# Development of a simple and easily interpretable cultivar identification system using 18 InDel markers in Japanese Pummelo (Citrus maxima)

**Riki Shimada**
Gifu University
**Tatsumi Netsu**
Shizuoka University
**Horiike Tokumasa**
Gifu University
**Tomoaki Watanabe**
NARO
**Keisuke Nonaka**
NARO
**Akiyoshi Tominaga**
tominaga.akiyoshi@shizuoka.ac.jp

Gifu University

**Research Article**

**Keywords:** Cultivar identification, Molecular marker, Breeders' rights, Parentage, Germplasm management

**Posted Date:** July 6th, 2026

**DOI:** https://doi.org/10.21203/rs.3.rs-10192350/v1



**Additional Declarations:** No competing interests reported.

## Abstract

Pummelo is one of the ancestral species involved in the origin and diversification of citrus and is also an important fruit crop cultivated mainly in Asia. In Japan, cultivar identification systems based on CAPS and InDel markers have been developed for mandarins and acid citrus and are used as practical tools for the protection of breeders' rights. However, a simple cultivar identification system for pummelo has not yet been sufficiently established. In this study, we designed 2,172 InDel markers potentially suitable for pummelo cultivar identification based on comparative analysis of whole-genome sequences and long-read data. Among these, 18 InDel markers showing clear biallelic PCR fragment patterns were selected, enabling cultivar identification using only standard PCR and agarose gel electrophoresis. These markers enabled major pummelo cultivars and their hybrids grown in Japan to be clearly distinguished using a minimum of five markers. In addition, a unique allele shared by 'Hondabuntan' and 'Hassaku' suggests that 'Hondabuntan' is a candidate parent of 'Hassaku'. The InDel markers developed in this study are expected to contribute not only to the protection of breeders' rights through cultivar identification but also to the elucidation of the genetic background of pummelo and provide useful knowledge for citrus breeding.

## Introduction

Pummelo (*Citrus maxima* (Burm.) Merr.) is characterized by large leaves with broad winged petioles, very large and fragrant flowers, and large fruits containing monoembryonic seeds (Ahmed et al., 2019; Uzun and Yesiloglu, 2012). Pummelo is considered to have originated in Yunnan Province, China (Huang et al., 2025), and is positioned, together with *C. medica* and *C. reticulata*, as one of the basic species involved in the origin and diversification of cultivated citrus. These three species gave rise to modern cultivated citrus species through repeated hybridization (Nicolosi et al., 2000; Omura and Shimada, 2016), making pummelo an important taxon for understanding citrus cultivar formation. In addition, the contribution of the pummelo genome has been demonstrated in the formation of major citrus cultivars grown in Japan (Fujii et al., 2016; Shimizu et al., 2016; Wu et al., 2021), suggesting that pummelo has played an important role in citrus breeding and cultivar diversification in Japan.

In Japan, pummelo is one of the important citrus fruit crops, with an agricultural output value of 4.5 billion yen, ranking fourth among citrus fruits after satsuma mandarin, Shiranui, and yuzu (Ministry of Agriculture, Forestry and Fisheries, Production Agricultural Income Statistics, e-Stat, https://www.maff.go.jp/j/tokei/kouhyou/nougyou_sansyutu/index.html, accessed March 22, 2026). In addition, various pummelo cultivars have long been grown in different regions of the country, including regional specialties such as 'Banpeiyu' in Kumamoto Prefecture and 'Tosa buntan' in Kochi Prefecture. Among these, 'Banpeiyu' grown in Yatsushiro has been registered as 'Yatsushiro Tokusan Banpeiyu' under the Geographical Indication (GI) Protection System of the Ministry of Agriculture, Forestry and Fisheries, and has attracted increasing attention in recent years (Ministry of Agriculture, Forestry and Fisheries. No. 94: Yatsushiro Tokusan Banpeiyu, https://www.maff.go.jp/j/shokusan/gi_act/register/0094/index.html, accessed March 22, 2026). Moreover, because pummelo is predominantly monoembryonic, zygotic seedlings can readily arise from open pollination, leading to the establishment of commercially important natural hybrid cultivars such as 'Kawachibankan' (*C. kawachiensis*) in Kumamoto Prefecture and 'Hassaku' (*C. hassaku* hort. ex Tanaka) in Hiroshima Prefecture. Thus, pummelo and its hybrid cultivars have played an important role in domestic citrus production and the formation of regional brands.

In contrast, recent reports have described cases in which nursery trees of newly bred citrus cultivars developed in Japan were taken overseas without authorization, leading to the production of pirated fruits and potentially causing economic losses to domestic producers (Endo et al., 2022). Therefore, cultivar identification systems based on various DNA markers have been developed to protect breeders' rights for registered Japanese citrus cultivars. First, CAPS (cleaved amplified polymorphic site) markers were developed for citrus (Shimada et al., 2014), and cultivar identification systems using these markers have been reported to enable reliable discrimination of citrus cultivars bred in Ehime Prefecture and at the NARO Institute of Fruit Tree and Tea Science (Fujii et al., 2019; Ninomiya et al., 2015; Nonaka et al., 2017). In addition, identification methods using SNP (single nucleotide polymorphism) markers and InDel (insertion/deletion polymorphism) markers have also been reported to enable the discrimination of Japanese citrus cultivars (Endo et al., 2020; Noda et al., 2021). These markers have also been applied to acid citrus cultivars (Niimi et al., 2021, 2022), demonstrating the continued development of cultivar protection technologies based on molecular markers. In fruit trees, molecular markers also play an important role in verifying parent–offspring relationships among cultivars and providing accurate pedigree information for breeding programs (Nonaka et al., 2017). Indeed, in several fruit crops, inconsistencies in the reported parentage of cultivars have been identified and revised using molecular markers (Kimura et al., 2003; Ninomiya et al., 2015; Yamamoto et al., 2003). Therefore, the establishment of reliable molecular markers is useful not only for cultivar protection but also for elucidating the genetic background of cultivars.

However, our previous application of InDel markers developed for mandarin cultivar identification to pummelo failed to discriminate major pummelo cultivars grown in Japan (Shimada et al., 2025). Many existing markers were developed mainly for cultivars derived from hybridization between two basic species, mandarin and pummelo, and may therefore not sufficiently capture the polymorphisms required to distinguish cultivars within ancestral citrus species such as pummelo. Thus, the development of new molecular markers with high discriminatory power for major pummelo cultivars is required. In this study, we developed InDel markers capable of distinguishing pummelo cultivars using LINDA, a marker design support tool. The markers developed in this study are expected to be useful not only for identifying major pummelo cultivars distributed in Japan but also for providing fundamental information for examining the genetic background and relationships among Japanese pummelo cultivars.

## Materials and Methods

## 1. Design and screening of InDel markers

InDel markers were designed using LINDA, a marker design support tool. LINDA was downloaded from GitHub (https://github.com/li3083200/LINDA) and used in this study. The LINDA analysis was performed using default parameters. LINDA designs InDel markers based on comparative analysis between

whole-genome sequences and long-read sequences. In this study, the whole-genome sequence of *C. maxima* (accession ID: GCA_002006925.1) was used as the reference genome (Wang et al., 2017), and the long-read sequencing data of 'Matobuntan' registered under BioProject PRJDB15974 and BioSample SAMD01597629 were used. Among the markers generated by LINDA, 18 markers showing clear biallelic PCR fragment patterns were selected, ensuring that at least one marker was located on each chromosome.

## 2. Plant materials and DNA extraction

In this study, a total of 24 pummelo cultivars and their hybrids maintained at the Citrus Research Division of the Institute of Fruit Tree and Tea Science, NARO (NIFTS), were used (Table 1). The plant materials included pummelo cultivars and their hybrids listed in the Fruit Tree Production and Shipment Statistics and in the Survey on Production Trends and Related Information for Specialty Fruit Trees conducted by the Ministry of Agriculture, Forestry and Fisheries of Japan, as well as a satsuma mandarin cultivar used as a breeding parent. Genomic DNA was extracted from mature leaves using a modified CTAB method (Doyle and Doyle, 1987).

Table 1
Cultivars and local varieties of pummelo and its hybrids used in the present study.

| Variety | Scientific name[z] | JP No.[y] | Parentage | Lane number[x] |
|---|---|---|---|---|
| Tosabuntan | *C. maxima* | 117280 | | 1 |
| Suishobuntan | *C. maxima* | 117279 | | 2 |
| Otachibana | *C. otachibana* hort. ex Yu.Tanaka | 117362 | | 3 |
| Banpeiyu | *C. maxima* | 171506 | | 4 |
| Anseikan | *C. maxima* | 117431 | | 5 |
| Chandler | *C. maxima* | 113449 | | 6 |
| Benimadoka | *C. maxima* | 117153 | Matobuntan×Hiradobuntan | 7 |
| Hayasaki | *C. maxima* | 117152 | Matobuntan×Hiradobuntan | 8 |
| Matobuntan | *C. maxima* | 117283 | | 9 |
| Hiradobuntan | *C. maxima* | 171507 | | 10 |
| Hogenbuntan | *C. maxima* | 117282 | | 11 |
| Banoukan | *C. maxima* | 117432 | | 12 |
| Hondabuntan | *C. maxima* | 117281 | | 13 |
| Natsumikan | *C. natsudaidai* Hayata | 117297 | | 14 |
| Hassaku | *C. hassaku* hort. ex Tanaka | 117286 | | 15 |
| Kawachibankan | *C. kawachiensis* | 117412 | | 16 |
| Summer Fresh | *Citrus* sp. | 168867 | Hassaku × Natsumikan | 17 |
| Naruto | *C. medioglobosa hort.* ex Tanaka | 117293 | | 18 |
| Grapefruit | *C. paradisi* Macf. | 168864 | | 19 |
| Yugehyokan | *C. yuge-hyokan* hort. ex Yu.Tanaka | 117507 | | 20 |
| Sweet Spring | *Citrus* sp. | 168866 | Uedaunshiu×Hassaku | 21 |
| Yellow Pummelo | *Citrus* sp. | 169661 | Hassaku × Hiradobuntan | 22 |
| May Pummelo | *Citrus* sp. | 169662 | Hassaku × Hiradobuntan | 23 |
| Uedaunshiu | *C. unshiu* Marcov. | 117333 | | 24 |

[z] Scientific names or parents' names are referenced from Iwahori (1999) and Shimizu et al. (2016).

[y] Description by Genebank（https://www.gene.affrc.go.jp/index_ja.php）.

[x] Indicates electrophoresis number.

## 3. PCR amplification and gel electrophoresis

PCR amplification was performed in a 10 μL reaction mixture containing 5 ng of genomic DNA, 5 pmol each of forward and reverse primers, and 5 μL of GoTaq® Green Master Mix (Promega, Madison, WI, USA). The PCR conditions followed the method of Endo et al. (2022). Briefly, an initial denaturation was performed at 95°C for 2 min, followed by 32 cycles of denaturation at 95°C for 30 s, annealing at 57°C for 30 s, and extension at 72°C for 1 min, with a final

extension at 72°C for 5 min. The PCR products were separated by electrophoresis on a 2.0% agarose gel (Agarose S; NIPPON GENE CO., Tokyo, Japan), stained with ethidium bromide (EtBr Solution; NIPPON GENE CO.), and visualized.

## 4. Genotyping using InDel markers

Based on the electrophoresis profiles obtained, the amplified fragment patterns of alleles for each marker were recorded. Allele bands were designated as “A” and “B” according to fragment size, with the larger fragment designated as allele A and the smaller fragment as allele B. Using this information, the minimum marker set required to distinguish the 24 cultivars was determined using MinimalMarker software (Fujii et al., 2013). Observed heterozygosity (Ho), expected heterozygosity (He), and polymorphism information content (PIC) were calculated using MarkerToolKit v1.0 (Fujii, 2008). The probability that two or more cultivars share identical genotypes was also calculated using MarkerToolKit v1.0, as described previously (Ukai, 2005). Parentage was assessed by examining whether each putative parent contributed one marker allele to the progeny at each marker locus.

## 5. Parentage analysis of ‘Hassaku’

Among the markers identified using LINDA, one primer set amplified a specific band shared by ‘Hondabuntan’ and ‘Hassaku’. The primer sequences were as follows: forward primer, 5'-AATGAACCAAGTCAAACAACTAGCTATT-3'; reverse primer, 5'-GATTCTATTCTACAGTGCATCTCAATCC-3'. For this marker, PCR amplification and gel electrophoresis were performed as described above. DNA fragments corresponding to the A, B, and C alleles were excised from the gel and purified using a gel extraction kit (NucleoSpin® Gel and PCR Clean-up; Takara Bio, Shiga, Japan). Sequencing was performed by FASMAC Co., Ltd. In addition, ‘Kunenbo’ (JP No. 117387), which was examined as a putative parent of ‘Hassaku’, was also genotyped using the 18 InDel markers developed in this study according to the same procedure.

# Results

## 1. Selection of InDel markers for pummelo cultivar identification

As a result of the analysis using LINDA, 2,172 candidate InDel markers expected to be polymorphic in pummelo were designed (Table S1). These markers were distributed across all chromosomes, with 162 to 372 markers designed per chromosome. Among them, InDel markers showing clear biallelic PCR fragment patterns were screened, and 18 InDel markers were selected (Table 2). The size difference between the A and B alleles was at least 150 bp for all selected markers, enabling straightforward visual discrimination of genotypes by electrophoresis.

Table 2
InDel markers developed in this study for cultivar identification

| Marker name | Chr number | InDel position | Forward primer (5' -3') | Reverse primer (5' – 3') |
|---|---|---|---|---|
| LID-1 | Chr1 | 10884932 | GTACCATGGTCAAGTTGCATACAATA | ATATTTTCCAAAGATTGAGGCACGAA |
| LID-2 | Chr1 | 31807844 | TGGCAGTCCATTTTTCCATTGTTATTA | AGAGAAGAAGAAAATGAAATGAAGCGA |
| LID-3 | Chr2 | 14292245 | CTTTTCCTTTGTAAATGAGAACTGGAGA | AATGAATCCTAGTATGCAACGAAGTATG |
| LID-4 | Chr2 | 51307140 | CAGCTAAGACTGGTCAATAAAATTACTGTA | AATAAATAAAAACCAGAGTCGAGAAGGATT |
| LID-5 | Chr3 | 3042270 | TGGCTGATCCAAATTTAATGATACGG | GCAATGTCATTGTACCTGTATTAGCA |
| LID-6 | Chr3 | 13061837 | CCACGCAATGAAAGTATTGAGATCTT | TAGGATCTTTAGGTGAGCGTGTTTTA |
| LID-7 | Chr4 | 1262506 | AGTAAAGGAAATGTTGAAATTTAGTACCGA | GCTTGCAGGTATGTTATTGTAAATCTTTAC |
| LID-8 | Chr4 | 1717946 | CTAAACCAACTAGATTTAGAGTATGGAACG | TGGAGGTTGATGATAAAAACTAATTACCTC |
| LID-9 | Chr4 | 1751601 | AAACACATATAGCTTACTTAAACCACGATT | GGTTCATTTCTTTTGTTTAGTTGTTGAAAC |
| LID-10 | Chr4 | 25546115 | CAGATGTTTTAGAAATATCACAAATGCCC | TATCCCTTACCTTTCCAAAATTTGAACTT |
| LID-11 | Chr5 | 106133 | TCATAAAATCTTCATTGGAGAAGGATTTGT | CGTTTTGGTATCTTTTCTGACATCATTATG |
| LID-12 | Chr5 | 29847343 | ATGGCTTTTTCCTAAGAATTCAATCAAATC | TTGTTTGTTAAATGGGAAATTCCAAAAGAA |
| LID-13 | Chr6 | 7988393 | TTTAACCAGTCAGTAATGATAGGCAAAG | TCAATACCAAACAAGACCTAAAAGTACC |
| LID-14 | Chr6 | 14848467 | ATTAAACTTTATCGCAACCTTTTGTTCTTT | AGTATCTTCCTTCAAATTCATTAACTAGGC |
| LID-15 | Chr7 | 2286392 | GAGATGCTTATTTTAATTGCCAACTTTGTA | ATATACACTACTCTTTAAACTTACAGCAGC |
| LID-16 | Chr7 | 12952826 | CGCCATTCATTCAATTTAGTGGATAAAAA | CCATCAAGATCATCAACGTTTAGTACAAA |
| LID-17 | Chr8 | 16268946 | ATGTCTTCAAGCAATTTGTTCTGTAGAA | CACTGAATGAAATTTATGGCATAAACCC |
| LID-18 | Chr9 | 34961861 | CTGGAAAATGTTTTCTGAATTTGCCTAA | CACGTATTGGATGATTACTTGTCTCATT |

The 18 selected InDel markers were applied to 24 cultivars, including pummelos, pummelo hybrids, and satsuma mandarin. As a result, clear biallelic PCR fragment patterns were obtained for each InDel marker using DNA extracted from leaves (Fig. 1, Table 3).

Table 3
Genotypes of pummelo and its hybrids based on 18 InDel markers

| Variety | LID-1 | LID-2 | LID-3 | LID-4 | LID-5 | LID-6 | LID-7 | LID-8 | LID-9 | LID-10 | LID-11 | LID-12 | LID-13 | LID-14 | LID-15 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Tosabuntan | AA | BB | BB | AB | AB | AA | AA | AB | AB | AB | AA | AA | BB | AA | AB |
| Suishobuntan | AB | AB | BB | AB | AB | AA | AA | AB | BB | AB | AA | AA | BB | AA | AB |
| Otachibana | AA | BB | BB | AB | AB | AA | AA | AB | AB | AB | AA | AA | BB | AA | AB |
| Banpeiyu | AB | BB | AB | BB | AA | AA | AA | AB | AB | AB | AA | AA | BB | AA | AA |
| Anseikan | AB | BB | BB | BB | AA | BB | AB | AB | AB | BB | AB | AB | BB | AB | AA |
| Chandler | AB | AB | BB | BB | AA | AA | AA | AB | AB | AB | AA | AB | AB | AA | AA |
| Benimadoka | BB | AB | BB | BB | AA | AA | AA | AB | BB | AB | AA | AB | BB | AA | AB |
| Hayasaki | BB | AB | BB | AB | AA | AB | AA | AB | BB | AB | AA | AB | BB | AA | AA |
| Matobuntan | AB | BB | BB | AB | AA | AB | AA | AA | BB | AB | AA | AB | BB | AA | AB |
| Hiradobuntan | AB | AB | AB | BB | AA | AA | AA | AB | AB | AB | AA | AA | AB | AA | AA |
| Hogenbuntan | AA | BB | BB | AB | AB | AA | AA | AB | AB | AB | AA | AA | BB | AA | AB |
| Banoukan | BB | BB | AB | BB | AB | AA | AB | AB | AB | AB | AB | AB | AB | AB | AB |
| Hondabuntan | BB | AB | AB | BB | AA | AB | AA | AA | BB | AB | AA | AB | AB | AA | AB |
| Natsumikan | BB | BB | BB | BB | AB | AA | AB | BB | AA | BB | AB | AB | BB | AB | AB |
| Hassaku | AB | AB | AB | BB | AB | AB | AB | AB | AB | AB | AB | AB | AB | AB | BB |
| Kawachibankan | AB | BB | BB | BB | AA | AB | AB | AB | AB | AB | AB | AA | BB | AB | AB |
| Summer Fresh | AB | BB | BB | BB | AB | AA | AB | BB | AA | BB | AB | AA | AB | AB | BB |
| Naruto | BB | BB | BB | BB | AB | AB | AB | AB | AB | AB | AB | BB | AB | AB | AA |
| Grapefruit | AB | AB | BB | BB | AB | AA | AB | AB | AB | BB | AB | AB | BB | AB | BB |
| Yugehyokan | AB | BB | BB | BB | AA | AB | AB | AB | AB | AB | AB | AB | BB | AA | AB |
| Sweet Spring | AB | BB | AB | BB | AB | AB | BB | BB | AA | BB | AB | AB | AB | AB | BB |
| Yellow Pummelo | BB | AA | AB | BB | AB | AA | AB | BB | AA | BB | AA | AB | AB | AA | AB |
| May Pummelo | AB | AA | BB | BB | AB | AB | AA | AB | BB | AB | AB | AB | AB | AA | AB |
| Uedaunshiu | AB | AB | AB | BB | BB | AA | BB | BB | AA | BB | AB | AB | BB | BB | BB |
| PIC[Z] | 0.368 | 0.317 | 0.239 | 0.195 | 0.337 | 0.291 | 0.328 | 0.371 | 0.375 | 0.353 | 0.305 | 0.353 | 0.275 | 0.291 | 0.375 |

[Z] PIC values were calculated using MarkerToolKit v1.0 (Fujii, 2008).

## 2. Minimum marker set for cultivar identification and parentage testing

Compilation of the InDel marker genotypes revealed that three cultivars, 'Tosabuntan', 'Otachibana', and 'Hogenbuntan', shared identical genotypes (Table 3). These three cultivars have also been identified as synonymous cultivars in previous studies (Nishimura et al., 2024; Shimizu et al., 2016). Therefore, 'Tosabuntan' was selected as the representative of these three cultivars. MinimalMarker analysis revealed that a minimum of five markers was required to discriminate the 22 cultivars and identified five possible combinations of such markers (Table 4).

Table 4
Minimum marker set required for discriminating 24 cultivars

| Set | Marker combination[Z] |
|---|---|
| S1 | LID-1(1), LID-4(2), LID-7(4), LID-12(5), LID-18(9) |
| S2 | LID-1(1), LID-5(3), LID-7(4), LID-12(5), LID-18(9) |
| S3 | LID-2(1), LID-4(2), LID-7(4), LID-12(5), LID-18(9) |
| S4 | LID-2(1), LID-5(3), LID-7(4), LID-12(5), LID-18(9) |
| S5 | LID-3(2), LID-4(2), LID-7(4), LID-12(5), LID-18(9) |

[z] The numbers in parentheses indicate chromosome numbers.

Using genotype data, MarkerToolKit calculates allele frequencies, heterozygosity, polymorphism information content (PIC), and the probability that two or more cultivars share identical genotypes (Table S2). The polymorphism information content ranged from 0.195 to 0.375, with an average value of 0.324 (Table 3). Furthermore, the probability that two or more cultivars share identical genotypes was highest in 'Yugehyokan', at $9.39 \times 10^{-4}$, whereas the lowest value among pummelos was observed in 'Anseikan', at $2.73 \times 10^{-7}$ (Table S3).

Among the 24 cultivars analyzed, six known parentage relationships were included: 'Hayasaki' ('Matobuntan' × 'Hiradobuntan'), 'Benimadoka' ('Matobuntan' × 'Hiradobuntan'), 'Yellow Pummelo' ('Hassaku' × 'Hiradobuntan'), 'May Pummelo' ('Hassaku' × 'Hiradobuntan'), 'Summer Fresh' ('Hassaku' × 'Natsumikan'), and 'Sweet Spring' ('Uedaunshiu' ×'Hassaku' ). Genotypes at the 18 InDel markers were analyzed in these cultivars to examine the inheritance of alleles from parents to offspring. The results confirmed that all alleles observed in 'Hayasaki', 'Benimadoka', 'Yellow Pummelo', 'May Pummelo', 'Summer Fresh', and 'Sweet Spring' could be consistently traced to either parent, with no contradictory inheritance patterns.

## 3. Parentage analysis of 'Hassaku'

In the marker screening for cultivar identification, we identified an allele that was shared exclusively by 'Hondabuntan' and 'Hassaku' (Fig. 2a). At this marker locus, the A and B alleles were widely observed among pummelos, whereas the C allele was shared only by 'Hondabuntan' and 'Hassaku'. If this shared C allele is identical by descent, 'Hondabuntan' could be a candidate parent of 'Hassaku'. Therefore, we performed sequence analysis of the alleles at this marker. The sizes of the three alleles at this marker were 392 bp for allele A, 214 bp for allele B, and 525 bp for allele C, and each contained multiple InDel polymorphisms and SNPs (Fig. 2b). More specifically, allele A differed from allele B by a 179 bp insertion, a 1 bp deletion, and multiple SNPs, whereas allele C differed from allele A by a 133 bp insertion. The sequence of the C allele was identical between 'Hondabuntan' and 'Hassaku'. To further examine this possibility, 'Kunenbo', a putative parent of 'Hassaku', was genotyped using the 18 InDel markers, and the genotypes of 'Hondabuntan', 'Kunenbo', and 'Hassaku' were compared (Fig. 2c). The genotype of 'Hassaku' was consistent with Mendelian inheritance from the putative parental combination of 'Hondabuntan' and 'Kunenbo' across all 18 InDel markers.

## Discussion

In this study, we aimed to develop DNA markers for cultivar identification of pummelos distributed in Japan. InDel markers designed using LINDA are codominant markers whose genotypes can be determined by PCR and electrophoresis alone. Moreover, unlike CAPS markers, they do not require restriction enzyme digestion. These easy-to-use InDel markers are expected to be adaptable to methods such as the chromatographic printed array strip (C-PAS) method (Monden et al., 2023), and may therefore be applicable even in facilities with limited testing resources, such as customs offices (Watanabe et al., 2025).

The set of InDel markers previously developed for mandarin cultivar identification and applied to pummelo included only 12 markers and showed a biased chromosomal distribution (Endo et al., 2022). Furthermore, when applied to pummelo, only a limited number of these markers showed polymorphism (Shimada et al., 2025). Consequently, no marker set has yet been established for discriminating major pummelo cultivars distributed in Japan. The 18 markers screened in this study were developed based on comparative analysis of pummelo sequences and were therefore expected to be capable of detecting polymorphisms among pummelo cultivars. These markers were located on all nine chromosomes, with at least one marker on each chromosome. PIC values are generally classified into three categories: highly informative (PIC ≥ 0.5), moderately informative (0.25 ≤ PIC < 0.5), and slightly informative (PIC < 0.25) (Botstein et al., 1980). Most of the markers used in this study were classified as moderately informative, with an average PIC value of 0.324. This value was comparable to the average PIC values reported for CAPS markers developed in mandarin, 0.309 (Ninomiya et al., 2015), and for InDel markers, 0.304 (Noda et al., 2021). These results indicate that the marker set developed in this study has sufficient discriminatory power for pummelo cultivar identification.

The three cultivars 'Tosabuntan', 'Otachibana', and 'Hogenbuntan', previously reported to be synonymous cultivars, showed identical genotypes across all 18 markers (Nishimura et al., 2024; Shimizu et al., 2016). In addition, previous records indicate that 'Hogenbuntan' from Kagoshima Prefecture was introduced to Kochi Prefecture and later became known as 'Tosabuntan' (Iwahori, 1999). The finding that these three cultivars showed identical genotypes in this study supports previous reports and historical records. When these three synonymous cultivars were represented by 'Tosabuntan', MinimalMarker analysis demonstrated that the remaining 22 cultivars could be discriminated using only five of the 18 markers. Furthermore, even the highest probability that two or more cultivars share identical genotypes was low, with the maximum value observed in 'Yugehyokan' at $9.39 \times 10^{-4}$. This suggests that the marker set developed in this study has sufficient discriminatory power to accommodate the future development of many additional pummelo and pummelo hybrid cultivars.

Verification of known parentage relationships based on InDel marker genotypes is desirable to demonstrate the reliability of these markers for cultivar identification. In addition, genotype information obtained from DNA markers is important for confirming correct cross combinations and clarifying genetic backgrounds (Khadari et al., 2003; Shimizu et al., 2016). The plant materials used in this study included six cultivars bred by NARO, namely 'Hayasaki', 'Benimadoka', 'Yellow Pummelo', 'May Pummelo', 'Summer Fresh', and 'Sweet Spring'. We therefore examined whether the marker genotypes of these cultivars were consistent with inheritance from their reported parents. As a result, no inconsistencies in parentage relationships were detected in these six combinations. This result indicates that the genotypes obtained using these InDel markers are consistent with the breeding pedigrees and supports the reliability of these markers. Furthermore, among Japanese cultivars, some are chance seedlings with proposed parentage relationships. A well-known example is 'Suishobuntan', which has been suggested to be derived from 'Tosabuntan' × 'Banoukan' (Shimizu et al., 2016). However, an inconsistency was observed at the LID-2 marker in 'Suishobuntan'. Because the breeding history of 'Suishobuntan' remains unclear, 'Tosabuntan' × 'Banoukan' has been proposed as the most plausible parental combination. Thus, this result suggests that the actual parental combination of 'Suishobuntan' may differ from the

currently proposed one. During marker screening for cultivar identification, we identified a unique allele that was shared exclusively by ‘Hassaku’ and ‘Hondabuntan’. ‘Hassaku’ is one of the major mid- to late-maturing citrus cultivars in Japan, producing fruit of excellent quality. It was discovered as a chance seedling around 1860, and ‘Kunenbo’ has been proposed as the pollen parent (Shimizu et al., 2016). However, the seed parent remains unknown, except for evidence that ‘Hassaku’ has pummelo-type cytoplasm. The C allele identified in this study, which was shared by ‘Hondabuntan’ and ‘Hassaku’, was characterized by a 133 bp insertion relative to the A allele. Because the independent occurrence of InDel variants with exactly the same length at the same genomic position is unlikely, shared InDels are likely to reflect common ancestry (Shedlock and Okada, 2000). In addition, the genotypes of ‘Hassaku’ were consistent with Mendelian inheritance from ‘Hondabuntan’ and ‘Kunenbo’ based on the 18 InDel markers and the additional marker carrying the shared C allele. However, when ‘Kunenbo’ was assumed to be one parent of ‘Hassaku’, both ‘Hondabuntan’ and ‘May Pummelo’ showed no Mendelian inconsistency as the other parent based on the 18 InDel markers. Nevertheless, ‘May Pummelo’ is a cultivar bred by NARO and registered in 1985, which is inconsistent with the historical background of ‘Hassaku’ as a chance seedling discovered around 1860. Thus, among the cultivars examined in this study, ‘Hondabuntan’ was considered the most plausible candidate for the other parent of ‘Hassaku’.

## Conclusions

In conclusion, the 18 InDel markers developed in this study provide a novel and simple marker system for identifying Japanese pummelo cultivars. These reliable markers are expected to support the Japanese citrus industry by contributing to the protection of breeders’ rights and the regional brand value of pummelo cultivars. Furthermore, the markers developed in this study may also be useful for elucidating breeding relationships among pummelo cultivars. The identification of previously unrecognized valuable parental cultivars may provide useful genetic resources for future breeding. In addition, the LINDA-based marker design and analysis methods used in this study are expected to be applicable to other horticultural crops for which genome sequence information is available.

## Declarations

# 1. Funding

The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.

# Author Contribution

All authors contributed to the study conception and design. Plant materials were prepared by T.W. and K.N. Marker development, genotyping, and data analysis were performed by R.S. and T.N. T.H. and A.T. supervised the study and contributed to the interpretation of the results. The first draft of the manuscript was written by R.S., and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

# Acknowledgements

We thank the Institute of Fruit Tree & Tea Science, NARO, for providing plant materials used in this study under a Material Transfer Agreement (MTA). We also thank the NARO Genebank for providing the citrus genetic resources used in this study.

# Data Availability

All data generated and/or analyzed during the current study are included in this published article and its supplementary information files.

Other data required for this study are provided in the Supplemental.

## References

1. Ahmed, S., Rattanpal, H. S. and Singh, G. 2019. Diversity, characterization and evaluation in Pummelo (*Citrus maxima* Merr.) cultivars using SSR markers and quality parameters. Indian J. Genet. Plant Breed. 79: 594–605. DOI: 10.31742/IJGPB.79.3.9
2. Botstein, D., White, R. L., Skolnick, M. and Davis, R. W. 1980. Construction of a Genetic Linkage Map in Man Using Restriction Fragment Length Polymorphisms. Am. J. Hum. Genet., *32*: 314–331.
3. Doyle, J. J. and Doyle, J. L. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemical Bulletin, *19*: 11–15.
4. Endo, T., Fujii, H. and Shimada, T. 2022. Development of Cultivar Identification System Using 12 InDel Markers for Widely Distributed Citrus Cultivars in Japan. Japan Agricultural Research Quarterly: JARQ, *56*: 329–340. DOI: https://doi.org/10.6090/jarq.56.329
5. Endo, T., Fujii, H., Yoshioka, T., Omura, M. and Shimada, T. 2020. TaqMan-MGB SNP genotyping assay to identify 48 citrus cultivars distributed in the Japanese market. Breeding Science, 70: 363–372. DOI: https://doi.org/10.1270/jsbbs.19142
6. Fujii, H. 2008. MarkerToolKit: An analysis program for data sets consists of DNA marker types obtained from various varieties. DNA Polymorphism, 16: 103–107.
7. Fujii, H., T. Ogata, T. Shimada, T. Endo, H. Iketani, T. Shimizu, T. Yamamoto and M. Omura. 2013. Minimal marker: an algorithm and computer program for the identification of minimal sets of discriminating DNA markers for efficient variety identification. Journal of Bioinformatics and Computational Biology

11: 1250022. DOI: https://doi.org/10.1142/S0219720012500229

8. Fujii, H., Narita, T., Oshino, H., Endo, T., Kawakami, T., Goto, H., Yoshioka, T., Omura, M. and Shimada, T. 2019. CAPS markers with stability and reproducibility for discriminating major citrus cultivars in Japan. DNA Polymorphism, 27: 71–79.
9. Fujii, H., Ohta, S., Nonaka, K., Katayose, Y., Matsumoto, T., Endo, T., Yoshioka, T., Omura, M. and Shimada, T. 2016. Parental diagnosis of satsuma mandarin (*Citrus unshiu* Marc.) revealed by nuclear and cytoplasmic markers. Breeding Science, 66, 683–691. DOI: https://doi.org/10.1270/jsbbs.16060
10. Huang, Y., Makkumrai, W., Fu, J., Deng, C., Wu, Q., Wang, S., Wang, L., Wu, X., Gao, J., Chen, C., Guo, L., Chen, P., Wu, F., Deng, X., Wang, X. and Xu, Q. 2025. Genomic analysis provides insights into the origin and divergence of fruit flavor and flesh color of pummelo. New Phytologist, 245, 378–391. DOI: https://doi.org/10.1111/nph.20223
11. Iwahori, S. 1999. Kankitsusouron (In Japanese). Yokendo, Tokyo
12. Khadari, B., Breton, C., Moutier, N., Roger, J., Besnard, G., Bervillé, A. and Dosba, F. 2003. The use of molecular markers for germplasm management in a French olive collection. Theoretical and Applied Genetics, 106: 521–529. DOI: https://doi.org/10.1007/s00122-002-1079-x
13. Kimura, T., Sawamura, Y., Kotobuki, K., Matsuta, N., Hayashi, T., Ban, Y. and Yamamoto, T. 2003. Parentage Analysis in Pear Cultivars Characterized by SSR Markers. Journal of the Japanese Society for Horticultural Science, 72, 182–189. DOI: https://doi.org/10.2503/jjshs.72.182
14. Monden, Y., Kakigi, M., Haque, E., Takeuchi, T., Takasaki, K. and Tanaka, M. 2023. Chromatographic printed array strip (C-PAS) method for cultivar-specific identification of sweetpotato cultivars 'Beniharuka' and 'Fukumurasaki.' Breeding Science, *73*: 313–321. DOI: https://doi.org/10.1270/jsbbs.22101
15. Nicolosi, E., Deng, Z. N., Gentile, A., La Malfa, S., Continella, G. and Tribulato, E. 2000. Citrus phylogeny and genetic origin of important species as investigated by molecular markers. Theoretical and Applied Genetics, 100, 1155–1166. DOI: https://doi.org/10.1007/s001220051419
16. Niimi, E., Fujii, H., Ohta, S., Iwakura, T., Endo, T. and Shimada, T. 2021. Development of Acid Citrus Cultivar Identification by CAPS Markers. Horticultural Research, 20: 17–27. (In Japanese). DOI: https://doi.org/10.2503/hrj.20.17
17. Niimi, E., Fujii, H., Ohta, S., Iwakura, T., Endo, T. and Shimada, T. 2022. Development of Acid Citrus Cultivar Identification System by SNP Markers. Horticultural Research, 21: 111–122. (In Japanese). DOI: https://doi.org/10.2503/hrj.21.111
18. Ninomiya, T., Shimada, T., Endo, T., Nonaka, K., Omura, M. and Fujii, H. 2015. Development of Citrus Cultivar Identification by CAPS Markers and Parentage Analysis. Horticultural Research, 14: 127–133. (In Japanese). DOI: https://doi.org/10.2503/hrj.14.127
19. Nishimura, K., Okuma, M., Kaneyoshi, J., Yamasaki, A., Nagasaka, K., Murata, K., Monden, Y., Kato, K., Nishida, H., Nakazaki, T. and Nakano, R. 2024. Workflow for development of CAPS markers with one type of restriction enzyme to identify citrus cultivars. Tree Genetics & Genomes, 20: 27. DOI: https://doi.org/10.1007/s11295-024-01661-x
20. Noda, T., Daiou, K., Mihara, T. and Nagano, Y. 2021. Potential application of simple easy-to-use insertion-deletion (InDel) markers in citrus cultivar identification. Breeding Science, 71: 601–608. DOI: https://doi.org/10.1270/jsbbs.21021
21. Nonaka, K., Fujii, H., Kita, M., Shimada, T., Endo, T., Yoshioka, T. and Omura, M. 2017. Identification and Parentage Analysis of Citrus Cultivars Developed in Japan by CAPS Markers. The Horticulture Journal, *86*: 208–221. DOI: https://doi.org/10.2503/hortj.OKD-026
22. Omura, M. and Shimada, T. 2016. Citrus breeding, genetics and genomics in Japan. Breeding Science, 66: 3–17. DOI: https://doi.org/10.1270/jsbbs.66.3
23. Shedlock, A. M. and Okada, N. 2000. SINE insertions: Powerful tools for molecular systematics. BioEssays, 22: 148–160. DOI: https://doi.org/10.1002/(SICI)1521-1878(200002)22:2%3C148::AID-BIES6%3E3.0.CO;2-Z
24. Shimada, R., Ohta, S., Tominaga, A., Shimada, T., Endo, T. and Fujii, H. 2025. Genotypic Characterization of Pummelo and its hybrids with mandarin based on InDel markers and PCA. DNA Polymorphism, *33*: 1–8. (In Japanese)
25. Shimada, T., Fujii, H., Endo, T., Ueda, T., Sugiyama, A., Nakano, M., Kita, M., Yoshioka, T., Shimizu, T., Nesumi, H., Ikoma, Y., Moriguchi, T. and Omura, M. 2014. Construction of a citrus framework genetic map anchored by 708 gene-based markers. Tree Genetics & Genomes, 10: 1001–1013. DOI: https://doi.org/10.1007/s11295-014-0738-9
26. Shimizu, T., Kitajima, A., Nonaka, K., Yoshioka, T., Ohta, S., Goto, S., Toyoda, A., Fujiyama, A., Mochizuki, T., Nagasaki, H., Kaminuma, E. and Nakamura, Y. 2016. Hybrid Origins of Citrus Varieties Inferred from DNA Marker Analysis of Nuclear and Organelle Genomes. PLOS ONE, 11: e0166969. DOI: https://doi.org/10.1371/journal.pone.0166969
27. Ukai, Y. 2005. Cultivar identification theories (Vol. 79). (In Japanese)
28. Uzun, A. and Yesiloglu, T. 2012. Genetic Diversity in Citrus. In M. Caliskan (Ed.), Genetic Diversity in Plants. InTech. DOI: https://doi.org/10.5772/32885
29. Wang, X., Y. Xu, S. Zhang, L. Cao, Y. Huang, J. Cheng, G. Wu, S. Tian, C. Chen, Y. Liu, H. Yu, X. Yang, H. Lan, N. Wang, L. Wang, J. Xu, X. Jiang, Z. Xie, M. Tan, R. M. Larkin, L.-L. Chen, B.-G. Ma, Y. Ruan, X. Deng and Q. Xu. 2017. Genomic analyses of primitive, wild and cultivated citrus provide insights into asexual reproduction. Nature Genetics, 49: 765–772. DOI: https://doi.org/10.1038/ng.3839
30. Watanabe, T., Endo, T., Kawahara, Y., Shirasawa, K., Nonaka, K., Isobe, S., Shimada, T., Goto, S. and Fujii, H. 2025. LAYLA: Development of a comprehensive and cross-sample program for detecting structural variants and its application to citrus cultivars. Breeding Science, 75: 442–454. DOI: https://doi.org/10.1270/jsbbs.25020
31. Wu, G. A., Sugimoto, C., Kinjo, H., Azama, C., Mitsube, F., Talon, M., Gmitter, F. G. and Rokhsar, D. S. 2021. Diversification of mandarin citrus by hybrid speciation and apomixis. Nature Communications, 12: 4377. DOI: https://doi.org/10.1038/s41467-021-24653-0
32. Yamamoto, T., Mochida, K., Imai, T., Haji, T., Yaegaki, H., Yamaguchi, M., Matsuta, N., Ogiwara, I. and Hayashi, T. 2003. Parentage Analysis in Japanese Peaches using SSR Markers. Breeding Science, *53*: 35–40. DOI: https://doi.org/10.1270/jsbbs.53.35

## Figures

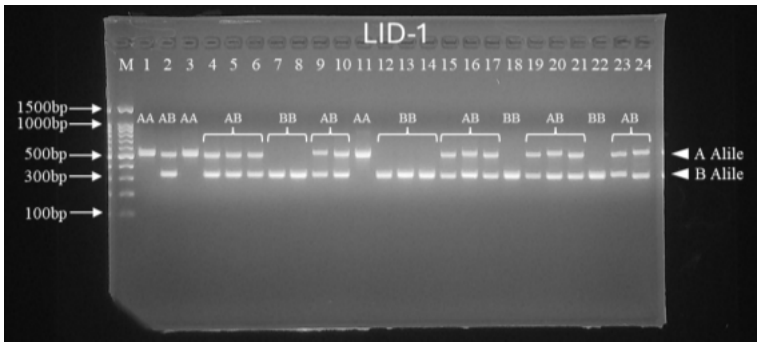


**Figure 1**

PCR fragment pattern of 24 samples using LID-1. M：100 bp DNA size ladder. The number indicates plant material; the same number as in Table 1 is used. The two-character alphabet above the band pattern indicates the genotype of the InDel marker. As indicated by the arrowheads, the larger fragment was designated as allele A and the smaller fragment as allele B.

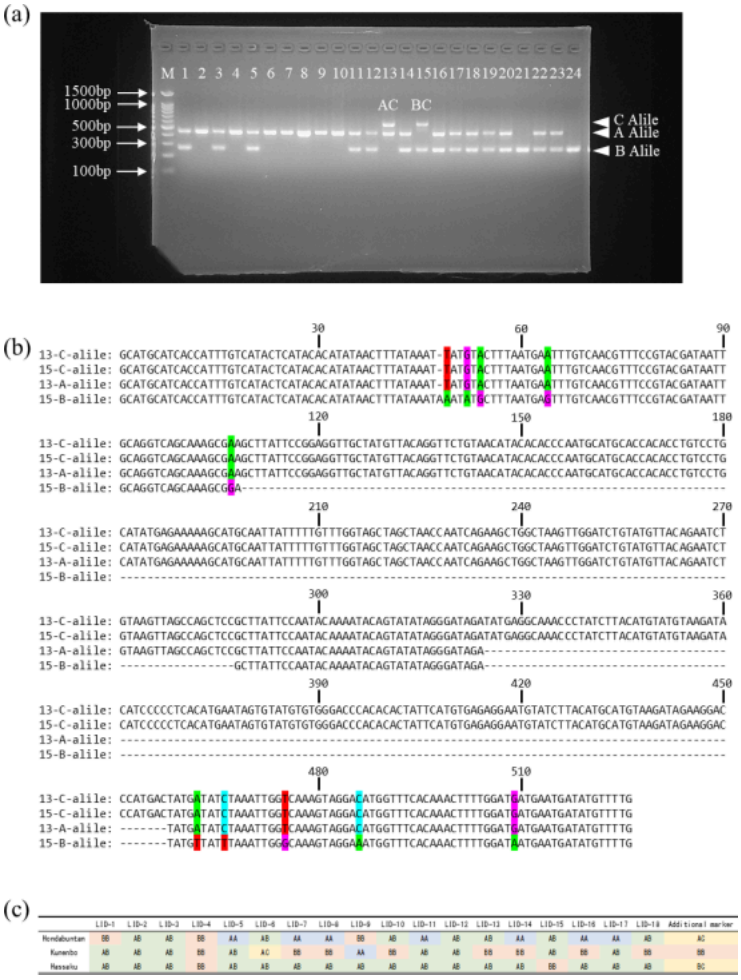


**Figure 2**

(a) An InDel marker showing the C allele shared exclusively by Hondabuntan (13) and Hassaku (15). M：100 bp DNA size ladder. The number indicates plant material; the same number as in Table 1 is used. As indicated by the arrowheads, the expected longer fragment was designated as the A allele, the shorter fragment as the B allele, and the allele found only in ‘Hondabuntan’ and ‘Hassaku’ was designated as the C allele. (b) Results of sequence analysis of the three alleles. Colored letters indicate SNPs, and hyphens (-) indicate InDels. The C allele sequence was obtained from ‘Hondabuntan’ (13) and ‘Hassaku’ (15), and the two sequences were identical. (c) Genotypes of ‘Hassaku’ and its putative parental combination, ‘Hondabuntan’ and ‘Kunenbo’, at the 18 InDel Marker and the additional marker carrying the shared C allele.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarytable.xlsx