothness between them (**Figure 2**). Fruit cross-sections also show the changes in flesh color, rind thickness, and the number of seeds. Although not apparent from the images, variation is also observed among these cultivars in many traits, such as sugar and acid content, coloring time and aroma, and flesh quality. These trait variations do not differ from those found in the population of a single cross in citrus. The probability of obtaining an elite candidate with superior traits, such as Satsuma, from a single cross between Kishu and Kunenbo is supposed to be very low. Therefore, with the current systematic breeding program, the possibility of selecting an individual with traits comparable to those of Satsuma is insignificant.

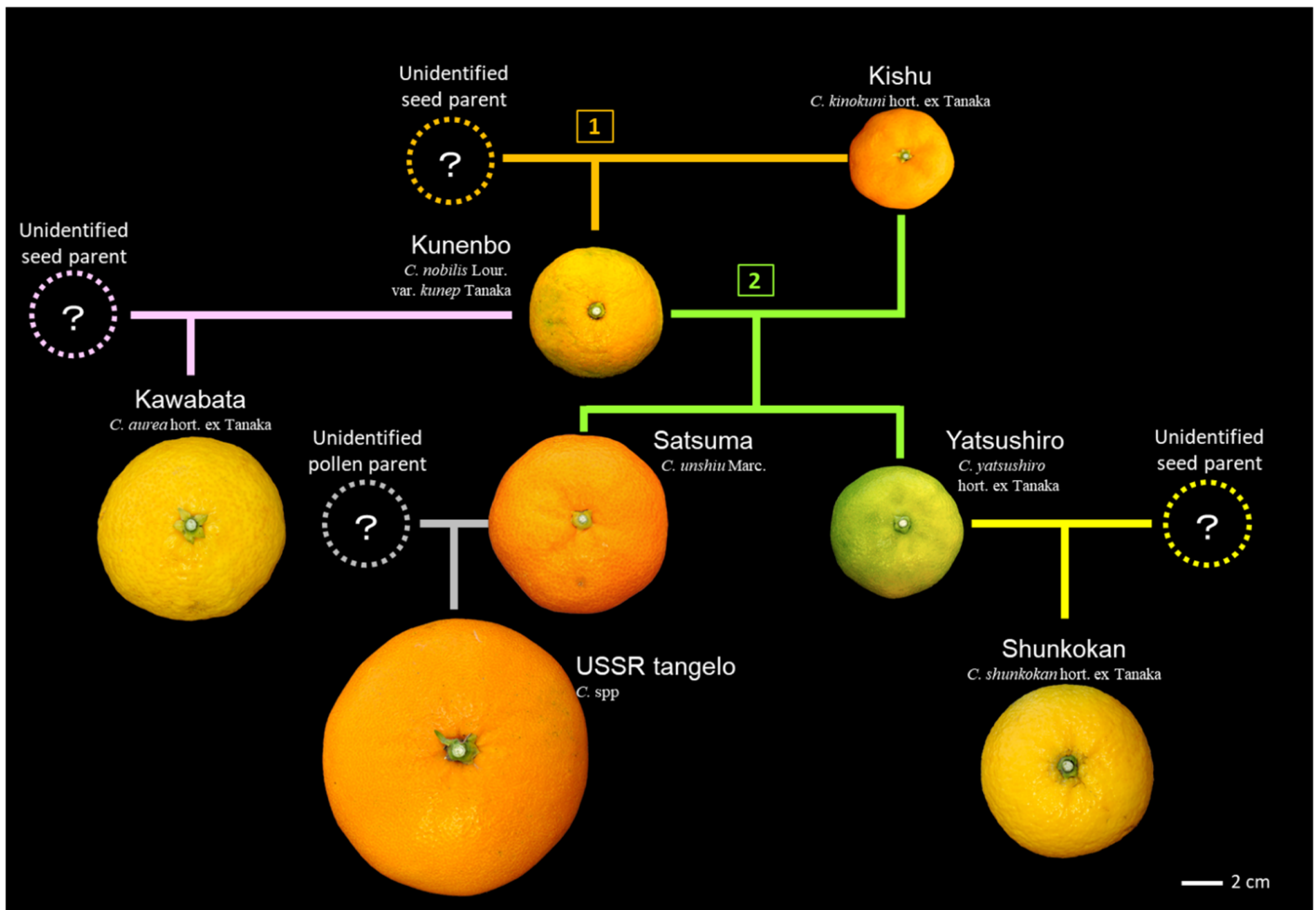


Figure 2. Pedigree of Kishu (*C. kinokuni* hort. ex Tanaka) and Kunenbo (*C. nobilis* Lour. var. *kunep* Tanaka). A question mark indicates an unidentified parent. The numbers in squares indicate the first cross of Kishu to produce Kunenbo (1), then the second cross of Kishu with Kunenbo to produce Satsuma and Yatsushiro (2).

Satsuma was discovered in Japan around 1648 [32]. One factor that enabled the selection of Satsuma from a cross between Kishu and Kunenbo when no systematic breeding system was available was adjacent cultivation of these that facilitate natural hybridization [30]. There were many such areas in Japan with seeds obtained and discarded every year. Satsuma would be selected over several decades from the seedlings germinated from these discarded seeds. This indicates that it is possible to select a high-quality elite hybrid within a single generation by taking sufficient time for selection, even without conducting modern systematic breeding.

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