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Research Paper

The use of a fertile doubled haploid apple line for QTL analysis of fruit traits

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Apple is an economically important crop, and various approaches to genetic analysis in breeding programs have been attempted, including the production of doubled haploid (DH) lines, which are genetically homozygous. In this study, we used a DH line for QTL analyses, for the first time in a fruit tree, expecting it to simplify the analysis of the inheritance of quantitative traits and thus to enhance QTL detection power. Using an F_1 population from 'Prima' × 'Apple Chukanbohon 95P6' (DH), we constructed a genetic map of 'Prima', and identified 19 QTLs for 13 traits. These QTLs had comparatively high LOD scores and explained a large part of the variation of the phenotypes. In particular, acidity, juice browning, and skin splitting clearly segregated at a 1:1 ratio, consistent with the segregation of the alleles at the detected QTLs in linkage group 16; these traits appeared to be regulated by single genes, despite general consideration that they are quantitative traits. Using this simple genetic composition of the F_1 population, we concluded that the skin splitting of apple fruit has recessive inheritance, and that the allele for splitting is tightly linked with those for high acidity and low juice browning in 'Prima'.

Key Words: acidity, detection power, doubled haploid 'Apple Chukanbohon 95P6', juice browning, QTL analysis, skin splitting.

Introduction

Genetically pure lines are indispensable in breeding of self-pollinated crops, and as parents of F_1 hybrid cultivars in cross-pollinated crops. They are conventionally produced by several generations of selfing. Doubled haploids (DH) could shorten the time for the generation of homozygous lines, and those derived from anther culture have been routinely used to develop new varieties in both self-pollinated (e.g., rice, tobacco, barley, and wheat) and cross-pollinated crops (e.g., maize, *Brassica*, eggplant, melon, and asparagus) (Germanà 2011, Veilleux 1994).

In fruit tree crops, the production of genetically pure lines by selfing is impractical because of their long reproductive cycle and frequent self-incompatibility. Because fruit crops are vegetatively propagated, homozygosity is not required for the propagation. However, to improve breeding programs or to simplify genetic analysis, large efforts have been made to produce DH lines of orange, apple, pear, peach, grape, olive, and kiwifruit by spontaneous development or by anther or microspore culture (Germanà 2006). Superior DH breeding parents would stably produce superior progeny because of no genetic segregation in meiosis. DH can also solve the problem of highly heterozygous genomes of fruit crops, which has been a barrier to smooth genetic analyses, in contrast to self-compatible crops like many cereals or vegetables. For example, in heterozygous crops, the *de novo* assembly of genomic sequence reads from next generation sequencing must deal with the unclear origin of reads with polymorphic bases (which can be derived from heterozygous alleles or paralogous sequences) and the unclear phase (coupling or repulsion) among polymorphic loci. Heterozygous genomes of fruit crops also complicate the genetic analysis of F_1 populations because of the variety of alleles to evaluate. In QTL mapping of quantitative traits, we reluctantly must use two-way pseudo-testcross mapping (Grattapaglia and Sederoff 1994), with a lower QTL detection power than analyses of backcrossed (BC) or DH populations (**Fig. 1**). The continuity of phenotype distribution of the quantitative traits in the test population is often magnified by the variety of alleles and hinders the use of simple genetic approaches such as map-based cloning.

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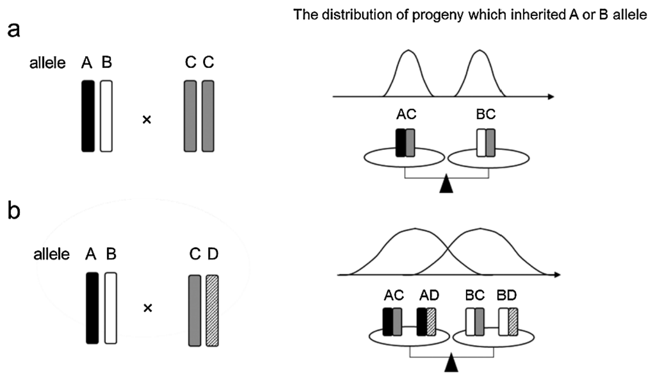

Fig. 1. QTL analysis in (a) a population generated by crossing with a double haploid (DH) line and (b) a population generated by crossing with a heterozygous apple line (conventional double pseudo-testcross). In *a*, all zygotes from the paternal DH parent are homozygous, and the alleles from this parent do not generate phenotypic variation in any trait. Therefore, the distribution of the F_1 population directly reflects that of alleles inherited from only maternal parent, and the effects of alleles A and B can be evaluated precisely in the absence of confounding effects of other alleles. In *b*, when the effects of alleles A and B are evaluated, the variance caused by alleles C and D would lower the sensitivity of QTL detection, especially in study of minor QTLs in a small population.

DH lines have been used for the whole-genome sequencing of fruit crops (e.g., banana, orange, and peach) by *de novo* assembly (D'Hont *et al.* 2012, Genome Database for Rosaceae 2012, Xu *et al.* 2013). The whole genome of the diploid apple cultivar 'Golden Delicious' (GD) sequenced in 2010 (Velasco *et al.* 2010) had problems in assembly caused by high heterozygosity and a recent whole-genome duplication. However, an improved apple genome sequence with remarkably longer contigs was obtained by using a DH line of 'GD' (Daccord *et al.* 2017). Yet the use of DH lines in fruit breeding programs or genetic family analysis is delayed, because almost all DH lines have weak vigor and low fertility and produce insufficient progeny (Höfer and Flachowsky 2015, Vanwynsberghe *et al.* 2005). However, some fertile DH lines showing pollen germination were reported in orange (Yahata *et al.* 2015) and apple (Höfer *et al.* 2008, Okada *et al.* 2009). 'Apple Chukanbohon 95P6' (95P6), produced by anther culture from 'Senshu', is a rare such resource (**Supplemental Fig. 1**). Although it bears fruits with half the size and a quarter of the seeds of 'Senshu', it has acceptable vigor and both female and male fertility, and its 33% pollen germination rate is sufficient to generate cross populations for breeding or genetic analysis (Okada *et al.* 2009).

Apple (*Malus* × *domestica* Borkh.) is one of the most economically important crops in most temperate regions of North and South America, Europe, East and West Asia, Oceania, and Africa. Its worldwide production is increasing year by year and reached 89 Mt in 2016 (FAOSTAT, http://faostat.fao.org). 'Red Delicious', 'GD', and 'Fuji' are giants among produced or traded cultivars (O'Rourke 2003). Breeders are also working to release new, more attractive cultivars. Marker-assisted breeding is a powerful tool for the efficient selection of any crop, especially of fruit tree crops, which can take more than 5 years to bear fruit for evaluation. To develop useful selection markers for apple, identification of causal genes for qualitative traits (e.g., ethylene production, skin color, apple scab resistance, columnar tree, and pale green lethal disorder in seedling) have been carried out mainly using linkage analyses and map-based cloning (Ban *et al.* 2007, Harada *et al.* 2000, Orcheski *et al.* 2015, Otto *et al.* 2014, Takos *et al.* 2006, Vinatzer *et al.* 2001, Wolters *et al.* 2013). For the many quantitative traits (e.g., soluble sugar content, acidity, phenolic compounds, volatile organic compounds, firmness, bitter pit, and water core), which are commercially important but more difficult to analyze, QTLs have been detected using QTL mapping, genome-wide association study, and pedigree-based analyses (Bink *et al.* 2014, Cappellin *et al.* 2015, Chagné *et al.* 2012, Costa *et al.* 2010, Kenis *et al.* 2008, King *et al.* 2000, Kumar *et al.* 2013, Kunihisa *et al.* 2014, 2016, Liebhard *et al.* 2003). However, these studies were carried out using heterozygous materials, which might have lowered the QTL detection power.

In this study, we obtained progeny of a cross between the DH line '95P6' and the normal heterozygous cultivar 'Prima', examined phenotypic distribution in the progeny, and performed QTL analysis based on the BC strategy, with the aim of demonstrating the application of DH lines to genetic analysis, for the first time in a fruit tree.

Materials and Methods

Plant materials

For the genetic map construction, and the evaluation of phenotypes for the QTL analysis, a population of 77 seedlings was generated from the cross between maternal 'Prima' and paternal 'Apple Chukanbohon 95P6' (95P6), which is a DH line (**Supplemental Fig. 1**) registered as No. 21629 in the Japan Plant Variety Registration System on 9 March 2012. The population was sown in 2008–2009 and grafted onto 'JM7' rootstocks (which had been planted in the field of the Fukushima Agricultural Technology Center) in 2009–2010. They were grown with a spacing of 2 m between trees in rows 5 m apart. The number of trees that bore fruits was 54 in 2013, 59 per year in 2014–2015, and 60 in 2016. The fruits were regarded as mature and harvested when the ground color at the calyx end was tinged with yellow. During 2013–2015, the 10 fruits of moderate quality per genotype were picked for phenotype evaluation of traits except skin splitting; if fewer than 10 fruits were produced (because of fruit drop or weak vigor), all harvested measurable fruits were evaluated. In 2016, only skin splitting in all harvestable fruits was evaluated.

Evaluation of phenotypes

The analyzed 15 traits and measurement methods are

Table 1. Fruit quality traits used for QTL analyses in the ‘Prima’ × ‘95P6’ F_1 population

		Trait	Description (assessment type)
Evaluation year	2013–2015	Harvest time (days)	Number of days after August 1, compensated by the starch index of harvested fruit
		Weight (g)	Mean fruit fresh weight
		Flesh color	Rank: 1 (green, green-white, white), 2 (green-yellow), 3 (white-yellow), 4 (yellow) (visual)
		Skin color intensity [L*, a*, b*]	L*, a* and b* values of the sunny side (color-difference meter)
		Firmness (lb)	Mean firmness of sunny and shaded sides of the fruit, measured after peeling (Magness-Taylor penetrometer)
		Acidity (%)	Acidity of juice obtained from a mixture of pieces from tested fruits (titratable acid content)
		Juice browning	Browning degree of juice. Rank: 1 (nil), 2 (slight), 3 (moderate), 4 (strong), 5 (severe), 6 (extreme) (visual)
		Sucrose content (%)	Sucrose content in juice (high-performance liquid chromatography, HPLC)
		Glucose content (%)	Glucose content in juice (HPLC)
		Fructose content (%)	Fructose content in juice (HPLC)
		Sorbitol content (%)	Sorbitol content in juice (HPLC)
		Soluble sugar content (%)	Total content of the above four sugar components in juice
	2016	Skin splitting (%)	The proportion of fruits with skin splitting on the shoulder

listed in **Table 1**. Harvest time was scored as the number of days after 1 August. On the day of harvest, starch index (*si*) was determined by staining the equatorial plane of fruits with I_2–KI solution (about 1% I_2, 5% KI) and measuring the proportion of stained area (rank: ‘0’, not stained; ‘1’, <10%; ‘2’, 20%; ‘3’, outside of fibrovascular bundle; ‘4’, outside of core; ‘5’, almost all area). The fruits with $1.5 < si < 2.5$ were regarded to be properly mature. Fruits with $si < 1.0$ were regarded as overripe, and their harvest day was empirically compensated by subtracting 4 days. The harvest day of fruits with $1.0 < si < 1.5$ was compensated by subtracting 2 days. The harvest day of immature fruits with $2.5 < si < 3.0$ was corrected by adding 3 days and that of fruits with $3.0 < si$ by adding 5 days. For *si*, weight, flesh yellowish color, skin color intensity, and firmness, the mean value of all measured fruits was regarded as the phenotype of the genotype in each year.

To measure acidity, juice browning, and sugar components, each fruit was cut into wedges from both sides, and the juice was squeezed from the pieces. Juice acidity was measured on the day of harvest, and the other two traits were evaluated later in samples stored in a freezer. For the measurement of juice browning, thawed and centrifuged juice was kept at room temperature overnight and visually ranked into six grades on its browning degree. The content of each sugar component was measured by HPLC according to Kunihisa *et al.* (2014).

The evaluations of 14 traits were carried out in 2013–2015, and the broad-sense heritability of each traits was calculated according to Kunihisa *et al.* (2014). Because the heritability is expected to be higher by averaging data from the repeated evaluations, the mean value of data obtained in multiple years was used as the phenotype data of each genotype. Skin splitting was quantified only in 2016 as the proportion of fruits with concentric splitting around the stem on the fruit shoulder, using harvestable 5–12 fruits per genotype.

The normality of data distribution for each trait was assessed in PASW Statistics 18 (IBM, Armonk, New York). Data with $p < 0.05$ by the Shapiro–Wilk test were judged to be non-normally distributed. The chi-square test was conducted to determine if the segregation of traits fit to Mendel’s law.

Construction of a genetic linkage map

All 77 seedlings from ‘Prima’ × ‘95P6’ were used for the analysis. A gene-specific marker (S-RNase) and 357 primer pairs for SSR detection, which could detect heterozygous loci in ‘Prima’ were used. Out of the 358 primer pairs, 353 were previously developed in apple or Japanese pear (**Supplemental Table 1**), and 5 were newly developed (**Supplemental Table 2**). DNA was extracted from the parents and progeny, and genetic polymorphisms were detected according to Kunihisa *et al.* (2014). Join Map 4.0 software (van Ooijen 2006) was used for map construction. The data on markers segregating only in ‘Prima’ were suitable for the software’s “BC” mode, and was grouped with LOD = 5.0 using the regression mapping module, followed by mapping with Kosambi’s method. The numbering and direction of LGs was defined with reference to the reported maps (Silfverberg-Dilworth *et al.* 2006).

QTL detection

QTL analysis was performed in MapQTL 6.0 software (van Ooijen 2009). The genetic map of ‘Prima’ and the phenotype data of 62 seedlings that bore fruits in 2013–2015 were used for the analysis, except skin splitting. The mean values obtained (max. 3 years) were used as phenotype data. As to skin splitting, the phenotype data in 60 seedlings in 2016 were used. Tentative QTLs were initially detected using interval mapping for all traits. The loci with maximum LOD score above the genome-wide threshold ($p = 0.05$), determined by permutation test (5000 cycles), were regarded as tentative QTLs. The markers nearest to the significant QTLs were used as cofactors for the subsequent multiple QTL mapping (MQM) to determine the final QTLs. If no other significant QTL was detected on the same LG in MQM, restricted MQM (rMQM) instead of MQM was used. For the analyses of traits with non-normal distribution, we combined the non-parametric Kruskal–Wallis test

with the above map-based analysis. The marker with the maximum K* value of the significance level ($p < 0.0001$, set as 0.05/400 markers) was selected and compared to the (r)MQM result.

Results

Phenotype data

The evaluated traits and phenotypes are summarized in **Table 1**. The broad-sense heritability (h^2) of them except skin splitting was shown in **Supplemental Table 3**. Skin color intensity, acidity, juice browning, glucose and fructose content were mainly regulated by genetic elements ($h^2 > 0.7$), while flesh color and soluble sugar content were largely affected by environmental elements ($h^2 < 0.4$). The distribution of phenotypes of F_1 seedlings and the data of their parents (‘Prima’ and ‘95P6’) are shown in **Fig. 2**. We did not investigate the proportion of fruits with skin splitting

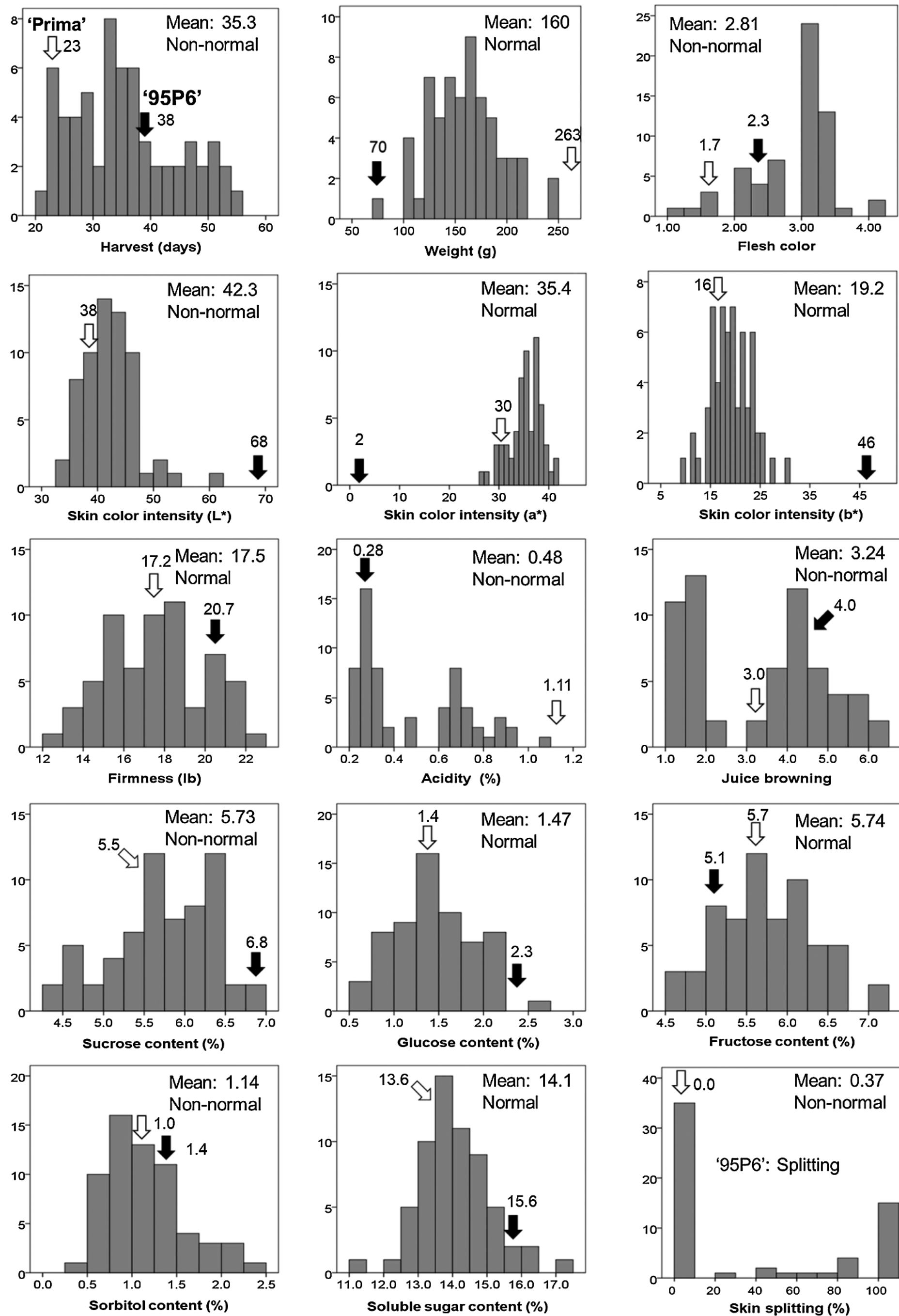

Fig. 2. Distribution of phenotypes in the F_1 population. *Y*-axes show frequency. The mean and distribution type by the Shapiro–Wilk test are shown. White arrows, ‘Prima’; black arrows, ‘95P6’. Splitting was observed in overripe ‘95P6’ fruits, but the proportion was not calculated.

Table 2. Significant QTLs detected on the linkage map

Trait	LG	Position (cM)	LOD	% exp.[a]	Significance[b]	Nearest marker
Harvest time	1	11.1	4.2	26.8	**	TsuENH101
Flesh color	16	17.0	6.3	37.2	****	Hi15g11
Skin color intensity (L*)	16	7.6	5.8	35.1	****	Hi02h08
(a*)	10	39.5	5.4	26.0	***	TsuENH008
	16	2.3	5.7	28.6	***	CH05b06
(b*)	16	7.6	6.1	36.6	****	Hi02h08
Acidity	16	5.6	24.8	84.2	****	Hi02h08
Juice browning	16	7.6	25.4	84.8	****	Hi02h08
Sucrose	1	17.3	4.1	26.1	**	Hi02c07
Glucose	2	11.4	6.2	13.4	****	CN493139SSR-1
	3	56.1	14.3	42.6	****	HGA8b-1
	11	62.5	3.3	6.3	*	NZmsEB153947
	15	22.9	4.2	8.3	**	MEST92
Fructose	1	39.4	9.3	49.9	****	NZmsCN879773
Sorbitol	1	16.3	4.1	19.8	**	Hi02c07
	16	6.6	5.3	26.6	***	Hi02h08
Soluble sugars	15	16.1	5.4	27.6	****	CH04g10
	16	6.6	4.2	20.7	***	Hi02h08
Skin splitting	16	6.6	23.3	83.3	****	Hi02h08

No significant QTLs for weight or firmness were identified.
The peak with the highest LOD is presented as the QTL.
[a] Percentage of the phenotypic variance that the QTL explained.
[b] Significance level: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

in the parents; in several years, 'Prima' produced no splitting fruits, but the overmature fruits of '95P6' were often split. According to the Shapiro–Wilk normality test, 8 traits out of 15 were non-normally (bimodal or skewed) distributed: harvest time, flesh color, skin color intensity (L* value), acidity, juice browning, sucrose content, sorbitol content, and skin splitting. Acidity, juice browning, and skin splitting had bimodal distributions. The segregation ratio of the population for acidity was 34:28 when the border was set at 0.4, and that for juice browning was 26:36 with a border of 3. The ratio of genotypes producing no splitting fruits to those producing at least one splitting fruit was 35:25. The segregation of these three traits fitted a 1:1 ratio by the chi-square test.

Remarkable correlations were found between some traits (**Supplemental Fig. 2**). Strong correlation was found between soluble sugar and sorbitol content, and between the skin color elements L* and b* (|correlation coefficients (CE)| > 0.7). Strong correlations were also found among juice browning, acidity, and skin splitting. These three traits were moderately correlated with L*, b*, and flesh color (0.4 < |CE| < 0.7).

Map construction

The genetic linkage map of 'Prima' consisted of 386 loci detected by 358 primer pairs, and contained 17 linkage groups (LGs), spanning a total length of 935.2 cM (**Supplemental Table 4**, **Supplemental Fig. 3**). Consistent with '95P6' being DH across the whole genome, all genome-wide SSR markers (approximately 800 tested) were homozygous in '95P6'; therefore, we did not produce a '95P6' map and no phenotypic segregation could be generated by this parent. Though the total length of constructed map was slightly shorter than reported ones consisting of many common markers (Kunihisa *et al.* 2014, Silfverberg-Dilworth *et al.* 2006), the marker order was consistent with that in them; no obviously lacking regions were found, except a region of about 10–20 cM missing at the top of LG6.

QTL analysis

The 19 QTLs for 13 traits detected on the map of 'Prima' by rMQM ($p < 0.05$) are listed in **Table 2**. No significant QTL was detected for fruit weight or firmness. The moderate effect QTLs for harvest time were detected on LG1 (LOD = 4.2, % of explained variance [exp.] = 26.8) and for flesh color on LG16 (LOD = 6.3, % exp. = 37.2), and QTLs for the three indexes of skin color intensity were found on LG10 and LG16 (LOD > 5.4, % exp. > 26.0). QTLs for sugar components were detected on LG1, LG2, LG3, LG11, LG15, and LG16 with LOD scores ranging from 3.3 to 14.3 (exp. = 6.3%–49.9%). Major QTLs for acidity, juice browning, and skin splitting were identified in adjacent to each other near Hi02h08 in the upper region of LG16, with remarkably high LOD scores (23.3–25.4; exp. = 83.3%–84.8%). The QTLs for flesh color, skin color intensity, acidity, juice browning, sorbitol content, soluble sugar content, and skin splitting were concentrated in the upper part of LG16. The phenotypes of these traits were more or less

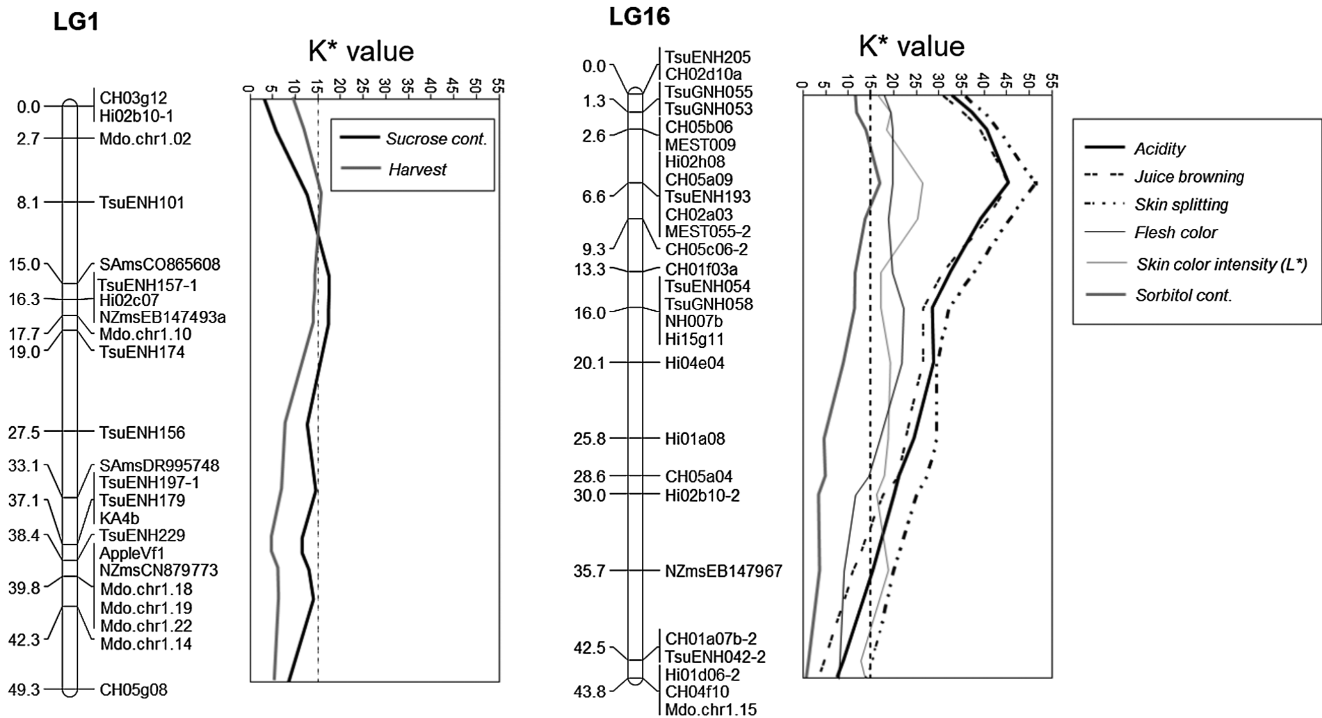

Fig. 3. Significant QTLs for non-normally distributed traits. QTLs ($p < 0.0001$) were detected by Kruskal–Wallis test. The threshold is shown by vertical dashed lines (K* value = 15.1).

Fig. 4. Distribution of genotypes with each allele at the Hi02h08 locus for 3 traits for which dominant QTLs were detected at the adjacent loci. The box plot displayed the maximum, third quartile, median, first quartile, and minimum value. Crosses, average; white circles, outliers. White boxes, genotypes with the 179-bp allele inherited from 'Prima'; gray boxes, genotypes with the 171-bp allele. Genotypes with different alleles were phenotypically separated, except No. 70.

correlated with each other (**Supplemental Fig. 2**), indicating the linkage of these QTLs. The QTLs for harvest time, sucrose, fructose, and sorbitol content were also concentrated on LG1, which partly explains the phenotypic correlations among these traits. For the eight non-normally distributed traits, the 1.5-LOD confidence interval of QTLs detected by rMQM included the peak of significant K* value calculated by the Kruskal–Wallis test; only the QTL for sorbitol content on LG1 did not exceed the threshold in the Kruskal–Wallis test (**Fig. 3**). In this non-parametric test, the predominant peaks for acidity, juice browning, and skin splitting were positioned at 6.6 cM on LG16, where five markers were located.

We examined the distributions of acidity, juice browning, and skin splitting, considering the allele type at the Hi02h08 locus inherited from 'Prima' (**Fig. 4**). For each trait, the segregation of the phenotype mostly corresponded to the segregation of the allele type across the same borders used in phenotype data analysis (0.4 for acidity, 3 for juice browning, presence or absence of skin splitting). We assumed that these three quantitative traits were regulated by single major genes in this population. The genotypes that inherited a 179-bp allele produced fruits with high acidity, skin splitting, and browning-resistant juice, in contrast to those with a 171-bp allele, which had fruits with low acidity, no skin splitting, and easily browning juice. No. 70 produced no splitting fruits, despite the presence of the 179-bp allele. No recombination occurred around these QTLs in No. 70,

according to its graphical genotype (data not shown); therefore, this mismatch might be caused by an environmental or unknown genetic effect.

Discussion

Comparison of phenotypic distribution

We compared the distribution of traits in the population from the 'Prima' × DH line '95P6' cross with published data. The phenotypes of harvest time, weight, firmness, content of sugar components, and soluble sugar content showed either normal or continuous single-peak distributions (even if they were statistically non-normally distributed), which is consistent with the published data (Kenis *et al.* 2008, King *et al.* 2000, Kunihisa *et al.* 2014). Flesh color also showed skewed continuous distribution, though a few class had no frequency. The L*a*b* values defining skin color intensity also had continuous single-peak distributions, whereas Chagné *et al.* (2016) reported bimodal or extremely skewed distributions of skin color intensity in some full-sib populations. The populations used by Chagné *et al.* (2016) segregated both green and red apples because of the cross of *MYB1/myb1* heterozygous parents, whereas the population we used bore only red fruits, probably because of a *MYB1/MYB1* ('Prima') × *myb1/myb1* ('95P6') cross. Skin color unaffected by *MYB1* is likely to be normally distributed.

Acidity showed a clear bimodal distribution in this study, whereas most of the previously evaluated populations showed a normal or skewed continuous distribution with a single peak (Kenis *et al.* 2008, Kunihisa *et al.* 2014, Morimoto *et al.* 2014, Visser and Verhaegh 1978). A population generated from a cross with *Malus sieversii*, which was optimized for the map-based cloning of *Ma* (the major gene regulating acidity), is a rare case with a bimodal distribution (Xu *et al.* 2012). Apple acidity is generally considered to be mainly regulated by only two QTLs on Chrs. 8 and 16 (Kenis *et al.* 2008, Kunihisa *et al.* 2014, Liebhard *et al.* 2003). Even for quantitative traits with only two causative genes, the phenotype would most likely show a continuous distribution. Juice browning, which had a clear bimodal distribution in this study, was reported as a quantitative trait which was distributed continuously and had at least two QTLs (Kunihisa *et al.* 2014). Skin splitting is also considered to be a quantitative trait in many fruits (Khadivi-Khub 2015), but the distribution data for this trait in an apple full-sib family is not available.

Acidity, juice browning, and skin splitting were generally regarded as quantitative traits controlled by multiple genes, as mentioned above. Therefore, it would be unusual that the phenotypes of these three traits simultaneously segregated at a 1:1 ratio (according to Mendel's law), and indicated the regulation by alleles of a single gene from one parent. The simplified inheritance of these traits shown in this study might be caused by the use of a DH line.

Comparison of detected QTLs

We wished to compare the QTLs detected on the map of 'Prima' crossed with the DH line (**Table 2**) with those detected on a 'Prima' map obtained by conventional double pseudo-testcross method. Using a 'Prima' × 'Fiesta' cross, King *et al.* (2000) reported a QTL for firmness on Chr. 1 of a 'Prima' map (exp. ~20%), but we detected no QTLs for firmness in our study, probably because of the difference in population size (152 vs. 62), different accuracy of phenotype data based on the replication of genotypes, or environmental factors. Other traits evaluated in the 'Prima' × 'Fiesta' family differed from those evaluated in this study, and were impossible to be compared.

The QTLs for harvest time (on LG1) and yellowish flesh color (on LG16) detected in this study were novel. The reported causal gene for yellowish flesh is a carotenoid cleavage dioxygenase gene (*CCD4*) in peach (Brandi *et al.* 2011), and apple *CCD4* is located between Hi15g11 and Hi04e04 (Apple Genome GDDH13v1.1; Daccord *et al.* 2017), where the LOD score of the QTL peaked. *CCD4* might control yellowish flesh even in apple, but it did not seem to have large effect.

The previously reported QTLs that are likely equivalent to those detected in this study are listed in **Supplemental Table 5**. Some of these QTLs were identified using data obtained in a single year; therefore, we reanalyzed QTLs for 14 traits using data in 2013, 2014 and 2015, respectively (**Supplemental Table 6**). Each QTL detected on LG16 for the L*a*b* values of skin color was in an identical or overlapping confidence interval region (**Table 2**), with positive effects in coupling phase (data not shown). This result indicates that the QTL for bright orangish red color intensity is on the top of LG16, which is consistent with previous report by Kunihisa *et al.* (2014). QTLs for fruit cortex color intensity and polyphenolic compounds have been identified in this region (Chagné *et al.* 2012, Kumar *et al.* 2013), which suggests that a key gene of the anthocyanin synthesis pathway might control these traits, including skin color intensity.

We detected a single major QTL for acidity at the top of LG16, where one of the causal genes for high acidity (*Ma*) is positioned (Xu *et al.* 2012). The equivalent QTLs were previously detected by many studies (Kenis *et al.* 2008, Kunihisa *et al.* 2014, Liebhard *et al.* 2003, Morimoto *et al.* 2014). We did not detect the QTL in LG8 previously reported (Kenis *et al.* 2008, Liebhard *et al.* 2003). The detection of a QTL explaining as much as 84.2% of variance was achieved owing to the simple composition of F_1 genotypes at the *Ma* locus (*Ma/ma* and *ma/ma*) because of the homozygosity of the DH line (*ma/ma*) and the non-segregated QTL on LG8 of 'Prima'. Though acidity also had a clear bimodal distribution in a 'Royal Gala' × 'PI613988' (*M. sieversii*) population (Xu *et al.* 2012), both parents were heterozygous (*Ma/ma*), and this might have lowered exp. (42.3%) of the QTL.

The QTL for juice browning detected at the top of LG16 in this study has been previously identified on the map of

'Akane' or 'Fuji' (Kunihisa *et al.* 2014, Morimoto *et al.* 2014), although no QTLs were detected on LG10 or LG17, previously reported loci. All of the phenotypes of genotypes that inherited different alleles at the QTL were separated by the border at 3.0 (**Fig. 4**). In previous studies, even though the F_1 genotypes had a bimodal distribution, the distributions of those with alternative alleles at the QTL overlapped in a non-negligible area (Morimoto *et al.* 2014). The population used in this study showed the most separated distribution and the simplest inheritance of juice browning so far.

Among the QTLs for sugar components detected in this study, four QTLs on LG1, LG3, LG15, and LG16 (for glucose, fructose, and sorbitol content) could be equivalent to reported ones (Guan *et al.* 2015, Kunihisa *et al.* 2014, Ma *et al.* 2016). The QTL detected for skin splitting is consistent with one reported by Kumar *et al.* (2013).

Many of the QTLs (for skin color intensity, acidity, juice browning, glucose, fructose, and skin splitting) were detected with comparatively high LOD scores, even though the population size (which is proportional to the LOD score) was the smallest level ($n = 62$). Although the QTL detection power is affected by several experimental conditions including analyzed populations, population size, the number of markers and so on, the clearer QTL detection for so many traits with high LOD scores in this study would be attributable to the population generated by the cross of DH line, where the phenotypic distributions of progenies inheriting alternative alleles at a QTL are expected to be more clearly separated than those in a usual pseudo-testcross population, as shown in **Fig. 1**.

QTL for skin splitting

In this study, skin splitting was likely to be regulated by a single gene located on LG16. The only reported apple gene for fruit splitting, *MdEXPA3* (Kasai *et al.* 2008), is positioned on Chr. 17 according to the apple genome v1.0 (Velasco *et al.* 2010). Skin splitting seemed to be recessively inherited at the major QTL on Chr. 16, as 'Prima' is a non-splitting cultivar heterozygous for splitting and non-splitting alleles, and '95P6' is a genetically homozygous splitting line.

Fruit splitting susceptibility was different between cultivars, depending on the genetic background. Moreover, the environmental conditions as temperature and humidity also greatly affect fruit splitting, as severe damage is observed in some locations in some years but not in others. To study splitting is difficult, because no method induces splitting reproducibly. We have few genetic information about fruit splitting because it is difficult to evaluate the phenotypes of genotypes adequately. Nevertheless, two QTL studies were attempted in sweet cherry (Khadivi-Khub 2015) and apple (Kumar *et al.* 2013). A QTL for apple fruit splitting on LG16 has been identified in a genome-wide association study (Kumar *et al.* 2013), but this QTL was for radial splitting from the stem end, whereas our QTL was for concentric splitting around the stem. Our results suggest that the same causal gene could regulate both types of splitting in apple. Our report on map-based QTL analysis is the first one to suggest the manner of inheritance of apple skin splitting and linkage with other traits such as acidity and juice browning.

In spite of the low reproducibility of this trait, in this study, almost all of the F_1 genotypes that inherited a splitting-inducing allele at the QTL on LG16 produced splitting fruits in a single year, probably because of optimal environmental conditions and the genetic homogeneity of the population.

Use of DH lines for future genetic analyses

The more QTLs that are related to a quantitative trait, the more normally the phenotypes are distributed and the more difficult the genetic analyses are. As the number of QTLs regulating a trait increases, the variation of generated genotypes to evaluate increases in geometric progression. Because no segregation occurs among the gametes of DH lines, DH crossing would theoretically lower the genotypic variation of the analyzed population and simplify the analyses of quantitative traits. As far as we know, this is the first report of the use of a DH line for QTL analysis in a fruit tree.

We observed a clear segregation of phenotypes consistent with that of the inherited alleles of QTLs for three quantitative traits (acidity, juice browning, and skin splitting). Even the positional cloning of causative genes for these quantitative traits might be possible, with a larger population.

To date, many DH lines of fruit crops have been produced with the aim of application to the efficient breeding program or genetic analyses. We need to sustain our efforts to obtain high-potential DH lines that could stably produce high-quality progeny, for the application to the breeding program. On the other hand, fertile DH lines such as '95P6' were demonstrated to be useful for genetic analyses. To analyze the cross between a normal heterozygous line and a DH line, we used BC model. In future, more precise genetic analyses would be possible using DH model, if a population consisting of DHs derived from the same donor could be generated following an improvement of the technique for DH cultivation.

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